Neutrophils in Atherosclerosis
From Mice to Man

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Abstract—Infiltration of leukocyte subsets is a driving force of atherosclerotic lesion growth, and during the past decade, neutrophils have received growing attention in chronic inflammatory processes, such as atherosclerosis. Equipped with various ready to be released mediators, evolved to fight invading pathogens, neutrophils may also hold key functions in affecting sterile inflammation, such as in atherosclerosis. Many of their secretion products might instruct or activate other immune cells (particularly monocytes) to, for example, enter atherosclerotic lesions or release proinflammatory mediators. Despite the emerging evidence for the mechanistic contribution of neutrophils to early atherosclerosis in mice, their role in human atherogenesis, atheroprogression, and atherosclerotic plaque destabilization is still poorly understood. This brief review will summarize latest findings on the role of neutrophils in atherosclerosis and will pay special attention to studies describing a translation approach by combining measurements in mouse and human. (Arterioscler Thromb Vasc Biol. 2014;35:00-00.)

Key Words: atherosclerosis ■ inflammation ■ leukocyte

Atherosclerosis: Current Understanding

Nowadays, increasing life expectancy, insufficient exercise, and an unhealthy diet foster a disease, which has been accompanying mankind already for a long time. Atherosclerosis, as revealed by computed tomography scanning of mummies from various ancient populations, already eliminated Egyptian pharaohs, but also Hungarian hunters.1 Despite this long history, it was only 100 years ago that Nikolai Anitschkow introduced the lipid theory after showing that rabbits fed with pure cholesterol established similar lipid-rich structures in arterial vessels like those seen in humans. Notably, in his additional work, he already described hallmarks of atherosclerotic disease pathophysiology like foam cell formation, white blood cell infiltration, and lipid accumulation.2

At present, cardiovascular diseases (CVDs) top the mortality statistics of developed countries, and myocardial infarction and ischemic stroke caused by atherosclerosis dominate among CVD. Atherosclerosis resides mainly within large arteries, where endothelial dysfunction caused by increased low-density lipoprotein titer and modification (eg, oxidized low-density lipoprotein) combined with an inadequate proliferative reserve at predilection sites with disturbed flow conditions3 is thought to be the initial trigger for lesion development. Thereafter, atherogenesis and plaque growth are driven by (chronic) inflammatory processes of the innate and adaptive immune system. Activation of endothelial cells results in increased expression of leukocyte adhesion molecules and the release of chemokines, which attract leukocytes, subsequently infiltrating the vascular wall.3,4 During the past decade, many cell types beyond monocytes and macrophages have gained attention in this onset, progression, and manifesta-tion of atherosclerotic lesions. In particular, neutrophils and their secretion products have entered the picture, and several excellent recent reviews have summarized this expanding field.5–11 As part of the innate immune response, neutrophils are highly efficient effector cells, which can readily be mobilized and recruited to sites of inflammation, carrying a multitude of weapons that are either primarily directed against an enormous variety of pathogens or serve as signal molecules for the subsequent recruitment of secondary immune cells. Furthermore, the neutrophil has been considered to be the main protagonist within the first line of defense under acute inflammatory conditions. However, in recent years, the neutrophil gained more attention with respect to chronic inflammatory processes, such as atherosclerosis, adipose tissue inflammation, or arthritis, and our appreciation has evolved, seeing neutrophils nowadays as key players, which either directly affect atherogenesis or might instruct immune cells (particularly monocytes) to enter atherosclerotic lesions in a second wave. Nevertheless, it still remains vague how neutrophils might act on atherogenesis, progression, and atherosclerotic plaque destabilization, predominantly in humans. This brief overview article will highlight the most recent findings on the role of neutrophils in atherosclerosis and will furthermore specifically emphasize studies applying a translational approach by transferring findings from mice to man.
Neutrophils in Atherosclerosis: An Update

Presence

Underestimation of neutrophils in atherosclerosis may have had several reasons, including their short life span, high turnover, phenotypic plasticity in tissue, and incomplete identification because of lack of specific markers. Until now, antibodies to Ly6G (expressed on mouse neutrophils) and CD66b (expressed on human neutrophils) are the 2 most specific markers described for mice and humans. Staining with an antibody to Ly6G identified neutrophils in early and advanced murine lesions, mainly in the plaque shoulder and in regions of high inflammatory activity. Furthermore, neutrophil depletion by repetitive injections of Ly6G antibody into mice lacking apolipoprotein E (Apoe−/−) significantly reduced lesion burden in atherogenesis. In humans, CD66b-positive neutrophils correlate with rupture-prone areas of the plaque and intraplaque hemorrhage (mostly occurring in human lesions), leading to neutrophil, and subsequent neutrophil protease accumulation further drive plaque instability. Notably, the degree and occurrence of intraplaque hemorrhage may directly correlate with future vascular events.

Entering the Lesion: The Microscopical View

Endothelial dysfunction accompanied by upregulation of adhesion molecules and the establishment of a chemotactic gradient attracts leukocytes and, in particular, neutrophils from the blood stream to the vascular wall. However, most knowledge about the (complex) leukocyte adhesion cascade stems from the microvasculature and should be extrapolated with caution. Fortunately, recent studies add to our understanding of leukocyte and neutrophil recruitment and arrest in larger arteries or under higher shear force. Sundd et al revealed that neutrophil rolling along the endothelium under higher shear (6–10 dyn/cm²) involves the formation of slings, which are distinct from tethers. Tethers are defined as nanotubes extruded from membranes of blood cells under the application of a pulling force, whereas slings are transformed tethers and are described as cell-autonomous adhesive structures extended at the front of rolling neutrophil. Both tethers and slings are important in enabling neutrophils to undergo slow rolling under high shear, which is particularly important in large arteries (Figure). Although the latter study introduces a new and exciting concept, the question still remains if the shear applied there (6–10 dyn/cm²) is enough to mimic arterial conditions because arterial shear rates range between 15 and 30 dyn/cm² or even higher. Further, high-resolution imaging of stabilized carotid arteries with the great advantage of reduced movement artifacts generated by heart cycle and respiration showed distinct differences of rolling dynamics between different cell subsets. Although a general increase of myeloid cell rolling and adhesion to the carotid bifurcation was already visible after 10 days of high-fat diet (HFD)–fed Apoe−/− mice, further recordings after 2- and 6-weeks HFD feeding revealed enhanced numbers of rolling neutrophils over time; however, monocyte numbers were similar at both time points. Additional analysis of rolling behavior showed neutrophils with high but similar rolling velocities at 2 and 6 weeks, whereas the rolling velocity of monocytes was reduced after 6-weeks HFD. Notably, rolling of T lymphocytes could only be recorded after 6-weeks HFD, underlining the importance of different kinetics of the individual cell types during atherogenesis and progression. However, secondary capture of activated platelets was mainly facilitated by neutrophils. Another study examining microvessel development in Apoe−/− mice receiving an HFD for 22 months, resulting in advanced atherosclerotic lesion formation, revealed the presence of complete microvascular networks with arterioles, capillaries, and venules in growing lesions. In contrast, less advanced lesions displayed no microvessels. As a conclusion, the author claims that early on leukocytes gain entry from the arterial endothelium, but with plaque growth, microvascular networks develop, enabling rolling, adhesion, and extravasation of leukocytes spontaneously via lesional venules. This venule recruitment pathway seems to be a 100-fold more efficient per endothelial area than invasion from the arterial lumen. In light of the neoangiogenesis being especially relevant in human lesions, observations made by the latter study might be particularly important for neutrophil entry to advanced human lesions side by side with the above mentioned intraplaque hemorrhage.

Mediators Affecting Lesional Neutrophil Recruitment

After selectin-mediated capture, neutrophils roll on the endothelium-sensing chemokines, which mediate integrin activation. Chemokines are released by the endothelium or are, for example, deposited by activated platelets. Neutrophil arterial recruitment specifically depends on CCL5 binding to CXCR1 and CXCR5, which, together with CXCR2 and CCR2, facilitate firm adhesion and subsequent neutrophil emigration (Figure). In this context, we could show that the α2,3-sialyltransferase IV (St3Gal4), important in, for example, the generation of functional selectin ligands, is also critical for CCL5 binding to and subsequent integrin activation on neutrophils and classical monocytes. St3Gal4-deficient neutrophils showed reduced CCL5-mediated arrest on inflamed endothelium, and CCL5-dependent neutrophil extravasation was markedly diminished in St3Gal4-knockout mice. Consequently, Apoe−/− St3Gal4−/− mice developed significantly smaller and less inflammatory atherosclerotic lesions. The latter highlights again that interference with early mechanisms triggering atherogenesis still holds promising therapeutic options. Along that road, it was also revealed that low-density lipoprotein receptor–deficient mice (Ldlr−/−) transplanted with CCL3-knockout bone marrow (CCL3 also binds to CCR1 and CCR5) display reduced aortic
sinus lesion formation, which seemed to be exclusively attributable to less neutrophil accumulation in plaque and reduced circulating neutrophil numbers in blood accompanied by a diminished responsiveness of CCL3-deficient neutrophils to CXCL1. In conclusion, CCL3 might establish a chemotactic gradient guiding neutrophils to atherosclerotic lesions.24 Interestingly, in humans, both CCL3 and CCL5 might serve as independent risk predictors of short-term mortality in patients with acute coronary syndromes.25 Although chemokines directly affect neutrophil attraction, adhesion, and emigration to atherosclerotic lesions, other players more indirectly influence neutrophil recruitment. For example, deficiency of the fatty acid amide hydrolase (FAAH), important for endocannabinoid degradation, leads to the development of smaller, but matrix metalloproteinase (MMP)-9 and neutrophil-rich, lesions in the aortic sinus of Apoe−/− FAAH−/− knockout mice after 10 and 15 weeks HFD. Notably, only neutrophil, but not monocyte, recruitment was increased in Apoe−/− mice treated with FFAH inhibitor. Further experiments revealed reduced regulatory T-cell numbers in spleen and increased secretion of tumor necrosis factor-α and interferon-γ from spleenocytes of Apoe−/− FAAH−/− knockout mice in vitro.26 In line, data from Hoyer et al27 also confirm increased neutrophil accumulation and MMP-9 expression in aortic roots of Apoe−/− mice receiving an HFD for 8 weeks when treated with the FAAH inhibitor URB597. In contrast, cannabinoid receptor type 2 (CB2) expression inversely correlates with MMP-9 expression in human symptomatic carotid specimens, whereas carotid and aortic root sections of Apoe−/− mice displayed CB2 expression in colocalization with MMP-9 and neutrophils. However, targeting CB2 with a specific agonist (JWH-133) diminished neutrophil numbers and MMP-9 expression significantly in murine aortic root lesions, and this effect was less pronounced in carotid lesions.28 Taken together, one has to carefully
discriminate between effects of endocannabinoids in general binding to CB1 and CB2 and a (putatively) sole anti-inflammatory function of CB2. Recently, FK866, a nicotinamide phosphoribosyltransferase inhibitor, has been shown to reduce neutrophil infiltration and MMP-9 content and increased collagen levels in Apoe−/− mice by reduction of systemic and intraplaque CXCL1. In line, treatment with Evasin-3, which neutralizes bioactivity of CXCL1 and CXCL2 in vivo and in vitro, selectively reduced neutrophilic inflammation in a mouse model of shear stress–induced atherogenesis and plaque vulnerability accompanied by less intraplaque MMP-9 and increased collagen content. Another study investigating the zinc finger transcription factor, Krüppel-like factor 2, in myeloid cells exclusively (Ly5Ccre KLF2b6os Ldlr−/−) reported enhanced lesion formation in Krüppel-like factor 2–deficient animals, which came along with increased CD11b expression on blood neutrophils and enhanced neutrophil adhesion to endothelial cells in vitro. Further, immunohistochemistry of aortic root sections displayed an increased number of lesional endothelial cells in vitro. Further, immunohistochemistry of animals, which came along with increased CD11b expression shown for human and mouse neutrophils.33
generating a proinflammatory neutrophil subset, as recently shown for human and mouse neutrophils.

Neutrophil’s Weapons
One of the most powerful tools of a neutrophil is the release of a diverse repertoire of granule proteins stored in 4 different compartments being discharged during different steps of neutrophil activation, transmigration, or tissue infiltration. Myeloperoxidase, for instance, abundantly expressed and secreted from neutrophils armory entered the stage within the past decade. Under necrotic conditions, neutrophil-derived Cramp/LL37–mediated monocyte adhesion turned out to be beneficial in re-endothelialization and could even abolish carotid artery restenosis when coated on a stent. Human neutrophil peptides or defensins have also been recently implied to exert proatherosclerotic properties by promoting monocyte adhesion, platelet activation, and foam cell formation. Another mediator in neutrophil-driven atherogenesis could be S100A9, which is constitutively expressed in neutrophils and other myeloid cell subsets. Indeed, mice deficient for Apoe−/− and S100A9 displayed reduced lesion areas in whole aortas. Furthermore, hyperglycemia has recently been shown to increase S100A8/9 plasma levels, leading to enhanced myelopoiesis and neutrophil counts under diabetic conditions, both of which are known risk factors for atherosclerosis.

However, another study did report no changes in the extend of atherosclerosis when S100A9-deficient bone marrow was transplanted into Ldlr−/− mice, which is somehow contradictory to the assumption that S100A9 protein, at least in an hyperlipidemic/atherosclerotic background, might stem from neutrophils solely. Despite granule proteins, another player from the neutrophil armory entered the stage within the past decade. Under certain conditions, neutrophils release neutrophil extracellular traps (NETs), which are mainly composed of fibrous chromatin structures decorated with granular components, histones, and some cytoplasmic proteins, all of which possess antimicrobial activities.

Primarily described to catch and neutralize invading pathogens, NETs were spotlighted in sterile inflammation recently. Confirming its hypothesized functional relevance in atherogenesis, the presence of luminal NETs under atherosclerotic conditions was proofed lately, and we could further reveal that Cramp/self-DNA complexes, putatively contained in neutrophil-expelled NETs, trigger activation of plasmacytoid dendritic cells, thereby fostering a proinflammatory type I interferon response, which drives atherogenesis and amplifies activation of newly recruited neutrophils (Figure). Mechanistic support stems from a study by Knight et al., demonstrating that inhibition of peptidylarginine deaminase playing a fundamental role in NET formation reduced atherosclerotic lesion sizes. Of note, markers of cell death and NET formation were associated with coronary artery disease, prothrombotic state, and occurrence of adverse cardiac events.

Hypercholesterolemia and Neutrophilia
Leukocytosis as a prognostic marker in CVD, in general, and neutrophilia as an independent predictor of cardiovascular outcomes (if analyzed together with general white blood
cell counts and C-reactive protein titers) has been described in various human clinical studies and was nicely summarized for neutrophils by Guasti et al.\textsuperscript{49} However, at least for neutrophilia, this seems to be independent of serum cholesterol levels in humans.\textsuperscript{49} In contrast, in animals, monocytosis and neutrophilia have mostly been correlated to hypercholesterolemia as evident in atherosclerotic models of swine, rabbit, and mouse.\textsuperscript{14,50–53} Mechanistically, although only investigated in mice to date, disturbed cholesterol efflux (introduced by ATP-binding cassette [ABC] transporter knockout) leads to increased cholesterol accumulation in cell membranes, thereby augmenting cell surface expression of the IL-3/GM-GSF receptor on hematopoietic stem cells, which increases their proliferative response, eventually resulting in monocytosis and neutrophilia.\textsuperscript{54} Further, systemic lack of ABC-transporters facilitates the development of splenic macrophages and dendritic cells, releasing elevated levels of granulocyte colony stimulating factor and IL-23–favoring hematopoietic lineage decisions toward granulocytes, thereby impairing the support for osteoblasts and the liberation of CXCL12 (Figure).\textsuperscript{55} Additionally, it was shown that proteoglycan-bound Apoe–/– significantly contributes to ABCA1- and ABCG1-mediated cholesterol efflux to high-density lipoprotein (HDL), explaining now why Apoe–/– mice fed an HFD exhibit such a pronounced myeloproliferation-fostering atherosclerosis.\textsuperscript{56,57} In line, treatment of neutrophils with HDL in vitro and in vivo reduces their activation status and their migratory and adhesive abilities.\textsuperscript{58} Interestingly, in Ldlr–/– mice, it was further revealed that already hematopoietic myeloid ABC-transporter deficiency (Lysm–/–Abca1–/–Abcg1–/– bone marrow into Ldlr–/– mice) drives neutrophilia (and monocytosis) and subsequent atherosclerotic lesion growth in the absence of hematopoietic stem cell and multipotent progenitor cell proliferation. The latter could be explained by increased expressions of macrophage colony stimulating factor and granulocyte colony stimulating factor in a specific population of splenic macrophages directly controlling monocyte and neutrophil production in the bone marrow (Figure). Ultimately also hyperglycemia, independently or synergistically of hypercholesterolemia, drives neutrophilia, monocytosis, and atherosclerotic lesion development by induction of S100A8/S100A9 titers directly correlate with neutrophil numbers in blood of humans and mice, suggesting a direct effect of hyperglycemia on neutrophil-derived S100A8/A9 plasma titers and enhanced proliferation of blood-derived CD34+ progenitor cells compared with type 1 diabetes mellitus without coronary artery disease. These data are phenocopied in a mouse model of diabetes mellitus and atherosclerosis. Further, S100A8/A9 titers directly correlate with neutrophil numbers in blood of humans and mice, suggesting a direct effect of hyperglycemia on neutrophil-derived S100A8/A9 release and subsequent myeloproliferation in both species. Yet, glucose control might be another important parameter side by side with lipid-lowering therapies as strategy to promote regression of atherosclerosis in diabetes mellitus.\textsuperscript{40} Endocannabinoids are a group of lipids involved in numerous immunologic responses via the cannabinoid receptors CB1 and CB2. Endocannabinoid signaling at CB2 receptors has been described to suppress immune cell functions, for instance, to control an inflammatory response. Comparing CB2 expression in upstream and downstream portions of symptomatic plaques in human carotid specimens revealed a correlation of decreased CB2 expression, but increased neutrophil numbers and MMP-9 accumulation. Correspondingly, stimulating CB2 with a specific agonist in Apoe–/– mice on HFD decreased neutrophil cellularity and MMP-9 release in murine atherosclerotic lesions, resulting in a more stable lesion phenotype. Thus, CB2 signaling may particularly decrease neutrophil accumulation in mouse and human plaque.\textsuperscript{59,60} CXCR4/CXCL12, another receptor/ligand axis, gains importance in CVD.\textsuperscript{61} Bot et al\textsuperscript{40} demonstrated that the inhibition of CXCR4 function enhanced adhesion and reduced apoptosis of neutrophils, resulting in increased neutrophil activation in murine atherosclerosis. Comparably, CXCR4 expression by

Translational Approach: From Mice to Man

Screening through the literature of neutrophils in atherosclerosis makes 2 things obvious: Studies are either placed in mice to investigate potential mechanisms rendering the neutrophil proatherogenic or performed with human material (blood, endarterectomy specimens) to correlate neutrophil numbers or accumulation of neutrophil secretion products with clinical outcomes of CVD. Despite the emerging evidence for the mechanistic contribution of neutrophils to early atherosclerosis in mice, the scientific community heavily doubts the involvement of neutrophils in late stage atherosclerosis in humans.\textsuperscript{13} Hence, the following section will particularly focus on recent studies that have combined measurements in mouse and man (Table). As already described above, hypercholesterolemia and malfunction of cholesterol efflux seem to play an important role in driving proliferation of myeloid progenitors and subsequent neutrophilia and monocytosis in mice. To evaluate whether this may partially be true also for human subjects, Tolani et al\textsuperscript{16} compared HDL titers and monocyte and neutrophil counts of children with familial hypercholesterolemia with $Ldlr^{+/−}$ Apoal1–/– mice on HFD and revealed a correlation of low HDL titers and enhanced myelocytosis in both species. However, the study has various limitations, most importantly low numbers for the human study group and the question whether the mouse model applied may be useful to directly mirror the human situation. Of note, patients with carotid stenosis and high anti-ApoA1 IgG titers display decreased collagen but increased MMP-9 content and neutrophil counts and, thus, may serve as predictors for future cardiovascular events.\textsuperscript{62} In line, the infusion of an anti-ApoA1 antibody into Apoe–/– mice led to increased aortic root lesion sizes, atherosclerotic lesions displaying a more vulnerable phenotype, and increased neutrophil and MMP-9 content.\textsuperscript{63} Complementary, others showed that infusion of HDL into patients with peripheral vascular disease reduced CD11b expression/activation of blood neutrophils, whereas injection of ApoA1 into tumor necrosis factor–treated mice diminished myeloid cell adhesion to activated endothelium (cremaster vessel).\textsuperscript{64} Again, the question remains how comparable these 2 approaches are. Diabetes mellitus, as a well-known risk factor for atherosclerosis, comes along with altered insulin signaling, higher adipose lipolysis, hyperglycemia, and leukocytosis. Hence, there is an ongoing debate as to whether leukocytosis is cause or consequence of diabetes mellitus–associated atherosclerosis. Notably, type 1 diabetes mellitus (T1DM) patients with coronary artery disease exhibit significantly increased neutrophil and monocyte counts along with increased S100A8/A9 plasma titers and enhanced proliferation of blood-derived CD34+ progenitor cells compared with type 1 diabetes mellitus without coronary artery disease. These data are phenocopied in a mouse model of diabetes mellitus and atherosclerosis. Further, S100A8/A9 titers directly correlate with neutrophil numbers in blood of humans and mice, suggesting a direct effect of hyperglycemia on neutrophil-derived S100A8/A9 release and subsequent myeloproliferation in both species. Yet, glucose control might be another important parameter side by side with lipid-lowering therapies as strategy to promote regression of atherosclerosis in diabetes mellitus.\textsuperscript{40}
circulating neutrophils was lowered in patients with acute cardiovascular syndromes. However, some of the expression data (mRNA, protein for CXCR4 and CXCL12) varies between human and mouse samples.

In conclusion, the contribution of neutrophils to human atherogenesis, -progression, and -growth requires continued scrutiny and intense research.

**Future Perspectives**

Myocardial infarction and ischemic stroke, as clinical manifestations of atherosclerosis, mostly affect human health in individuals aged ≥50 years; however, atherosclerotic lesion development already starts during adolescence. Even if available, a lifelong medication would be cost-intensive and might come at a considerable risk of side effects, which are challenging to avoid. Hence, besides a healthy lifestyle, which everybody should ensure for themselves, treatment strategies may preferably target late-stage lesion development. Thus, investigating mechanisms forcing plaque instability (such as the release of myeloperoxidase, ROS, and others, eg, by activated neutrophils) may deserve increasing attention. However, awaiting more appropriate models of plaque destabilization and rupture in mice, involvement of neutrophils in such processes is to date rather speculative and translation into the human situation still needs to be approved. Moreover, because neutrophils belong to the first line of defense cell, depletion or other long-term treatment strategies directly affecting neutrophils function have to be viewed with caution. Hence, nanomedical methodologies directly applying drugs at the side of inflammation (eg, atherosclerotic lesions) could represent promising future therapeutic approaches.

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None.

**References**


Atherosclerosis is a chronic inflammatory disease of the arterial wall. With its major clinical complications, such as stroke or myocardial infarction, atherosclerosis is still the leading cause for morbidity and mortality in western societies. To successfully identify future therapeutic targets, in-depth understanding of underlying mechanisms and cellular key players is needed. Until recently, the involvement of neutrophils in atherosclerosis was somehow underestimated, a perspective which changed within the past couple of years. To date, most of our knowledge about neutrophils in atherosclerosis stems from studies performed in mice, and the major question to be answered is whether these findings can directly be translated into the human situation. This review highlights latest advances in uncovering the role of neutrophils in atherosclerosis and pays special attention to studies describing a translational approach by combining measurements in mouse and human, hence providing a state of the art update in this field.
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