

Neutrophils in atherosclerosis

A brief overview

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Summary

Atherosclerosis is a chronic inflammation of the arterial wall and the continuous infiltration of leukocytes into the plaque enhances the progression of the lesion. Because of the scarce detection of neutrophils in atherosclerotic plaques compared to other immune cells, their contribution was largely neglected. However, in the last years studies have accumulated pointing towards the contribution of neutrophils to atherogenesis. In addition, studies are emerging implying a role for neutrophils in advanced atherosclerosis and/or plaque destabilization. Thus, this brief review delivers an overview of the role of neutrophils during early and late stage atherosclerosis.

Zusammenfassung

Atherosklerose ist eine chronische Entzündung der Arterienwand. Die kontinuierliche Infiltration von Leukozyten fördert ihr Fortschreiten. Aufgrund der geringen Anzahl neutrophiler Granulozyten in atherosklerotischen Läsionen wurde die Rolle dieser Zellen bisher weitgehend vernachlässigt. Studienergebnisse der vergangenen Jahre deuten darauf hin, dass neutrophile Granulozyten zur Initiation und Progression der Atherosklerose wesentlich beitragen. Außerdem lassen neue Untersuchungen auf eine Rolle neutrophiler Granulozyten in der Destabilisierung atherosklerotischer Läsionen schließen. In dieser Übersicht sind die wichtigsten Funktionen neutrophiler Granulozyten in verschiedenen Phasen der Atherosklerose zusammengefasst.

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Neutrophile Granulozyten und Atherosklerose Eine kurze Übersicht

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Atherosclerosis is a chronic inflammatory disease characterized by the progressive neointimal lesion formation and lumen narrowing of affected arteries. This multifactorial inflammatory process results in diverse cerebrovascular and cardiovascular complications associated with high mortality and morbidity where acute myocardial infarction, stroke and peripheral artery disease are the main clinical manifestations (1).

Much effort has been committed to elucidating the contributory role of various immune cells including monocytes and macrophages, lymphocyte subsets, dendritic cells (DCs) and platelets (2). However, recent studies point at an important causal role of neutrophils, the most abundant white blood cell subset in humans, during atherosclerotic lesion formation. Former underappreciation of neutrophils in atherosclerosis may be due to the sparse

detection in both, human and murine atherosclerotic lesions. This may be a result of their limited lifespan and rapid clearance by macrophages compared to other immune cells. Thus, the low number of detected neutrophils in atherosclerotic plaques may in fact reflect a significant invasion and turnover in lesions in vivo. Additionally, due to the phenotypical changes of neutrophils displaying markers typically expressed on antigen presenting cells and the lack of adequate detection methods, most attempts failed to detect neutrophils in larger numbers.

In spite of that, studies on experimental mouse models and human atherosclerotic plaque biopsies suggest an important role of neutrophils in the onset, progression and instability of atherosclerotic lesions.

In line, specific neutrophil depletion in atherosclerotic Apoe^{-/-} mice receiving a high fat diet resulted in a significantly reduced plaque size in early stages of lesion formation (3). In humans, neutrophils localize in rupture-prone areas and accumulation of neutrophil mediators leads to plaque instability (4, 5).

In this review we will briefly illustrate the most recent findings of the role of neutrophils in atherosclerosis, with special emphasis on the stage-specific contribution to initiation, progression and destabilization of the atherosclerotic plaque.

Neutrophilia triggers atherosclerosis

The fact that peripheral neutrophil counts and atherosclerotic lesion size positively correlate in mice and men (3, 6) indicates

that mediators regulating neutrophil homeostasis effect development of atherosclerosis by altering peripheral neutrophil counts, especially under hyperlipidaemic conditions. Neutrophil homeostasis is regulated at different levels, including

- production in and release from the bone marrow,
- survival in the circulation,
- homing and clearance of senescent neutrophils.

Neutrophil production is primarily regulated by granulocyte colony stimulating factor (G-CSF) (7), while retention of neutrophils in the bone marrow is controlled by the chemokine receptors CXCR2 and CXCR4 (8, 9). CXCR2 is constitutively expressed in high levels, which relay mobilization signals like CXCL1 (KC in mouse/Gro- α in human) and CXCL8 (IL-8). In contrast, CXCR4 expression is low on newly generated neutrophils and recognizes retention signals like CXCL12 (SDF1 α) (8).

In hyperlipidaemia, the bone marrow concentration of CXCL12 and expression of CXCR4 are reduced, thus promoting neutrophil mobilization and impairing neutrophil homing to the bone marrow (3) (► Fig. 1). The importance of CXCR4-dependent neutrophil retention has been demonstrated via disruption of the CXCL12/CXCR4 interaction in atherosclerotic mice which leads to neutrophilia and enlarged atherosclerotic lesion size (10, 11). In line, reduced CXCR4 expression on circulating neutrophils could also be observed in patients with CVD (11).

In contrast in mice, hyperlipidaemia increases neutrophil-presented CXCR2 and circulating CXCL1, promoting neutrophil release from the bone marrow into the circulation (3). Interestingly, it has recently been shown in mice that neutrophils regulate the haematopoietic niche under homeostatic conditions (12). An increase in neutrophil count on one hand positively correlated with released haematopoietic stem and progenitor cells (HSPCs), and on the other hand negatively with CXCL12-producing niche cells and total protein-level of CXCL12 in the bone marrow. Thus leading to the conclusion that senescent neutrophils (CD62L^{LO} CXCR4^{HI}) return to

the bone marrow, where they are engulfed by macrophages. These in turn regulate via interaction with CXCL12 producing stromal cells the CXCL12 level and though modify the release or retention in the haematopoietic niche (12) (► Fig. 1). Besides facilitated mobilization, hypercholesterolaemia and hyperglycaemia lead to increased neutrophil counts not just in the blood but also at sites of production, i.e. the bone marrow and the spleen (3, 13). In this context the group of Dr. Tall performed several studies to elucidate a link between lipid metabolism and neutrophil production in the bone marrow. A lack of the ABC cassette transporter genes *Abca1* and *Abcg1* resulted in an increase in the number of neutrophils and monocytes in the blood. This effect was due to a defect in the cholesterol efflux of macrophages and DCs, leading to an activation of the IL-23/IL-17 axis with subsequent production of G-CSF (► Fig. 1). Furthermore, the data demonstrated a significant increase of HSPCs in the bone marrow, which express both ABCA1 and ABCG1 (14–16). Moreover, increased production of S100A8/S100A9 in the blood by neutrophils stimulates RAGE on common myeloid progenitor cells (CMPs) and macrophages in the bone marrow and thus activates the release of colony stimulating factors inducing proliferation of GMPs (granulocyte-monocyte progenitor) leading to neutrophilia (17, 18) (► Fig. 1).

Besides hyperlipidaemia and hyperglycaemia chronic stress has been suggested to enhance atherosclerosis.

However, this potential mechanism has long been unclear. Recently, it has been shown that chronic stress acts on the bone marrow via the sympathetic nervous system to increase inflammatory leukocyte supply to the atherosclerotic lesion (19). Neutrophil counts were found to be significantly higher after exposure to chronic stress in both mice and humans. The increased neutrophil content in the plaque of Apoe^{-/-} mice after stress correlated positively with the noradrenaline levels. This prototypical stress hormone has been shown to regulate the CXCL12 synthesis (19) (► Fig. 1).

Taken together, factors like chronic stress, hyperlipidaemia and hyperglycaemia disturb the cytokine-driven control of neutrophil homeostasis and triggers an increase in peripheral neutrophil counts.

Role of neutrophils during initiation and progression of atherosclerosis

As described, neutrophil counts increase with metabolic changes associated with atherosclerosis development and closely correlate with the extend of early atherosclerosis formation under hyperlipidaemic conditions (3). This effect was reversed after specific antibody depletion of neutrophils in mice. Similar effects were also observed when neutrophilia was induced by CXCR4 antagonist administration or by lack of the transcription factor IRF8, the latter mimicking chronic myelogenous leukemia (10, 20). Neutrophils play an important role in inflammation due to their ability to perform a variety of effector functions (21–23). They are capable to instruct and activate other immune cells, notably monocytes, to enter sites of inflammation or additionally release of pro-inflammatory mediators.

Neutrophil recruitment into the lesion

Dysfunction of endothelial cells covering luminal arterial surface occurs in predisposed areas, exposed to

- hyperlipidaemia,
- high shear stress, and
- pro-inflammatory cytokines.

Endothelial dysfunction is characterized by reduced vasodilatation, increased leakiness and a pro-inflammatory state characterized by enhanced expression of chemokines and adhesion molecules (24).

Neutrophil recruitment and adhesion is regulated by a wide array of chemokine receptors and cell adhesion molecules (CAMs), e.g. intracellular adhesion molecule-1 (ICAM-1), E- and P-selectin (25). After capturing via the selectins and following rolling along the endothelium,

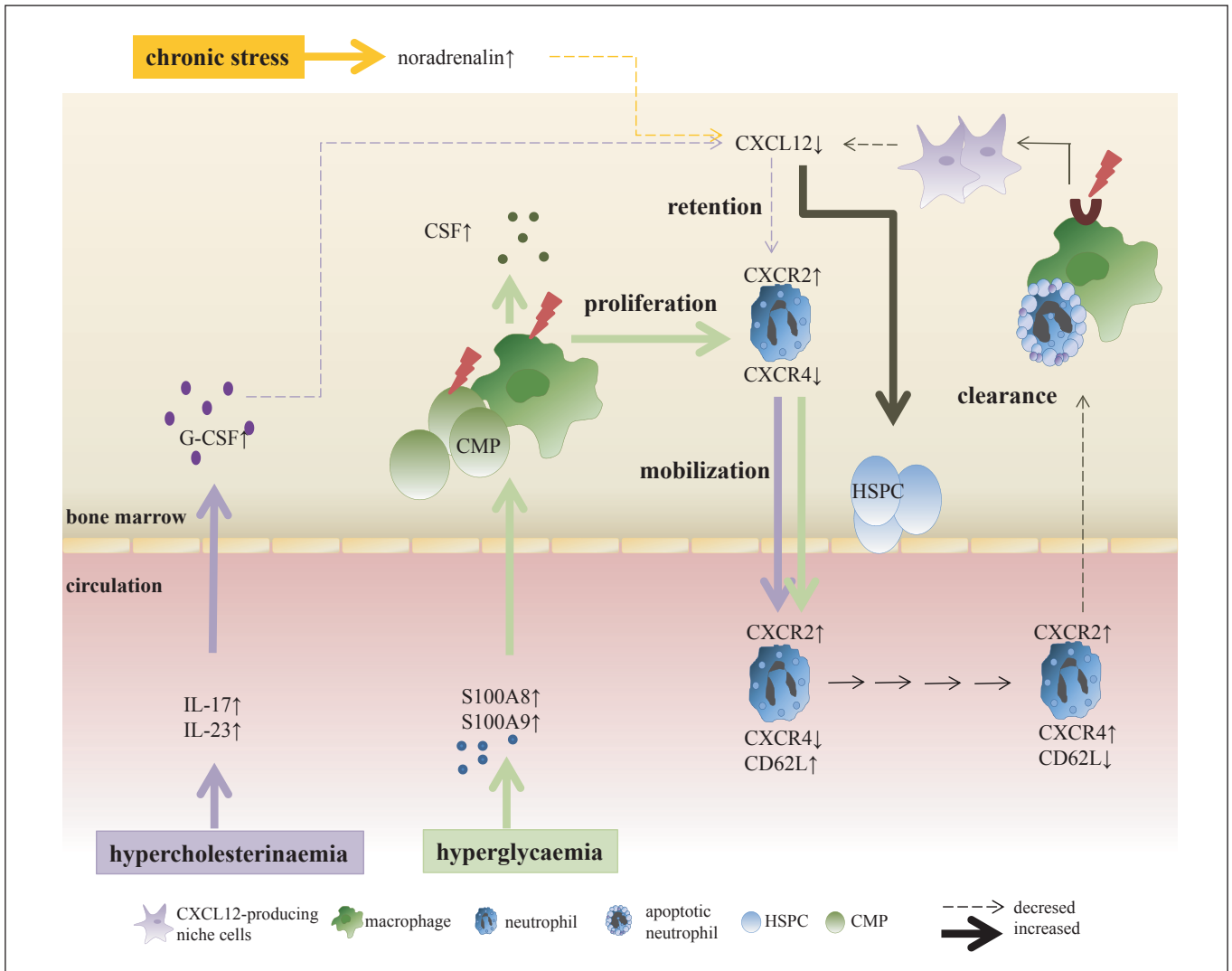


Fig. 1 Atherosclerotic risk factors disturb neutrophil homeostasis. Neutrophil production is regulated by granulocyte colony stimulating factor (G-CSF), neutrophil retention in the bone marrow is mediated via CXCR4 and CXCL12, and neutrophil mobilization is controlled by CXCR2 and its ligands CXCL8 and CXCL1. Under hypercholesterolaemia (lilac arrows) CXCL12 in the bone marrow is reduced thus promoting neutrophil release and impairing neutrophil homing to the bone marrow. Hyperglycaemia induces S100A8/S100A9 release from neutrophils (green arrows). These activate the

RAGE receptor on macrophages and CMPs in the bone marrow to stimulate GMP proliferation by CSF release. Chronic stress is an additional factor causing neutrophilia (orange arrows). Enhanced noradrenalin levels cause decrease of CXCL12 and thus boost the mobilization of neutrophils. In addition, neutrophils themselves regulate the haematopoietic niche (black arrows). Senescent neutrophils return to the bone marrow and after engulfment by macrophages induce a decrease in production of CXCL12.

neutrophils are activated through chemokine receptors followed by firm adhesion.

One of the important chemokines herein is CCL5 deposited by activated platelets (26). CCL5 acts through CCR1 and CCR5 and induces arterial recruitment of neutrophils and classical monocytes alike (3, 27). Interestingly, in a recent mouse study it has been shown that posttranslational modification of CCR5 via α 2,3-sialyltransferase IV (St3Gal4) plays an important role in

CCL5 binding and arterial recruitment of neutrophils and classical monocytes (28) (► Fig. 2). Besides CCL5, platelets release a vast amount of additional chemokines, whereof CXCL4 is the most abundant one (29). Furthermore, it has been demonstrated that CXCL4, in a not yet understood mechanism, mediates the delay of human and mouse neutrophil apoptosis and may therefore contribute to lesional accumulation of neutrophils (30). More-

over, CCL5 and CXCL4 form functional heteromers which induce recruitment of classical monocytes and neutrophils (31, 32).

Thus, platelet-borne chemokines deliver important signals for arterial accumulation of neutrophils which may be one explanation for their importance during early atherosclerosis (33, 34).

Neutrophils boost monocyte recruitment and activation

Monocytes and macrophages are the most abundant leukocytes found within the atherosclerotic plaque. The complex process of their recruitment involves a broad range of mediators like chemokines, activated complement components, and lipid mediators (35, 36). Neutrophils act as accelerators of early monocyte recruitment as absence of neutrophils was shown to significantly decrease the number of monocytes and macrophages within the arterial wall (3). Once bound to the endothelium neutrophils release soluble components including proteinase-3 (PR3), azurocidin and α -defensins. Azurocidin, which is cationic in nature and therefore favours deposition on the endothelium, is presented to rolling monocytes and promotes their firm adhesion (37) (► Fig. 2). PR3 drives the expression of adhesion molecules, contributing to monocyte adhesion and further stimulates endothelial CCL2 expression, thus amplifying monocyte recruitment (38). Alpha-defensins (aka HNPs) activate the endothelium to up regulate the CCL2 expression (39). In addition, neutrophils release the monocyte-attracting granule proteins cathepsin G and cathelicidin (LL-37 in human, CRAMP in mouse) (40).

Mice lacking CRAMP displayed a reduced lesion size in early atherosclerosis, caused by diminished inflammatory monocyte recruitment (41). In a subsequent study it has been shown that neutrophil-released CRAMP is reversely transported through the endothelium where it induces integrin activation when recognized by FPR2 expressed on classical monocytes (42). Alternatively, neutrophil-triggered monocyte recruitment is promoted via the IL-6 trans-signalling (43). IL-6 released by macrophages and endothelial cells (ECs) binds to its soluble receptor sIL-6R and can promote the activation of monocytes. sIL-6R can be generated by alternative splicing or shedding of the membrane bound IL-6R. Neutrophils mediate the sIL-6R shedding via matrix metalloproteinases (MMPs) (44), resulting in upregulation of CCL2 and VCAM on ECs and thereby enhanced monocyte recruitment. This effect could be disrupted

under the treatment with an IL-6 transsignalling inhibitor and resulted in reduced atherosclerotic plaque burden (43, 44).

Once migrated into the arterial wall, monocytes undergo differentiation into macrophages and foam cells following uptake of oxidized LDL (45). Studies investigating the neutrophil and macrophage relationship in this process suggest that neutrophils trigger foam cell formation thus contributing to atheroprogession. Herein, it was demonstrated in experimental studies that macrophages in presence of α -defensins produce nitrotyrosine, which is known as a crucial molecule contributing in oxidation of LDL (► Fig. 2). In the same study it was suggested that macrophages respond to stimulation with α -defensins with increase in CD36 and CD68 expression. Both are main receptors for uptake of oxLDL (39, 46). Furthermore, in mice and humans, macrophages exhibit defects in their phagocytic capacity under conditions of neutrophil-specific granule deficiency. The effect can be rescued by the supernatant of activated neutrophils, which mechanistically enhances expression of receptors involved in phagocytosis. (46–48).

Besides granule proteins, oxygen radicals are another mediator in macrophage activation. Neutrophils produce large amounts of reactive oxygen species (ROS) via MPO, lipoxygenases, and NADPH oxidase. Neutrophil-derived MPO is phagocytosed by resident macrophages through the macrophage mannose receptors. MPO and macrophage mannose receptor interaction leads to release of ROS along with pro-inflammatory cytokines (i. e. TNF- α , IL-1, IL-6, IL-8 and GM-CSF), thus further contributing to the inflammation progression within the lesion. On the other hand, it is well established that ROS released either by neutrophils or activated macrophages oxidize LDL thereby promoting foam cell formation (49).

Taken together, several studies suggested a partnership between neutrophils and macrophages in early atherosclerosis wherein neutrophils initiate monocyte recruitment to the developing atherosclerotic lesion. Furthermore, neutrophils activate macrophages to release pro-inflammatory cytokines and enhanced expression of

phagocytosis receptors. These latter effects may also be relevant in the role of neutrophil-driven plaque destabilization.

Plaque instability and neutrophils

Vulnerable plaques are associated with the accumulation of a vast amount of inflammatory cells, enlarged necrotic core (NC) and fibrous cap thinning (50, 51). Based on their activation, adhesion and infiltration, neutrophils might play a crucial role in promoting all these processes. Indeed, in mice and humans neutrophils have been shown to be present not just in early atherosclerotic lesions but also in later stages primarily located in highly inflamed regions (4, 52). Moreover, neutrophils and neutrophil-derived granule proteins accumulate on atherosclerotic culprit lesions and positively correlate with vulnerable plaque traits (4, 52). Similarly, patients affected by acute coronary syndromes exhibit increased circulatory neutrophil counts with reduced apoptosis and increased activation suggesting prolonged life and inflammatory status (53).

Neutrophil proteases promote plaque destabilization

Mechanical weakening of the fibrous cap can lead to events like plaque rupture and subsequent thrombotic events. Numerous factors mediate the structural changes but the degradation of the extracellular matrix (ECM) is a key process (54). MMPs degrade a broad range of matrix substrates like elastin, collagen and fibronectin (54) (► Fig. 2). Besides macrophages, neutrophils are an important source of MMP-9 that was found to positively correlate with neutrophil intraplaque recruitment in experimental studies (55). Furthermore, other neutrophil-derived MMPs such as MMP-8 and MMP-13 have been shown to play a role in a pro-destabilizing mechanism by affecting plaque macrophages and collagen content (56–58). Inhibition of MMP-8 or MMP-13 (56, 58) resulted in

- decreased lesion size,
- reduced numbers of lesional macrophages, and
- increased collagen content within the plaque.

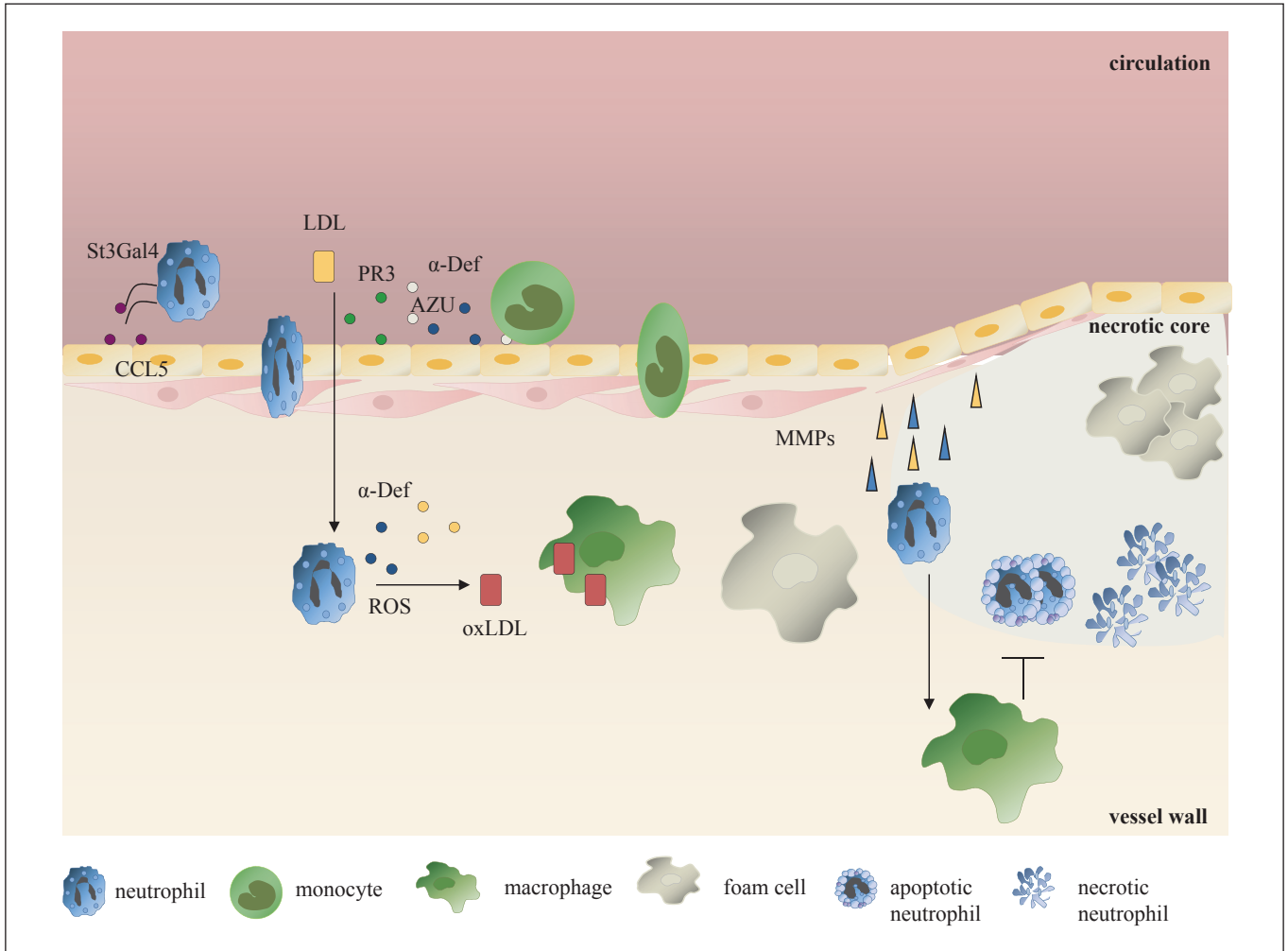


Fig. 2 Role of neutrophils in atherosclerosis. Neutrophil recruitment is mediated via adhesion molecules on inflamed endothelium and CCL5 released by activated platelets. St3Gal4-mediated sialylation of CCR5 on the neutrophil surface critically controls the function of CCR5. After binding to the endothelium neutrophils release soluble mediators (PR3, azurocidin and α -defensin) which promote monocyte adhesion. After migration to the lesion

site neutrophils contribute to oxidization of LDL, thus facilitating macrophage foam cell formation. In addition, neutrophils release MMPs involved in the thinning of the fibrous cap. Through cleavage of receptors involved in apoptotic cell clearance, neutrophil proteases reduce efferocytosis and subsequently feed necrotic core formation.

Besides ECM degradation, MMPs can process chemokines to increase their activity. Indeed, MMP-9 cleaves CXCL1, CXCL5, CXCL6 and CXCL8, though enhances their activation (59). Additionally, MMP-8 uses also CXCL5 and CXCL8 as substrates, which promote further leukocyte recruitment into the plaque (60).

MPO, highly abundant in primary granules of neutrophils, is released after activation and serves as a biomarker for atherosclerosis in mouse and humans (61). Furthermore, human rupture-prone atherosclerotic plaques were associated with infiltrating MPO-positive neutrophils (62). MPO can promote

plaque vulnerability via stimulation of further cell infiltration shown in experimental studies, by e.g. activating leukocyte integrins or the endothelium to increase the expression of adhesion molecules (63, 64). On the other hand, MPO-generated ROS enhance the activity of MMP-8 and MMP-9, leading to enhanced leukocyte recruitment and ECM degradation (65). Further plaque weakening is mediated by neutrophil elastase (NE).

A recent experimental study demonstrated that NE has the ability to cleave the macrophage receptor for haemoglobin-haptoglobin complexes (CD163). Macrophages remove haemoglobin through

CD163 and its accumulation during intra-plaque haemorrhages is a key factor during plaque destabilization. Inactivation of this receptor through NE may therefore contribute to plaque vulnerability (5).

Role of neutrophils in necrotic core formation

A crucial feature of an unstable plaque is the large NC. Apoptosis and secondary necrosis of foam cells and SMCs are thought to be a major contributor to NC development (50, 51). However, with the studies showing that neutrophils in both mouse

and humans accumulate also in advanced lesions (4, 52), it is possible that neutrophils play an important contributory role in NC formation.

1. Neutrophils are short lived cells and may, when not cleared after undergoing apoptosis, quickly undergo secondary necrosis and hence feed the NC.
2. By the mechanisms described, neutrophils contribute to oxidative modification of lipoproteins. These modified lipoproteins compete for uptake by macrophages of a set of receptors that is also utilized during apoptotic cell clearance and hence neutrophils may feed a competitive mechanism.
3. Neutrophils may be involved in the inhibition of apoptotic cell recognition that leads to hampered efferocytosis in late stage atherosclerosis (51) (► Fig. 2).

MFG-E8 acts as a molecule bridging between an apoptotic cell and the phagocyte during efferocytosis. Its absence leads to accumulation of apoptotic debris in atherosclerotic lesions (66). It has been shown that neutrophil-released HMGB1 (high mobility group box 1) binds to integrins on macrophages and thus inhibits the recognition of MFG-E8 (66). Moreover, HMGB1 binds to RAGE on macrophages and though inhibits the recognition of phosphatidylserine expressed by apoptotic cells (66).

The metalloproteinase ADAM17, expressed by neutrophils, induces Mer tyrosine kinase shedding, an integral membrane protein that is preferentially expressed by phagocytic cells, where it promotes efferocytosis and inhibits inflammatory signaling. Following the shedding it comes to a release of a soluble mediator that inhibits efferocytosis (67, 68). Moreover, ADAM17 sheds the macrophage scavenger receptor CD36. As previously described it is involved in LDL uptake but also in thrombospondin-mediated efferocytosis, leading to deficient apoptotic cell clearance (68, 69). In line, in ADAM17-deficient mice macrophages display an increase in their phagocytosis capacity (69). Furthermore, neutrophil serine proteases (NE, cathepsin G, PR3) have been shown to cleave the complement component 5a receptor (C5aR) (70). C5aR recognizes not only complement component 5a but also ribosomal protein S19 (RP S19) oligomers. RP S19 oligomers are released

from apoptotic cells and serve as a “find-me” signal for macrophages (71). Due to the non-responsiveness following C5aR cleavage, recognition of apoptotic cells is reduced.

Conclusions

Over the preceding years the hitherto underappreciation of neutrophils in atherosclerosis has started to change. Studies in mice lend support to the notion that neutrophils are vital during early inflammatory responses in the artery thus fostering atherogenesis.

At this early stage, it appears important that neutrophils promote continuous monocyte attraction and infiltration to the side of the inflammation.

However, associative studies also suggest a possible role for neutrophils during plaque progression and ultimately plaque destabilization. Since mechanistic evidence for the latter is scarce, additional studies are required to provide firm evidence for a role of neutrophils in plaque destabilization.

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Conflict of interest

The authors declare no conflict of interest.

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