Update on selective treatments targeting neutrophilic inflammation in atherogenesis and atherothrombosis

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Summary
Atherosclerosis is the most common pathological process underlying cardiovascular diseases. Current therapies are largely focused on alleviating hyperlipidaemia and preventing thrombotic complications, but do not completely eliminate risk of suffering recurrent acute ischaemic events. Specifically targeting the inflammatory processes may help to reduce this residual risk of major adverse cardiovascular events in atherosclerotic patients. The involvement of neutrophils in the pathophysiology of atherosclerosis is an emerging field, where evidence for their causal contribution during various stages of atherosclerosis is accumulating. Therefore, the identification of neutrophils as a potential therapeutic target may offer new therapeutic perspective to reduce the current atherosclerotic burden. This narrative review highlights the expanding role of neutrophils in atherogenesis and discusses on the potential treatment targeting neutrophil-related inflammation and associated atherosclerotic plaque vulnerability.

Keywords
Atherosclerosis, inflammation, neutrophil

Introduction
Atherosclerosis is the most common pathological process underlying cardiovascular (CV) diseases, currently considered as the leading cause of mortality worldwide (1). This chronic and smouldering disorder of large- and medium-sized arteries is characterised by endothelium dysfunction followed by the trans-endothelial migration of immune cells and lipids from to circulation to the arterial wall where they accumulate, giving rise to atherosclerotic lesions covered by a fibrous cap mostly derived from vascular smooth muscle cells (2, 3). Atherosclerotic plaques generally cause clinical manifestations either by provoking thrombosis that can acutely interrupt the blood flow or embolise and lodge in distal arteries, or by producing flow-limiting stenosis that leads to tissue ischaemia (4). If atherosclerosis was initially considered mostly as a lipid-related disease, inflammatory responses are now believed to underlie all key steps in atherogenesis, from the initial modification of healthy arterial endothelium to thrombus formation at the site of plaque rupture (5). Driven by this paradigm evolution and corroborated by the fact that even good adherence to contemporary therapies does not eliminate the risk of suffering a recurrent CV events, recent therapeutic developments lead to the production of compounds targeting the inflammatory processes, with the hope that they may help to further reduce the residual risk of major adverse cardiovascular events (MACE) despite actual standards of care (6-8). The involvement of neutrophils in inflammatory-related processes underlying atherogenesis is an emerging field, where evidence for their causal contribution in the various stages of atherosclerosis is accumulating (5). This review will highlight the expanding role of neutrophils in atherogenesis and discusses on the treatments targeting neutrophilic inflammation. This narrative review is based on the material found on MEDLINE and PubMed up to August 2013. We looked for the terms “neutrophil, atherosclerosis, vulnerability” in combination with “pathophysiology, chemokine, integrin”.

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Role of neutrophils in the pathophysiology of atherosclerosis

Neutrophil biology has received a growing interest since they have emerged as key effectors and regulatory cells of the immune response, extending their roles far beyond acute inflammation and resistance against extracellular pathogens (9, 10). Gene expression profiling has revealed that neutrophils, once considered transcriptionally static, express a variety of key inflammatory mediators, previously known to be expressed only in other cells, such as monocytes/macrophages (11). Similarly to other vascular and immune cells in the atherosclerotic microenvironment, neutrophil survival might be altered by different pro-inflammatory signals (such as adhesion, transmigration, hypoxia, cytokines and neutrophil granule protein) (12). Although it is still not clear the role of neutrophil apoptosis in atherogenesis in vivo, several pro-atherosclerotic mediators have been shown to potentially prolong neutrophil lifespan in vitro and ex vivo in both humans and mice (13-15). A prolonged lifespan, combined with an acquired ability to synthesise and release immuneregulatory cytokines, might be essential for the efficient elimination of damaging agents and for the potential interactions with other cell subsets (e.g. macro-

Figure 1: Neutrophils are recruited within atherosclerotic plaques. Hyperlipidaemia induces neutrophilia as a result of enhanced granulopoiesis and mobilisation from the bone marrow and promotes neutrophil activation by enhancing expression of CD11b and CD66b. Neutrophil recruitment within atherosclerotic plaques depends on CCR1, CCR2, CCR5 and CXCR2, CXCR4 and their interactions with CC and CXC chemokines (mainly CXCL1 and CCL5). CD: cluster of differentiation; CCR: chemokine (C-C motif) receptor; CXCR: chemokine (C-X-C motif) receptor.
phages, dendritic cells, natural killer cells, lymphocytes and mesenchymal stem cells). It is therefore not surprising to see that neutrophils are involved in the pathogenesis of numerous inflammatory disorders, such as infections, autoimmunity, cancer and chronic inflammation, including atherosclerosis (10, 16).

The rare detection of neutrophils in atherosclerotic lesions has contributed to their underappreciated role in the pathophysiology of this disease; however, refined staining techniques have allowed sensitive detection of neutrophils in murine and human atherosclerotic plaque specimens (8, 17). Data from human studies have shown a positive correlation between increased circulating neutrophil counts and cardiovascular risk (18, 19) and have also indicated that neutrophils are present at sites of atherosclerotic plaque rupture/erosion or in thrombi from patients with acute coronary syndromes (19, 20). Studies using Apolipoprotein E-deficient (ApoE−/−) mice have highlighted the existence of neutrophils in atherosclerotic lesions being present both in luminal and adventitial plaque regions, and those studies indicated that their depletion protected from atherosclerosis (5). Recently, Döring et al. showed that a chronic myelogenous leukemia-like phenotype in atherosclerotic mice was associated with expansion of neutrophils and promoted neutrophil-driven atherosclerosis (21). Interestingly the depletion of neutrophils in these ApoE−/− mice with a bone marrow deficient in interferon regulatory factor 8 (IRF8) abrogated the increase in atherogenesis (21), suggesting a critical role for circulating neutrophil count in mouse atherosclerotic models.

The importance of neutrophils in this context has been also demonstrated through experiments that interfered with the CXCL12/CXCR4 axis. Blockade of CXCR4 was shown to cause leukocytosis, increase neutrophil intraplaque content and aggravate atherosclerosis in ApoE−/− or LDL receptor-deficient (Ldlr−/−) mice, whereas depletion of neutrophils prevented the exacerbation of the disease (22). In addition, haematopoietic deficiency of CCL3 results in attenuated plaque development by altering neutrophil half-life and reducing neutrophil adhesion and accumulation in the plaque (23) (Figure 1). Neutrophils were shown to contribute to monocyte recruitment and their phenotypical shift (Figure 2). In both humans and mice, two subsets of monocytes exist and there is accumulating evidence that hyperlipidaemia-mediated monocytosis is restricted to classical (inflammatory, Gr1+) rather than non-classical (resident, Gr1−) monocytes and that classical monocytes preferentially give rise to foam cells. Neutrophil secretory products promote monocyte/macrophage phenotypic changes towards an inflammatory phenotype, which may prevail during later stages of atherosclerosis. Neutrophils’ participation on monocyte recruitment can be deduced from experiments with neutropenic mice, in which the extravasation of inflammatory monocytes is markedly reduced compared with mice with intact white blood cells. Neutrophil granule proteins (such as α-defensins and LL-37) are chemotactic for both human and murine monocytes. The relevance of these findings is highlighted in patients with specific granule deficiency, lacking granule proteins (such as α-defensins and LL-37). These patients present decreased recruitment of monocytes to skin blister chambers (5, 24). In mice, the research group of Weber and Soehnlein recently clarified that the neutrophil granule protein cathelicidin (called CRAMP in mouse and LL37 in human) was essential to promote early atherogenesis by increasing the adhesion of classical monocytes to the endothelium (25). The molecular mechanism underlying this effect was partially mediated by the activation of the formylpeptide receptor 2 on classical monocytes and consequent up regulation on their surface membrane of β1- and β2-integrins (26). The inhibition of this neutrophil granule protein has been recently indicated as a potential treatment strategy against re-endothelialisation and neo-intima formation after stent implantation in atherosclerotic mice (27), suggesting an active role of CRAMP in mouse vascular pathophysiology.

Furthermore, different associations between neutrophils and several metabolic abnormalities related to cardiovascular risk factors (such as hyperlipidaemia, hyperglycaemia, and hypercoagulability) have been reported and will be discussed in the following paragraphs.

**Neutrophils and hyperlipidaemia**

Hyperlipidaemia and inflammation are important players in the pathophysiology of atherosclerosis (Figure 1). Clinical studies and observations from animal models indicate that hypercholesterolaemia increases counts of circulating neutrophils and monocytes, which then accumulate in lesions and increase atherosclerosis (28). It has been reported that hypercholesterolaemia induces neutrophilia in ApoE−/− mice as a result of enhanced granulopoiesis and mobilization from the bone marrow. The degree of hypercholesterolaemia-induced neutrophilia positively correlated with the extent of early atherosclerotic lesion formation. The recruitment of neutrophils to large arteries was found to depend on CCR1, CCR2, CCR5, and CXCR2, whereas peripheral venous recruitment required CCR2 and CXCR2 only. This difference corresponds to the endothelial deposition of the platelet-derived chemokine CCL5 in arteries, but not in veins (29). In addition, the majority of transient leukocyte-endothelial cell contacts in atherosclerosis are attributable to neutrophils (30). In atherosclerotic lesions in ApoE−/− mice both under normal chow or high-cholesterol diet, Rotzius et al. also showed that neutrophils mainly accumulate in shoulder regions and co-localise with monocyte high-density areas (30). Although it is not known if neutrophil infiltration might precede monocytes, recent studies showing that neutrophil granule products might favour monocyte recruitment (25–27) strongly support the hypothesis of neutrophils as pivotal players in sustaining monocyte infiltration in the late stages of atherogenesis. Interestingly, hyperlipidaemia was shown to change both the neutrophil phenotype and functionality. Alipour et al. showed that acute post-prandial hypertriglyceridaemia was associated with an increased surface expression of CD11b and CD66b on circulating neutrophils from healthy volunteers (31). However, these post-prandial pro-inflammatory responses involving neutrophils were not reduced by pre-treatment with rosuvastatin in hyperlipidaemic patients with premature coronary sclerosis (32), indicating that the direct interaction between neutrophils and triglycerides might require a selective treatment to be efficaciously inhibited. Chronic hyperlipidaemia might be also considered as a priming condition for circulating polymorphonuclear leukocytes
Neutrophil granule proteins strongly promote the recruitment of monocytes within atherosclerotic plaques in mice. These proteins have been shown to induce a shift toward a pro-inflammatory phenotype of monocyte (G1+) and enhance adhesion molecule expression on endothelial surface (33). In fact, in hyperlipidaemic patients, myeloperoxidase serum levels were increased and neutrophils presented CD11b up regulation as compared to healthy controls (33). In addition, the severity of hyperlipidaemia was positively correlated with the level of neutrophil activation (33), indicating a potential direct effect of lipids on neutrophil changes in morphology and bioactivity.

**Neutrophils and hyperglycaemia**

Hyperglycaemia promotes neutrophilia and monocytosis, through enhanced myelopoiesis, and impairs atherosclerosis regression. In diabetic mice, neutrophils were shown to induce damage-associated molecular patterns in response to hyperglycaemia (notably S100A8/S100A9), which interact with glucose-inducible receptor for advanced glycation end-products (AGEs) on common myeloid progenitors, resulting in the release of inflammatory Ly6-Chi cells. On the other hand, both S100A8 and S100A9, which are myeloid-related proteins (MRPs) modulated by calcium, were shown as critical players in the regulation of myeloid cell function via Toll-like receptor-mediated pathways (34). Mice deficient for S100A9 (also named MRP-14) were characterised by relevant reduction in leukocyte accumulation within vascular injured tissues or atherosclerotic plaques (34) as compared to controls. In particular, in a mouse model of thrombo-haemorrhagic vasculitis, MRP-14−/−
mice were shown to have a reduced vascular infiltration of neutrophils as compared to wild type, suggesting that S100A8 and S100A9 might promote neutrophil recruitment within atherosclerotic lesion of vascular injured areas (34).

These observations demonstrated a potential crosstalk between neutrophils and myeloid progenitor cells in diabetes, leading to an increased production of monocytes and neutrophils, which might provide a mechanistic link between diabetes and coronary artery disease (CAD) (35).

**Neutrophils and hypercoagulability**

Neutrophils might be an important link between coagulation and inflammatory pathways through mutual interactions with platelets, proteolysis of tissue factor pathway inhibitor and neutrophil extracellular trap (NET) formation. Activated platelets play an important role in neutrophil recruitment and activation, and in turn activated neutrophils stimulate platelets, leading to the development of prothrombogenic state. Conversely, unstimulated or weakly activated neutrophils attenuate platelet aggregation, whereas resting platelets inhibit leukocyte chemotaxis and adhesion (36). Neutrophil serine proteases promote intra-vascular thrombus growth in vivo by enhancing tissue factor- and factor XII-dependent coagulation through proteolysis of the tissue factor pathway inhibitor (37). The role of coagulation proteases in atherosclerosis has been characterised by contradictory results. Borisshoff et al. recently investigated the potential role of the inhibition of thrombin formation on plaque morphology using different transgenic atherosclerotic mice with diminished or increased coagulability (38). FII-/WTapoE−/− mice with genetically reduced prothrombin levels were characterised by reduced leukocyte infiltration, collagen and smooth muscle cell content as compared to single ApoE knockout animals. On the other hand, hypercoagulability in mice with thrombomodulin gene mutation (TMPro/ProApoE−/−) was associated with increased circulating neutrophil count and activation. This study provided interesting insights on the potential association among neutrophils, their proteases and the activation of coagulation cascade during atherogenesis (38).

NETs have been identified in atherosclerotic lesions in mouse and in human plaques obtained by endarterectomy; however, their contribution to atherogenesis is poorly understood (39). Recently, they have been also found within fresh and lytic thrombi from patients after acute myocardial infarction (40). NETs have been shown to trap microorganisms and promote their subsequent disposal (10). During NET formation, intracellular membranes become disintegrated, granule proteins get access to the nucleus, plasma membrane ruptures and nucleosomes are released (41). NETs might also promote thrombus formation by interacting with the endothelium, platelets, coagulation factors and red blood cells (42). In addition, neutrophils may promote atherothrombosis by facilitating plaque rupture or superficial erosion of the endothelial monolayer because of their large content of matrix-degrading proteases and production of reactive oxygen species (ROS). Furthermore, it is also possible that neutrophils account for an important source of apoptotic and necrotic cells, thus contributing to the necrotic core formation, a critical feature of unstable plaques (5).

As the importance of neutrophils in atherogenesis had been under-appreciated, some previous data that could be related to neutrophil action were attributed to other cells and may need to be reconsidered. For example, increased atherosclerotic lesion sizes in mice lacking molecules involved in the leukocyte adhesion cascade were preferentially related to modified monocyte recruitment (43). Therefore, the understanding of neutrophil participation in the onset and progression of atherosclerosis could identify these cells as a new therapeutic target for both prevention and treatment of atherosclerosis (19).

**Treatments targeting neutrophil-related inflammation in atherogenesis**

Targeting the inflammatory processes underlying atherosclerosis has become subject of intense research, which so far provided...
The selectin family consists of the three closely homologous glycoproteins, E-selectin, P-selectin, and L-selectin, that all bind glycoproteins and glycolipids bearing sialyl Lewis X (sLeX) in a calcium-dependent manner (67). Selectins mediate initial rolling and tethering of inflammatory cells at sites of activated endothelium (68). P-selectin is constitutively present in the α-granules of platelets and in the Weibel-Palade bodies of endothelial cells, and can be rapidly translocated to the cell surface on activation (69). E-selectin is specifically expressed by activated endothelial cells, it is detectable either after or concurrently with P-selectin and recognises several diverse and structurally distinct glycol-conjugates and differs from the other lectins in its preference for ligands other than, and in addition to, PSGL-1 glycoprotein ligand-1 (PSGL-1) (47, 69). L-selectin is constitutively expressed in leukocytes. In addition to its role in the initial steps of the adhesion cascade, L-selectin is also responsible for the recirculation and homing of lymphocytes to lymph nodes via high endothelial venules (70).

P-selectin is up regulated in human atherosclerotic plaques (43, 71). In ApoE−/− mice, P-selectin expression is also increased and its level of expression correlates well with the extent of lesion development (72). P-selectin−/− ApoE−/− mice have a protective effect in atherosclerotic lesions development; however, the reduction of lesion size depends on diet type and duration. A fat diet for a prolonged period neutralises the beneficial effects of P-selectin deficiency (73). Additionally, blockade of P-selectin decreases the deposition of CCL5 on atherosclerotic endothelium, which is accompanied by reduced adhesion of neutrophils (29, 43).

Reduced atherosclerotic lesion sizes have also been observed in E-selectin-deficient mice (74). Coincidence of E-selectin and neutrophil presence in the endothelial lining of atherosclerotic mice has been demonstrated (75). By contrast, absence of L-selectin augments early stages of atherosclerosis. After six weeks of normal or high cholesterol diet, aortic lesions in ApoE−/− L-sel−/− mice were increased compared with ApoE−/− controls. After 12 weeks of high-cholesterol diet, however, there was no difference in atheroma formation between ApoE−/− L-sel−/− and ApoE−/− mice. These data indicate that L-selectin protects from early atherosclerosis (68).

PSGL-1 is the most important and best-characterised ligand for L-selectin or P-selectin and, if appropriately glycosylated, PSGL-1 may also bind E-selectin. PSGL-1 is the only known selectin ligand capable of binding all three selectins. Recognition of PSGL-1 by E-selectin is insensitive to sulfation of PSGL-1, whereas recognition by L-selectin or P-selectin is particularly sensitive to this modification, indicating that E-selectin recognises a distinct epitope (67). PSGL-1 is constitutively expressed on all leukocytes; however, its expression level and degree of glycosylation varies significantly in the different subtypes of leukocytes, which contribute to their selective recruitment to atherosclerotic lesions (76). Deficiency in PSGL-1 protects against atherosclerosis in animal models. Psgl-1−/−, ApoE−/− mice presented reduced leukocyte rolling and firm attachment on endothelial cells compared to Psgl-1+/−, ApoE−/− controls, on both standard chow and Western diet.

Blocking selectin actions could represent an alternative to prevent or treat cardiovascular disease, by interrupting an early event in atherosclerotic lesion growth, development and instability (77). Inclacumab, a novel human monoclonal antibody against human P-selectin, is currently in phase II trial for atherosclerosis treatment (6). In preclinical studies, inclacumab has demonstrated to specifically bind human P-selectin and inhibit P-selectin mediated functions, with no impact on platelet activation and aggregation in in vitro assays. In addition, inclacumab was able to reduce the elevated circulating levels of platelet-leukocyte aggregates in patients with peripheral arterial disease (66). Despite still unclear, these aggregates might play a critical role in inflammation and atherothrombosis, and potentially be increased in prothrombotic states. Platelet P-selectin and PSGL-1 are crucial for this interaction, that results in firm adhesion between the two cell types, up regulation of tissue factor on leukocytes, and biosynthesis of several cytokines (78, 79).

The blockade of PSGL-1 by an antibody was able to reduce leukocyte rolling and protected against atherosclerosis ApoE−/− mice (80). In addition, the concentration of recombinant PSGL-1-immunoglobulin (PSGL-1-Ig) that is requested to dampen system inflammation is 30-fold lower than the dose necessary to inhibit selectin-mediated rolling, suggesting that PSGL-1-Ig inhibits inflammation by mechanisms other than leukocyte rolling. Indeed, it has been reported that PSGL-1-Ig binds CXC and CC chemokines (such as CXCL1 and CCL21, respectively). Titration of chemokines with PSGL-1-Ig inhibited chemotaxis of mouse neutrophils in response to CXCL1. Interaction of P-selectin with PSGL-1 also participates to the regulation of vascular thrombosis and blocking of PSGL-1 dramatically inhibited the growth of arterial neointima after wire-induced arterial injury in ApoE−/− mice. The inhibition of PSGL-1 and P-selectin is therefore considered as potential therapeutic target to reduce CAD complications such as atherothrombosis or restenosis after angioplasty (81, 82), but unfortunately, the costs related to the production of PSGL-1-Ig are likely to prohibit the development of dedicated clinical trials (67).

Other strategies to modulate selectins include: i) the use of competitive inhibitors, like sLex oligosaccharides, sLex mimetics, multivalent sLex ligands or different molecular weights heparins; ii) the inhibition of glycosyltransferases; iii) the deviation of Lewis antigens glycosylation by carbohydrate decoys that act as substrates for glycosyltransferases. These glyco-mimetics or glycosylation modifiers that target selectin–selectin ligand interactions could modulate structural carbohydrate features, resulting in suppression of pathologic phenotypes with less toxicity than existing treatment modalities (67).
Table 1: Summary of the experimental pharmacological modulation of neutrophilic inflammation in atherogenesis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Status</th>
<th>Study outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclacumab</td>
<td>Human monoclonal antibody against human P-selectin</td>
<td>Phase II trial for atherosclerosis treatment</td>
<td>Specifically binds human P-selectin and inhibit P-selectin mediated functions. Reduced levels of circulating platelet-leukocyte aggregate in patients with peripheral arterial disease</td>
<td>Berman et al. (2013) [6]; Kling et al. (2013) [66]</td>
</tr>
<tr>
<td>cM7</td>
<td>Small cyclic peptide mimicking the sequence of the binding site of CD40L on Mac-1</td>
<td>Preclinical studies</td>
<td>Decreased atherosclerosis in Ldr−/− mice</td>
<td>Wolf et al. (2011) [48]</td>
</tr>
<tr>
<td>7H</td>
<td>Human antibodies against human VCAM-1</td>
<td>Preclinical studies</td>
<td>Attenuated atherosclerosis in ApoE−/− mice</td>
<td>Park et al. (2013) [49]</td>
</tr>
<tr>
<td>TAK779</td>
<td>Small-molecular inhibitor of CCR5</td>
<td>Clinical development was discontinued due to local reactions at injection sites</td>
<td>Reduction atherosclerosis in Ldr−/− mice</td>
<td>Briz et al. (2006) [50]; van Wanrooij et al. (2005) [51]</td>
</tr>
<tr>
<td>Met-RANTES</td>
<td>CCL5/RANTES receptor antagonist</td>
<td>Preclinical studies</td>
<td>Reduced the progression of atherosclerosis in ApoE−/− mice</td>
<td>Veillard et al. (2004) [52]</td>
</tr>
<tr>
<td>MKEY</td>
<td>Peptide antagonist of CCL5-CXCL4 interactions</td>
<td>Preclinical studies</td>
<td>Attenuated CCL5-CXCL4 heterodimerisation, reducing atherosclerotic lesion in ApoE−/− mice</td>
<td>Koenen et al. (2009) [54]</td>
</tr>
<tr>
<td>MLN-1202 (Millennium Pharmaceuticals)</td>
<td>Humanized monoclonal antibody that binds to CCR2 and thus inhibits CCL2 binding</td>
<td>Completed phase II trial</td>
<td>Improved high-sensitivity C-reactive protein (hsCRP) levels, a surrogate marker of coronary disease, in patients at high risk for atherosclerosis and that had documented elevations in CRP</td>
<td>Gilbert et al. (2011) [55]</td>
</tr>
<tr>
<td>CCX-140 (ChemoCentrx)</td>
<td>Small molecule inhibitor of CCR2</td>
<td>Completed in phase II trials in diabetics</td>
<td>Pre-clinical testing for treatment of atherosclerosis</td>
<td>Berman et al. (2013) [6]</td>
</tr>
<tr>
<td>Propagermanium</td>
<td>CCR2 antagonist</td>
<td>Clinically used in Japan for the treatment of chronic hepatitis B</td>
<td>Attenuated atherosclerosis via the inhibition of macrophage infiltration in ApoE−/− mice.</td>
<td>Yamashita et al. (2002) [57]</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>CCR5 antagonist</td>
<td>Currently approved by the FDA for the treatment of HIV.</td>
<td>Inhibited atherosclerotic progression ritonavir-induced atherosclerotic and in a late phase of spontaneous atherosclerosis model, both in in ApoE−/− mice</td>
<td>Koenen et al. (2011) [58]; Cipriani et al. (2013) [59]</td>
</tr>
<tr>
<td>TLK-19705 (Telik)</td>
<td>CCR2 antagonist</td>
<td>Preclinical studies</td>
<td>Reduced the areas of atherosclerotic lesion in ApoE−/− mice and inhibited human CCL2-induced chemotaxis of monocytes/macrophages</td>
<td>Okamoto et al. (2012) [56]</td>
</tr>
<tr>
<td>Evasin-3</td>
<td>Chemokine-binding proteins, only to CXCL1 and CXCL8 and their murine counterparts, KC and MIP-2, respectively</td>
<td>Preclinical studies</td>
<td>Inhibited neutrophil-mediated inflammation in myocardial ischaemia/reperfusion, carotid atherosclerosis and ischaemic stroke</td>
<td>Copin et al. (2013) [60]; Montecucco et al. (2010) [61]</td>
</tr>
<tr>
<td>PMX53</td>
<td>C5a antagonist</td>
<td>Preclinical studies</td>
<td>Abrogated atherosclerotic lesion progression in ApoE−/− mice under normal diet (early atherosclerosis)</td>
<td>Manthey et a. (2011) [115]</td>
</tr>
<tr>
<td>GW3965</td>
<td>Agonist of LXRss</td>
<td>Preclinical studies</td>
<td>Induced regression of atherosclerotic lesions in Ldr−/− mice and in LXRα−/−ApoE−/− mice</td>
<td>Levin et al. (2005) [62]; Bradley et al. (2007) [44]</td>
</tr>
<tr>
<td>LXR-623</td>
<td>Agonist of LXR-α</td>
<td>Preclinical studies</td>
<td>Reduced atherosclerosis progression in LDL−/− mice and in New Zealand White Rabbits</td>
<td>Katz et al. (2009) [63]; Quinet et al. (2009) [64]; Giannarelli et al. (2012) [65]</td>
</tr>
<tr>
<td>JWH-133</td>
<td>Agonist of cannabinoid receptor type 2 (CB2)</td>
<td>Preclinical studies</td>
<td>Reduced carotid intraplaque content of matrix metalloprotease-9</td>
<td>Montecucco et al. (2012) [45]</td>
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</table>
Inhibition of integrin interactions with their ligands

Integrins are heterodimeric transmembrane receptors regulating cell-to-cell and cell-extracellular matrix interactions (83). The velocity of the leukocyte rolling is reduced by both selectin- and integrin-dependent adhesive interactions. Subsequent firm arrest and crawling are mediated by leukocyte surface integrins, such as very late antigen (VLA)-4, lymphocyte function-associated antigen 1 (LFA-1) or macrophage-1 antigen (Mac-1) that interact with endothelial ligands, including vascular cell adhesion molecule (VCAM)-1 or intercellular adhesion molecule (ICAM)-1. The integrin-dependent adhesive interactions depend on their avidity to strongly bind to their endothelial ligands (84).

VCAM-1 and ICAM-1 are up regulated by hyperlipidaemia and pro-inflammatory cytokines in regions predisposed to lesion formation (85). However, VCAM-1 expression is restricted to the predisposed lesion area, whereas ICAM-1 expression extends to regions beyond atherosclerotic lesions (86). ICAM-1 interacts with β2 integrins, whereas VCAM-1 is the main counter-receptor for β1-integrins. The presence of several members of the β1-integrin family on neutrophils including VLA-4 (α4β1), VLA-5 (α5β1) and VLA-9 (α9β1) has been reported (87). Neutrophils express three different β2 integrins namely LFA-1, Mac-1 and gp150/95, of which LFA-1 and Mac-1 are the most relevant β2 integrins for their trafficking (88).

Studies on mice deficient in ICAM-1 or VCAM-1 have shown anti-atherogenic effects, such as reduced rolling and smaller lesion size. Additionally, blocking antibodies against, VLA-4, ICAM-1, or VCAM-1 protect against atherosclerotic responses (85). Mice treated with anti-Mac-1 antibody have also developed smaller atherosclerotic lesions than respective controls, demonstrating a functional role of Mac-1 in atherogenesis (89). Mac-1 is a receptor for CD40 Ligand (CD40L), which has been associated with atherosclerosis and its related complications (90). Treatment of Ldr4/- mice with a small cyclic peptide (cM7) that mimics the sequence of the binding site of CD40L on Mac-1 resulted in the decrease of intraplaque inflammation, a key determinant of vulnerability (48).

Ldr4/- mice with disrupted fourth Ig domain of VCAM-1 (homozygous VCAM-1 domain 4-deficient mice) presented reduced area of early atherosclerotic lesions. In contrast, deficiency of ICAM-1 either alone or in combination with VCAM-1 deficiency did not alter nascent lesion formation. Therefore, although expression of both VCAM-1 and ICAM-1 is up regulated in atherosclerotic lesions, evidence from basic research indicates that mainly VCAM-1 plays a dominant role in the initiation of atherosclerosis (91). Furthermore, two fully humanised antibodies against human VCAM-1, H6 and 7H, were shown to effectively inhibit inflammatory cell adhesion to this molecule. The 7H, which presented binding affinity to both murine and human VCAM-1, attenuated atherosclerosis in ApoE/- mice, improved plaque inflammation and stability, as well as inhibited the adhesion of inflammatory cells (CD45+ cells) (85).

Targeting the chemokine system

Chemokines constitute a family of structurally related proteins involved in the migration of innate and adaptive immune cells through the activation of a broad range of receptors belonging to the seven transmembrane G-protein-coupled receptor family (92). Accordingly, targeting the chemokine system may offer various different and complementary therapeutic options to reduce atherosclerosis. Hence different approaches (such as small-molecule receptor antagonists, blocking antibodies, structurally modified chemokines and chemokine-neutralizing proteins) have been considered to manipulate the chemokine system in atherogenesis and its acute complications (7, 58). These treatments might not only reduce neutrophil recruitment and activation within atherosclerotic plaques, but also affect the microvascular recruitment of these cells in other organs, such as the lung. For instance, acute lung injury (ALI) in mice caused by gram-negative bacterial infection has been shown to involve certain chemokines, such as CCL5 and CXCL4. The disruption neutralisation of the bioactivities of these chemokines (that are also upregulated in atherogenesis) has been shown to potently reduce neutrophil influx within the injured lung (93). Interestingly the genetic deletion or pharmacologic inhibition of CCR5 (a receptor of CCL5) induced similar protective effects on neutrophil pulmonary infiltration in a mouse model of ALI (94). The in vivo studies importantly contributed to highlight a critical role of these platelet-derived chemokines as non-specific targets for several diseases, suggesting a potential therapeutic interference.

As previously mentioned, the recruitment of neutrophils to the sub-intimal space in large arteries depends on CCR1, CCR2, CCR5, and CXCR2 (28). Genetic deletion of Ccr5 but not Ccr1 in ApoE/- mice protects from diet-induced atherosclerosis and is associated with a more stable plaque phenotype with a reduced mononuclear cell infiltration and Th1-type immune responses, and upregulation of the protective cytokine interleukin-10 (IL-10) (95). The chemokine CCL5 regulated on activation, normal T cell expressed, and secreted (RANTES) interacts with the chemokine receptors CCR1, CCR3, and CCR5 and is secreted by many different cell types, such as endothelial cells, smooth muscle cells, activated T cells, macrophages, and platelets. When CCL5 is produced recombinantly in prokaryotic cells, the initiating methionine residue is retained (Met-RANTES), resulting in a CCL5 receptor antagonist with nanomolar potency that does not retain high affinity for the murine chemokine receptors CCR3. Met-RANTES reduces the progression of atherosclerosis in ApoE/- mice with advanced atherogenesis and also regulates several vascular-cell functions related to acute manifestations of atherosclerosis (52).

TAK779 (Takeda) potently blocks CCR5 (96). Small-molecular receptors inhibitors, like TAK779, act as allosteric modulators, offering the opportunity to overcome the promiscuity of ligands since they can affect the binding affinity and/or the efficacy of all orthosteric ligands (97). In Ldr4/- mice, TAK779 effectively reduced atherosclerotic lesion formation due to a decrease of Th1 cells in the lesions (81). TAK779 was originally conceived as an inhibitor for human immunodeficiency virus (HIV) entry into host
cells; however, it has little oral bioavailability and its clinical development was discontinued due to local reactions at injection sites which made its management difficult (50).

Maraviroc is the only CCR5 antagonist currently approved by the FDA for the treatment of HIV (58). Maraviroc treatment in ritonavir-induced atherosclerosis in ApoE<sup>-/-</sup> mice was found to reduce atherosclerotic lesion size, to decrease macrophage infiltration, to down regulate the local expression of VCAM1, ICAM-1, CCL2, IL-17A, tumour necrosis factor α and CCL5. Furthermore, in a late phase of spontaneous atherosclerosis in which dyslipidaemia plays the major pathogenic role, maraviroc also inhibited atherosclerotic progression by reducing macrophage infiltration and lowering the expression of adhesion molecules and CCL5 within the atherosclerotic plaques (59).

Another possibility to interfere with the chemokine CCL5 is to disrupt the pro-atherogenic heteromers of CCL5 and CXCL4. Heterodimerisation of CCL5 with CXCL4 enhances monocyte arrest, and attenuation of CCL5-CXCL4 interactions by the peptide antagonist CKEY2 or its mouse ortholog MKEY reduced monocyte chemotaxis in <i>vitro</i> and atherosclerotic lesion in ApoE<sup>-/-</sup> mice, respectively (54). CCL2 and CCR2 play important roles in atherosclerosis progression as shown in both knock-out mice and human studies (98). Therefore, CCR2 antagonists are under investigation of their anti-atherosclerotic potential. MLN-1202 (Millennium Pharmaceuticals, Cambridge, MA, USA) is a humanised monoclonal antibody that binds to CCR2 and thus inhibits CCL2 binding. In a small phase II trial, MLN-1202 improved high-sensitivity C-reactive protein (hsCRP) levels, a surrogate marker of CAD, in patients at high risk for atherosclerosis and that had documented elevations in CRP (75). A small molecule inhibitor of CCR2, CCX-140 (ChemoCentryx, Mountain View, CA, USA), has recently been tested in phase II trials in diabetic nephropathy (99), and it is in pre-clinical testing for treatment of atherosclerosis (6). Propagermanium is a CCR2 antagonist clinically used in Japan for the treatment of chronic hepatitis B (56). Propagermanium attenuates atherosclerosis via the inhibition of macrophage infiltration in ApoE<sup>-/-</sup> mice (44). TLK-19705 (Telik, Palo Alto, CA, USA) is another CCR2 antagonist that has reduced the atherosclerotic lesion size in ApoE<sup>-/-</sup> mice. In addition, TLK-19705 inhibits human CCL2-induced chemotaxis of monocytes/macrophages in a concentration-dependent manner both in <i>vitro</i> and in <i>vivo</i> (56).

Chemokine-binding proteins isolated from parasites have been cloned and expressed, and present potent anti-inflammatory action in animal models (7). Evasin-3, from the saliva of the tick <i>Rhipicephalus sanguineus</i>, presents stringent chemokine selectivity, binding only to CXCL1 and CXCL8 and their murine counterparts (KC and MIP-2, respectively). Evasin-3 demonstrated potent ability to inhibit neutrophil-mediated inflammation in mouse models of antigen-induced arthritis, chemokine-induced neutrophil recruitment to the knee joint (100). In myocardial ischaemia/reperfusion model, Evasin-3 significantly reduced infarct size by preventing CXC chemokine-induced neutrophil recruitment and ROS production (61). Furthermore, Evasin-3 potently inhibited both chemokine bioactivity and related neutrophilic inflammation in mouse models of advanced carotid atherosclerosis. This was followed by the reduction of intraplaque neutrophil and matrix metalloproteinase-9 (MMP-9) content. Conversely, in the ischaemic stroke model, Evasin-3-mediated reduction of ischaemic brain neutrophil infiltration was not associated with any improvements in other clinical and histological outcomes (60). Recently, Evasin-3 and Evasin-4 have been shown to reduce infarct size in mice, which was accompanied by a decrease in post-infarction myocardial leukocyte infiltration, ROS release, and circulating levels of CXCL1 and CCL2. Treatment with Evasin-4, which binds to CCL5 and CCL11, induced a more potent effect, abrogating the inflammation already at one day after ischaemia onset and improving survival at 21-day follow-up (101).

The role of CXCR2 in atherogenesis was firstly investigated in monocyte/macrophages instead of neutrophils, showing a critical role in recruitment and activation of pro-atherosclerotic pathways (102, 103). In 2007, the research group of Weber identified the CXCR2 as one functional receptor of the cytokine macrophage migration inhibitory factor (MIF), a critical trigger for integrin-dependent arrest and migration for both monocytes and T cells (104). In a mouse model of advanced atherosclerosis, only the direct inhibition of MIF, but not of other canonical ligands of CXCR2 was able to induced atherosclerotic plaque regression (104), suggesting a specific pro-atherosclerotic role of the cytokine instead of receptor-triggered pathways. More recently, Soehnlein et al. identified CXCL1-CXCR2 as a key axis in mobilisation of classical monocytes from bone marrow and spleen in hypercholesterolaemic mice (104). Thus, the inhibition of this pathway might represent a promising target for selective medications to reduce atherogenesis and potentially its acute complications. However, recent evidence did not support anti-CXCL1 selective treatments as effective approaches to reduce post-infarction inflammatory response, wound healing and scar formation (101, 105). On the other hand, also MIF blockade has been also shown as detrimental in a mouse model of myocardial ischaemia and reperfusion (106). Thus, additional studies are needed to clarify if selective CXCR2 antagonism might be useful in the clinical setting. To date only two chemokine receptor antagonists have currently achieved the FDA approval: Maraviroc for the treatment of HIV and the low-molecular-weight CXCR4 antagonist Plerixafor (AMD3100) for aiding stem cell mobilisation (58). A common explanation to the lack of success of other chemokine-related trials is represented by the redundancy of chemokine system. In <i>vitro</i>, receptor-ligand interactions are not specific since several chemokines are able to bind to more than one receptor, and few receptors bind only a single ligand. However, that might not be exactly the same case in <i>vivo</i> since receptor and/or ligands production in different cell types are triggered by different transcription factors, in a non-redundant pattern (107). Additionally, the reasons for the failure in most clinical trials of chemokine receptor antagonists could have been inappropriate target selection, as in the case of CCR2 for rheumatoid arthritis, and also insufficient receptor coverage (97).
Targeting inflammatory molecules activating neutrophils, ROS and proteases

Evidence from clinical studies and basic research showed that Leukotriene B4 (LTB4) might be a relevant pro-atherosclerotic mediator in cardiovascular risk subjects and animals models characterised by hypoxic conditions (108, 109). The selective pharmacologic inhibition of interaction between LTB4 and its receptor (i.e. BLT-2) was shown to improve endothelial function and oxidative stress in mice with advanced atherosclerosis (110). However, no significant amelioration in atherosclerotic plaque size was observed (110). In New Zealand White rabbits, treatment with the BLT receptor antagonist BIIL284 for two weeks was able to reduce in-stent intimal hyperplasia and MMP content in carotid arteries as compared to vehicle-treated controls (111). This study showed a potential benefit of LTB4 inhibition in the interventional cardiology domain to potentially reduce the risk of stent restenosis.

In hypoxic ApoE-/- mice with advanced atherosclerosis, the genetic deletion of ApoT1 receptor was associated with reduction in atherosclerotic lesion size as compared to controls (109), suggesting that LTB4 inhibition might be particularly useful in subjects with intermittent hypoxia during sleep, such obstructive sleep apnea syndrome (OSAS). Considering that neutrophils are potently activated and migrate towards LTB4, the selective inhibition of this mediator might be promising as a therapeutic approach targeting neutrophil inflammation (112). Another neutrophil activator is represented by the activated complement fractions, which is particularly increased in heart diseases after an acute myocardial infarction (113). In particular, C3a and C5a bind oxidised low-density lipoproteins (ox-LDL) and co-localise with them within atherosclerotic lesions (114). In aortas of 25-week-old ApoE-/- mice under normal chow diet (characterised by early atherogenesis), C5a expression was found increased as compared to age-matched wild-type mice (115). The chronic inhibition of C5a with the selective antagonist PMX53 in these ApoE-/- mice was shown to abrogate atherosclerotic lesion progression as compared to vehicle-treated animals (115). Unfortunately, the authors did not investigate neutrophil infiltrate in these mouse models. Thus, potential anti-complement treatments to selectively inhibit neutrophil-mediated pro-atherosclerotic activities remain to be elucidated. Considering that increased levels of C3d and neutrophil granule products (i.e. MPO) were recently co-detected in atherosclerotic human aortic valves from autopsy (116), we recommend investigating neutrophil pathophysiological aspects in future studies using treatments targeting complement.

The pharmacological inhibition of neutrophil products (such as ROS or proteases) in atherosclerosis has been strongly suggested as critical therapeutic approach to reduce the risk of plaque rupture. Considering ROS, despite some evidence from animal models of early and advanced atherogenesis (117, 118), in humans it is still not clear in human carotid plaques if the compounds generated from the infiltrated neutrophils might actively contribute to the increase in plaque vulnerability (119). The investigations on anti-oxidant compounds targeting neutrophil ROS remain quite difficult considering that the majority of drugs developed are toxic and non-selective for neutrophil NADPH oxidases. On the other hand, treatments inhibiting matrix metalloproteases (MMPs) released by neutrophils (such as MMP-8 and MMP-9) might be particularly useful to reduce atherosclerotic plaque vulnerability in both humans and mice (45, 120). The enzymes are key players in degradation of the stabilising collagen and extracellular matrix within atherosclerotic plaques. Treatments targeting the endocannabinoid system (particularly targeting cannabinoid type 2 receptor [CB2] activation) were able to reduce both neutrophil and MMP-9 content within atherosclerotic plaques in mice with advanced carotid atherosclerosis (45). On the other hand, in mice deficient for the endocannabinoid degrading enzyme fatty acid amide hydrolase ([FAAH], characterised by with increased levels of endocannabinoids), neutrophils and MMP-9 were increased and nicely co-localised within atherosclerotic plaques (121). Not only MMP-9 expression, but also its activity has been targeted in vitro (122, 123).

Targeting LXR receptors

The two liver X receptors, LXRα and LXRβ, are emerging therapeutic targets for diseases as diverse as lipid disorders, atherosclerosis, chronic inflammation, autoimmunity, cancer and neurodegenerative diseases (124). The potential role of liver X receptors (LXR) pathway on both neutrophil homeostasis and atherosclerotic pathophysiology has been also established. LXRs (LXRα and LXRβ) are members of the nuclear receptor superfamily that regulate both lipid metabolism and inflammatory gene expression (28). LXR signalling contributes to the control of neutrophil homeostasis, regulating the efficient clearance of senescent neutrophils (125). Transplantation of bone marrow from Lxrα-/- and Lxrβ-/- mice into ApoE-/- or Ldlr-/- mice strongly increased atherosclerotic lesion development (126), and overexpression of LXRα led to reduced atherosclerosis in the absence of changes in plasma lipid levels (127). agonists of LXRs have presented anti-atherosclerotic effects in mouse models of atherosclerosis. GW3965 has induced regression of atherosclerotic lesions in Ldlr-/- mice, reducing macrophage content and altering lesions characteristics from vulnerable to stable (62). GW3965 treatment was able to reduce atherosclerotic lesion size in both ApoE-/- and Lxrα-/-/ApoE-/- mice submitted to a protocol of advanced atherogenesis (44). Since Lxrα-/-/ApoE-/- mice were shown to not only exhibit an accelerated atherosclerosis, but also an extreme cholesterol accumulation in peripheral tissues (44), GW3965 treatment might be considered as a particularly promising drug in individuals with advanced atherosclerosis.

Safety concerns such as increased hepatic lipogenesis, leading to hypertriglyceridaemia and liver steatosis, have prevented clinical trials to be performed with LXR agonists. However, it has been suggested that LXR-α activation is mainly responsible for these adverse effects, while LXR-β activation accounts for the anti-atherosclerotic effect. Therefore, specific targeting of LXR-β has been proposed to attain anti-atherosclerotic benefits, while avoiding hepatic lipogenesis and consequent hepatosteatosis (28, 65).
LXR-623 (WAY-252623) is a novel oral active synthetic ligand with preferential potency for LXR-β (63) that has reduced atherosclerosis progression in LDL-/- mice with no increase in hepatic lipogenesis (64). LXR-623 has also reduced the progression of atherosclerosis and induces plaque regression in combination with simvastatin in New Zealand White Rabbits (65).

Conclusion

The understanding of neutrophil-mediated mechanisms involved in atherogenesis opened new therapeutic perspectives interfering either with neutrophil recruitment or their activity. Different strategies directly or indirectly related to neutrophils, such as blocking cell adhesion molecule or chemokine activities, interfering with lipid metabolism are currently evaluated. After promising results in basic research studies, the therapeutic benefits of those selective compounds require to be replicated in humans.

Conflicts of interest

None declared.

References

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