BJCP British Journal of Clinical Pharmacology

DOI:10.1111/j.1365-2125.2012.04211.x

Therapeutic strategies to deplete macrophages in atherosclerotic plaques

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Keywords
adhesion molecules, atherosclerosis, cell death, macrophages, reverse cholesterol transport

Received
5 October 2011

Accepted
31 January 2012

Accepted Article Published Online
6 February 2012

Macrophages can be found in all stages of atherosclerosis and are major contributors of atherosclerotic plaque development, progression and destabilization. Continuous recruitment of monocytes drives this chronic inflammatory disease, which can be intervened by several strategies: reducing the inflammatory stimulus by lowering circulating lipids and promoting cholesterol efflux from plaque, direct and indirect targeting of adhesion molecules and chemokines involved in monocyte adhesion and transmigration and inducing macrophage death in atherosclerotic plaques in combination with anti-inflammatory drugs. This review discusses the outlined strategies to deplete macrophages from atherosclerotic plaques to promote plaque stabilization.

Macrophages in atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial wall and is the foremost cause of death in developed countries. This progressive disease is characterized by a pathological lipid deposition within the vessel wall of medium-sized and large arteries. Over the years, studies have implicated an important role of macrophages in plaque development, progression and destabilization [1–3]. Initially, monocytes selectively bind on adhesion molecules, such as vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and selectins on endothelial cells. These adhesion molecules are induced by mediators associated with risk factors (hypertension, diabetes, hyperlipidaemia, smoking, obesity and inflammation) [4]. Once bound, transmigration of inflammatory cells into the vessel wall is mediated by the endothelial release of chemokines, such as monocyte attractant protein 1 (MCP-1), which binds to its receptor on monocytes [5–7]. Infiltrated monocytes mature into macrophages by macrophage colony-stimulating factor and express scavenger receptors that allow them to engulf modified lipoproteins [7, 8]. Accumulation of lipid-laden macrophages and to a lesser extent T-lymphocytes beneath the endothelium constitutes a fatty streak [2, 9].

The evolution of a fatty streak to an atherosclerotic plaque involves activation of macrophages by T lymphocytes through interferon-γ (IFN-γ) and CD40 [2, 7]. Once macrophages are activated, they produce cytokines, chemokines and growth factors, promoting smooth muscle cell (SMC) migration and proliferation in the intimal layer, even though they also induce focal necrosis [10]. The collagen-synthesizing SMCs form a fibrous cap that surrounds the lipid and necrotic core. The fibrous cap prevents exposition of the thrombogenic lipid core to arterial blood and provides mechanical strength and stability to the plaque [11]. Accumulation of activated inflammatory cells, such as macrophages and T lymphocytes, in the shoulder region of the plaque amplifies the inflammatory process by the release of pro-inflammatory cytokines. Macrophages also release matrix metalloproteinases (MMPs) [12, 13] that degrade the extracellular matrix, and excessive amounts of nitric oxide (NO) [14] that induce SMC apoptosis. All these events weaken the fibrous cap and render the plaque rupture-prone [15]. Another mechanism that contributes to plaque destabilization is apoptosis, which occurs more frequently in regions with a high density of macrophages and foam cells, and is also strongly related to macrophage infiltration [16]. Vulnerable plaques typically display a large lipid core, a thin fibrous cap, a reduced number of SMCs and large numbers of macrophages and T cells [17, 18].

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Clinical manifestations of atherosclerosis are determined by cellular plaque composition and risk of thrombus formation. Stenotic fibrous lesions without thrombus risk restrict blood flow and provoke symptoms such as stable angina. Acute coronary syndromes, such as unstable angina and acute myocardial infarction, are caused by plaque rupture or endothelial erosion with mural thrombus formation [19]. Histopathological comparison between symptomatic and asymptomatic endarterectomy specimens reveals that the number of inflammatory cells is significantly increased in symptomatic plaques *vs*. asymptomatic ones [20–23]. Removal of macrophages from the plaque could attenuate the inflammatory response and subsequent plaque destabilization. This review focuses on current and possible new therapeutic targets and drugs that provoke macrophage elimination in different stages of atherosclerosis, either by reducing lipids in the circulation or from the plaque, decreasing monocyte recruitment, inhibiting macrophage activation or inducing macrophage death in atherosclerotic plaques.

Systemic LDL lowering

Statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors block the conversion of acetoacetyl-CoA and acetyl-CoA to mevalonate, a key intermediate in cholesterol synthesis in liver and other tissues (Figure 1 (1)). The resulting decrease in hepatic cholesterol content leads to a compensatory increase in hepatic low density lipoprotein (LDL) receptors, which clear LDL cholesterol from the circulation [24]. Accordingly, statins reduce the incidence of acute coronary syndromes in primary and secondary prevention in patients with high cholesterol concentrations as well as those at increased risk for cardiovascular events, independent of their cholesterol concentrations, suggesting that they also exhibit beneficial effects beyond lipid lowering (pleiotropic effects) [25–30]. A further reduction of major vascular events is safely produced by more intensive statin therapy (40–80 mg statin compared with 20–40 mg statin daily) [31]. Despite the efficacy of statins, a subpopulation of patients does not benefit from current lipid lowering strategies [32]. Therefore, overall morbidity and mortality from atherosclerotic vascular disease remain substantial and development of new strategies is still essential to reduce disease burden.

Other cholesterol reducing agents, such as ezetimibe, have not yet been proven to reduce the risk of cardiovascular events [33–36]. Ezetimibe inhibits intestinal cholesterol absorption via inhibition of the Niemann-Pick C1-Like 1 (NPC1L1) sterol transporter leading to a compensatory upregulation of hepatic LDL receptors and clearance of circulating LDL (Figure 1 (2)) [37].

Experimental studies

Preclinical studies demonstrate that the lipid lowering properties of ezetimibe as monotherapy or on top of statin therapy inhibit atherogenesis or reduce plaque burden [38–42].

Clinical studies

Clinical trials confirm the LDL cholesterol lowering effects of ezetimibe added to statin treatment, but whether ezetimibe enhances plaque regression and reduces clinical events has been controversial [34, 35, 43, 44]. The ongoing IMPROVE-IT trial attempts to detect benefits of ezetimibe as a second LDL cholesterol lowering drug added to aggressive statin therapy [45]. However, a recent study with statin-naïve patients shows no change in plaque volume between patients treated with simvastatin or simvastatin plus ezetimibe despite greater LDL cholesterol lowering in the latter group [46]. Moreover, adding ezetimibe to patients previously on statin therapy reduces LDL cholesterol but paradoxically induces plaque progression [46].

Interestingly, proprotein convertase subtilisin kexin 9 (PCSK9) regulates circulating LDL concentrations by inhibiting hepatic LDL receptor-mediated LDL uptake. In humans and mice, loss of function mutations in the gene encoding PCSK9 is associated with lifelong low LDL concentrations [47, 48]. Clinical trials with anti-PCSK9 antibody are ongoing [49].

Increased cholesterol efflux from macrophages

Recent studies focus on pathways to reverse cholesterol transport from atherosclerotic plaques to the liver for excretion into bile and faeces, whereby high density lipoprotein (HDL) and its major apolipoprotein, apolipoprotein A-I (apoA-I) play key roles in these processes. HDL is formed as discoidal pre-β_1-HDL by addition of phospholipid to lipid poor apoA-I, which is synthesized in liver and intestines. These particles take up free cholesterol from cells through adenosine triphosphate-binding cassette transporter-A1 (ABCA1). The free cholesterol is converted to cholesteryl ester (CE) by lecithin cholesterol acyltransferase (LCAT) to form spherical α-HDL particles, which can take up more free cholesterol through ABCG1 and ABCG4 that in turn is converted to CE by LCAT. The CEs are returned to the liver either directly via scavenger receptor B1 or via LDL receptors after transfer to apolipoprotein B lipoproteins in exchange for triglycerides by the cholesteryl ester transfer protein (CETP) (Figure 1). Studies have shown a strong inverse correlation between plasma HDL concentrations and atherosclerotic cardiovascular risk in humans [50, 51]. Several strategies have been explored to increase cholesterol efflux in order to stabilize atherosclerotic plaques including (i) raising HDL concentrations, (ii) inhibiting CETP, (iii) promoting expression of cholesterol transporters and (iv) other mechanisms (e.g. probucol).

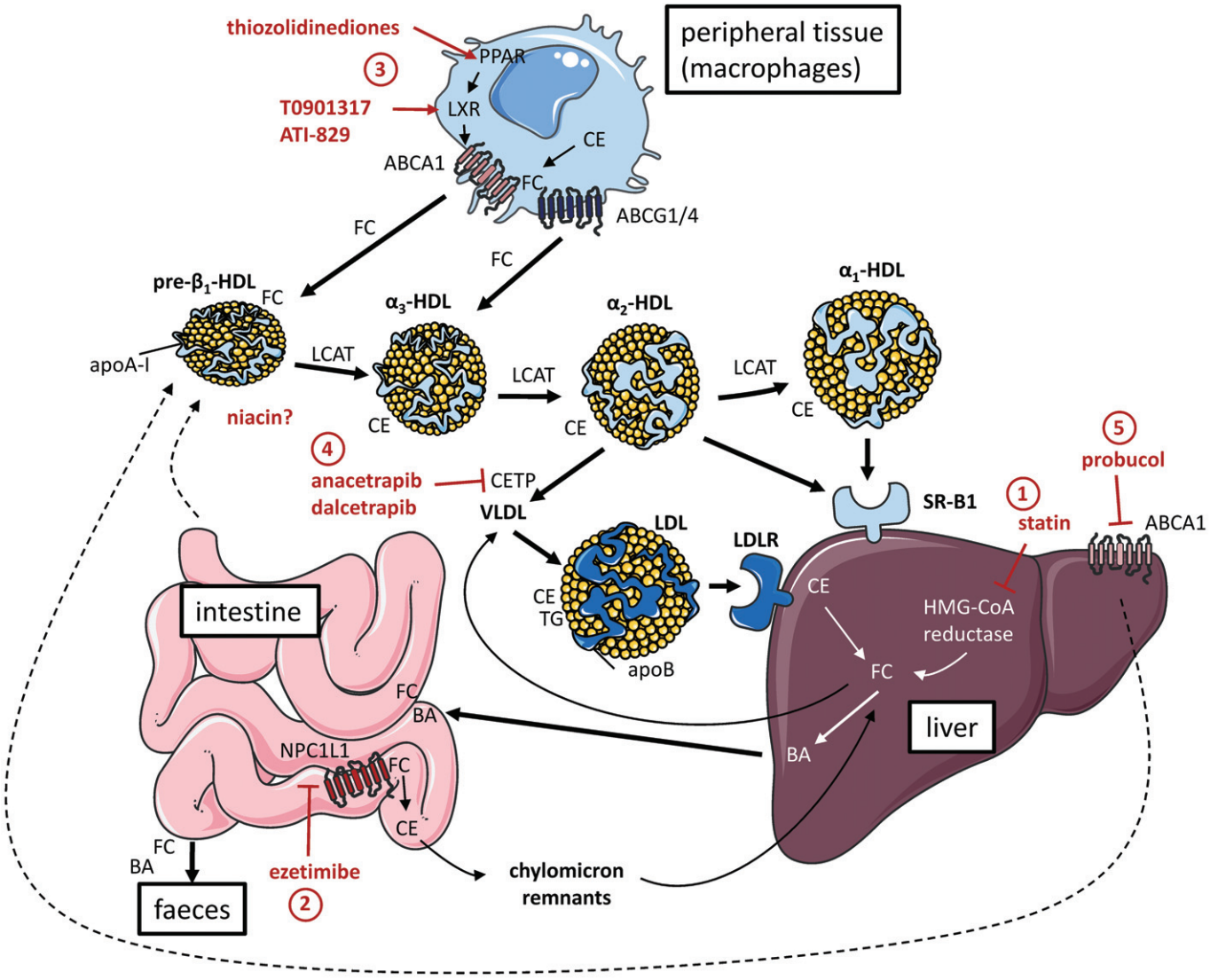

Figure 1

Schematic overview of pharmacological targets involved in cholesterol uptake (solid line) and reverse cholesterol transport (bold line). Cholesterol is synthesized in the liver via HMG-CoA reductase, a pharmacological target of statins (1). Ezetimibe inhibits cholesterol uptake in the intestines by blocking sterol transporter NPC1L1 (2). Chylomicrons transport dietary triglycerides and cholesterol from the gastrointestinal tract to the peripheral tissues, whereby chylomicron remnants are taken up by the liver. Lipid-poor pre-β_1-HDL (origin indicated with dashed line) promotes efflux of free cholesterol of macrophages via ABCA1. The expression of this cholesterol transporter is up-regulated by PPAR ligands thiozolidinediones and LXR agonists T0901317 and ATI-829 (3). In α-HDL particles, cholesterol is converted to cholesteryl esters by LCAT and further cholesterol efflux from macrophages is promoted by ABCG1 and ABCG4. Cholesterol is returned to the liver by SR-B1 or transferred to apoB lipoproteins VLDL and LDL via CETP. HDL concentrations are effectively raised by CETP inhibitors, torcetrapib and anacetrapib, and niacin (4). Finally, hepatic cholesterol is oxidized to bile acids or secreted in the bile unaltered where it is secreted in the faeces unless it is reabsorbed by the intestine. Probucol promotes cholesterol efflux by enhancing faecal excretion of HDL-derived cholesterol (5). ABCA1, adenosine triphosphate-binding cassette transporter-A1; apoA-I, apolipoprotein A-I; BA, bile acids; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LCAT, lecithin cholesterolacyltransferase; LDL, low density lipoprotein; LXR, liver X receptors; NPC1L1, Niemann-Pick C1-Like 1; PPAR, peroxisome proliferator-activated receptor; SR-B1, scavenger receptor B1; TG, triglycerides; VLDL, very low density lipoprotein

HDL infusion/raising

Experimental studies Animal studies support the concept of raising HDL by administering recombinant HDL and apoA-I Milano or overexpressing apoA-I for inhibition of atherosclerosis and show significant reductions in lesion and macrophage area by mobilizing tissue cholesterol and by reducing plaque lipids [52–55].

Clinical studies Short term infusion of reconstituted HDL containing recombinant apoA-I Milano in humans induces a substantial regression of coronary atherosclerosis after only 5 weeks of treatment [56]. Despite the positive outcome, the small sample size and dissimilarities in plaque burden at baseline are limitations of this study. In another investigation, infusion of reconstituted HDL

containing plasma-derived apoA-I significantly improves plaque characteristics as demonstrated by intravascular ultrasound, but there is no significant difference in lesion size [57]. This study was hampered by a small sample size and presence of liver abnormalities in the high dose group. In the search for HDL-raising drugs, fibrates, such as gemfibrozil, demonstrate improved cardiovascular outcomes in human clinical trials [58, 59], whereas subsequent trials with bezafibrate and fenofibrate have disappointing results with regard to amelioration in cardiovascular mortality outcomes [60–62]. The ARBITER-6-HALTS trial shows that HDL-raising by niacin as add-on therapy in patients receiving long term statin therapy is superior to ezetimibe with respect to changes in carotid intima-media thickness and the incidence of major adverse cardiovascular events [36]. These beneficial effects of niacin along with statin therapy on atherosclerosis development are further investigated in the AIM-HIGH study [63], but this study was prematurely discontinued in 2011 due to lack of benefits in reducing cardiovascular events of combined treatment and due to unexpectedly increased ischemic stroke rates.

CETP inhibition

CETP tends to influence negatively the reverse cholesterol transport pathway by lowering HDL and consequently scavenging of cholesterol from tissues and macrophages declines (Figure 1).

Clinical studies CETP inhibitors are capable of producing higher HDL concentrations than niacin, but clinical studies with the CETP inhibitor torcetrapib dramatically failed due to production of dysfunctional HDL and off-target toxicity [64]. Clinical trials with other CETP inhibitors, anacetrapib and dalcetrapib, are ongoing and these drugs are so far well tolerated (Figure 1 (4)) [65, 66]. Nevertheless, increased HDL concentrations as such are not a sensitive indicator of removal of lesional cholesterol to the liver, but rather the distribution of functional HDL particles. Recently, it has been shown that sera of patients with similar HDL concentrations have different cholesterol efflux capacities in macrophages because of differences in concentration of pre-β_1-HDL [67].

Up-regulation of cholesterol transporters

Besides the composition and concentration of extracellular cholesterol acceptors, the cholesterol efflux capacity is also dependent on the level of expression of cholesterol transporters ABCA1 and ABCG1. Expression of cholesterol transporters can be modulated by liver X receptors (LXR), which are ligand-activated transcription factors that regulate cholesterol transport and lipid metabolism in liver, intestines and macrophages in response to oxysterols (Figure 1 (3)). Furthermore, synthetic ligands of peroxisome proliferator-activated receptor (PPAR), fibrates (PPARα) and thiozolidinediones (PPARγ), are clinically used for treatment of dyslipidaemia and type II diabetes, respectively. In addition, these drugs are thought to have anti-atherogenic functions due to cholesterol efflux.

Experimental studies Bone marrow transplantations of ABCA1$^{-/-}$ and ABCG1$^{-/-}$ mice in hypercholesterolaemic mice demonstrate a cooperative role of ABCA1 and ABCG1 in macrophage reverse cholesterol transport since deficiency of both transporters in macrophages increases foam cell formation and accelerates atherosclerosis to a greater extent than bone marrow from either single knockout alone [68]. The natural LXR agonist 24(S), 25-epoxycholesterol induces selective upregulation of ABCA1, ABCG1 and apoE in macrophages thereby affecting the macrophage cholesterol transport [69]. Synthetic LXR agonists, such as T0901317, increase ABCA1 and ABCG1 expression, inhibit plaque progression, induce regression of established lesions and alter plaque composition favouring plaque stability, i.e. reducing macrophage content and increasing collagen content [70]. Moreover, studies in macrophage-selective LXR-deficient hypercholesterolaemic mice confirm that macrophage LXR expression is necessary for the atheroprotective actions of an LXR agonist [70]. The major drawback of these drugs is the effect on the liver resulting in severe hypertriglyceridaemia and hepatic steatosis. The tissue selective LXR agonist ATI-829 has overcome these side effects and is a potent inducer of ABCA1 expression in macrophages, supporting the possibility of using LXR agonists for the treatment of atherosclerosis [71]. PPAR activation augments cholesterol efflux from macrophages by controlling the transcriptional activity of genes regulating LXR, which induces ABCA1 expression, and raises HDL concentrations by stimulating hepatic apoA-I expression (Figure 1 (3)) [72, 73]. Bone marrow transplantation of PPAR-$\gamma^{-/-}$ into atherosclerotic mice results in a significant increase in atherosclerosis. PPAR-γ agonists in atherosclerotic mice attenuate atherosclerosis [74] and PPAR-α activation by fenofibrate in hypercholesterolaemic rabbits is associated with improvements in lipoprotein levels by decreasing triglyceride concentrations, plaque regression and changes in plaque composition towards a stable plaque phenotype [75].

Clinical studies In a small human study, patients treated with the PPAR-γ ligand pioglitazone show a significant enhanced cholesterol efflux capacity [76]. Although cholesterol efflux from macrophages represents only a small fraction of reverse transport cholesterol, it is probably the component most relevant to atheroprotection [77].

Other mechanisms

Probucol is a potent anti-oxidant drug that has been used in the past for the treatment and prevention of cardiovascular events, but was withdrawn from the European and American market due to its effects in reducing HDL con-

centrations and possible QT interval prolongation. Nevertheless, recent studies show that probucol promotes reverse cholesterol transport [78, 79].

Experimental studies In animal models probucol reduces atherosclerosis despite reducing HDL cholesterol concentrations [80, 81]. The anti-atherogenic effects of probucol are attributed to the enhancement of macrophage reverse cholesterol transport and faecal excretion of HDL-derived cholesterol. Probucol inhibits hepatic ABCA1, thereby reducing HDL concentrations but promoting reverse cholesterol transport without the involvement of scavenger receptor B1 (Figure 1 (5)) [78]. The mechanism of hepatic ABCA1 inhibition remains to be investigated but it is possible that probucol inhibits ABCA1 translocation to the plasma membrane, as shown in cultured macrophages [82].

Clinical studies A recent study in Japan shows a reduction in cardiovascular risk with long term probucol treatment in patients with familial hypercholesterolaemia [79]. In a very small study of 10 hypercholesterolaemic patients it was shown that probucol increases CETP activity, suggesting that reduced HDL concentrations are a direct consequence of CETP stimulation [83].

Decreased monocyte recruitment

Decreased monocyte recruitment at the level of adhesion molecules

Adhesion molecules (selectins and integrins) are locally up-regulated on the endothelium and leucocytes by cytokines due to risk factors like oxLDL, smoking, hypertension and diabetes, and are modulated by haemodynamic stress including arterial pressure and shear stress. E- and P-selectins mediate the initial monocyte tethering and rolling along endothelium, whereas vascular adhesion molecules VCAM-1 and ICAM-1 are involved in leucocyte adhesion by binding to the activated integrins on leucocytes, very late antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1), respectively.

Experimental studies Studies with atherosclerotic knockout mice have shown that altering the expression of E-selectin, P-selectin, VCAM-1 and ICAM-1 reduces lesion size as well as the number of macrophages in these lesions without affecting lipoprotein profiles and numbers of circulating monocytes (Table 1) [84–89]. Moreover, blocking antibodies towards leucocyte integrin VLA-4 or towards their endothelial counterparts VCAM-1 and ICAM-1 is effective in reducing macrophage recruitment to atherosclerotic plaques in mice [90–92]. Evidence for VCAM-1 expression on the endothelium preceding atherosclerotic plaque formation and most pronounced inhibition of mononuclear cell accumulation and reduction in lesions by VCAM-1 blockade further imply a dominant role of VCAM-1 in monocyte recruitment as well as in lesion initiation, progression and expansion.

Clinical studies Despite the promising results in animal studies, clinical anti-VCAM-1 therapy directed at lymphocyte trafficking with AGI-1067 in patients with acute coronary syndrome shows no significant cardiovascular benefits [93]. Clinical trials with VCAM-1 inhibitors, CAM741 and HUN-7293, are ongoing to assess the possible beneficial effects.

The anti-inflammatory effects of statins are in part due to inhibition of formation of mevalonate, a precursor of isoprenoids that prenylate small signal transducing guanosine triphosphatases Ras, Rho, Rab and Rae. Consequently, activation of nuclear factor-κB, expression of adhesion molecules and vascular inflammation is inhibited [94, 95]. Other anti-atherogenic effects of statins include direct binding of these drugs to the pro-inflammatory mediator LFA-1 on leucocytes, thereby blocking ICAM-1 for leucocyte adhesion [96]. Thus, statins may also inhibit leucocyte recruitment in atherosclerotic plaques by an HMG-CoA independent pathway.

Decreased monocyte recruitment at the level of junctional proteins

Transmigration of leucocytes across endothelial cells, also known as diapedesis, is determined by junctional proteins, such as junctional adhesional molecules (JAM) and connexins.

Experimental studies Enhanced expression of JAM-A is already present in the early stages of atherosclerosis in the endothelium of apoE-deficient mice on a high cholesterol diet, and the soluble form of JAM-A significantly reduces mononuclear cell recruitment to *ex vivo* perfused atherosclerotic carotid arteries [97]. Genetic deletion of JAM-A in apoE-deficient mice corroborates that JAM-A is implicated in leucocyte recruitment since JAM-A deficiency is associated with a decrease in macrophage content without altering the SMC content and endothelial cell recovery after wire injury of carotid arteries [98]. Moreover, JAM-A deficiency diminishes the deposited delivery of the chemokine regulated upon activation, normal T cell expressed and secreted (RANTES) on activated endothelium and subsequent monocyte recruitment and plaque formation [98]. Connexins are proteins forming gap junctions that play a role in direct intercellular communication whereby connexin 43 is upregulated in atherosclerotic lesions. Reducing connexin 43 by a genetic or pharmacological approach using statins in hypercholesterolaemic mice results in a decreased neointimal formation with concurrent reduced number of atheroma-associated inflammatory cells and increased thickness of the fibrous cap covering the lesions [99]. This suggests that gap junction communication between endothelial cells and leucocytes

Table 1

Molecular targets affecting monocyte recruitment and macrophage accumulation in atherosclerotic plaques

Molecular target	Mechanism	Model	Effect	Reference
P-selectin	Tethering and rolling	P-selectin$^{-/-}$ on C57BL/6 background on western diet or apoE$^{-/-}$ double knock out	Reduces macrophage number, reduces lesion size at initial stages of atherosclerosis and less development to advanced lesions	85–87
		P-selectin$^{-/-}$ on C57BL/6 background	Reduces neointimal formation without infiltration of inflammatory cells	84, 89
VCAM-1	Adhesion	VCAM-1$^{-/-}$ on LDLR$^{-/-}$ or apoE$^{-/-}$ background	Reduces lesion size and less adherence of mononuclear cells at initial stages of atherosclerosis	88, 169
		Monoclonal antibody against VCAM-1	Reduces monocyte infiltration and neointimal formation	91
ICAM-1	Adhesion	ICAM-1$^{-/-}$ on C57BL/6 background on western diet	Slightly reduces lesion size at initial stages of atherosclerosis	85
		Monoclonal antibody against ICAM-1 in apoE$^{-/-}$ mice	Reduces macrophage homing by 65%	90
VLA-4	Adhesion	Monoclonal antibody against VLA-4 in apoE$^{-/-}$ mice	Reduces macrophage recruitment by 48% and reduces neointimal formation by 72%	92
		Monoclonal antibody against VLA-4 in apoE$^{-/-}$ mice	Reduces macrophage homing by 75%	90
JAM-A	Transmigration	Ex vivo perfused carotid arteries by soluble JAM-A in apoE$^{-/-}$ mice on western diet	Inhibits mononuclear cell recruitment upon pretreatment	97
		JAM-A$^{-/-}$ on apoE$^{-/-}$ mice background on western diet	Decreases macrophage content and neointimal formation after wire injury	98
Connexin 43	Transmigration	Cx43 $^{\pm}$ on LDLR$^{-/-}$ mice background on western diet	Decreases development of atherosclerosis with concurrent decrease in macrophage number	99
CCL2(MCP-1)/ CCR2	Chemotaxis	CCL2$^{-/-}$ on LDLR$^{-/-}$ mice background on western diet	Reduces macrophage recruitment by 54%	5
		CCR2$^{-/-}$ on apoE$^{-/-}$ mice background	Reduces development of atherosclerosis from fatty streak to more advanced lesions	101
CCL5(RANTES)/ CCR5	Chemotaxis	Receptor antagonist Met-RANTES in LDLR$^{-/-}$ mice on western diet	Reduces macrophage content by 43%	105
		Mutant [^{44}AANA^{47}]-RANTES in LDLR$^{-/-}$ mice on western diet	Limits plaque formation and reduces macrophage infiltration	106
CX3CL1(fractal-kine)/CX3CR1	Chemotaxis	CX3CL1$^{-/-}$on apoE$^{-/-}$ or LDLR$^{-/-}$ background	Reduces accumulation of macrophages and dramatically reduces lesion area in brachiocephalic artery in apoE$^{-/-}$ mice as compared with aortic root and LDLR$^{-/-}$ mice	104
		CX3CR1$^{-/-}$ and apoE$^{-/-}$ double knockout mice on western diet	Decreases atherosclerotic lesion formation and macrophage infiltration by 40%	103
		CX3CR1$^{-/-}$ and apoE$^{-/-}$ double knockout mice on chow diet	Decreases atherosclerotic lesion formation and macrophage infiltration by 50%	102
MIF	Arrest and migration	Monoclonal antibody against MIF in apoE$^{-/-}$ mice on western diet	Reduces neointimal macrophage infiltration after wire injury and inhibits transformation of macrophages into foam cells	115
		Monoclonal antibody against MIF in apoE$^{-/-}$ mice on chow diet	Strongly reduces intimal macrophage content and inflammatory mediators	116

promotes leucocyte extravasation and may represent a therapeutic target for atherosclerosis treatment (Table 1).

Decreased monocyte recruitment at the level of chemokines

Chemokines are chemotactic proteins that are secreted by SMCs and endothelial cells in response to pro-inflammatory stimuli. These proteins interact with cell surface receptors on leucocytes and, to a lesser extent, with glycosylaminoglycans of the extracellular matrix and endothelial surfaces.

Experimental studies Several chemokines and their receptors have been identified to take part actively in atherogenesis, including CCL2(MCP-1)/CCR2, CCL5(RANTES)/CCR5 and CX3CL1 (fractalkine)/CX3CR1 (Table 1) [100]. Genetic deletion of these ligands or their receptors drastically reduces monocyte recruitment and development of diet-induced atherosclerosis [5,101–106]. Simultaneously targeting three chemokine/chemokine receptor pairs almost abolishes atherosclerosis [107]. *In vivo* antagonism of RANTES, either by the receptor antagonist Met-RANTES or by mutant [^{44}AANA^{47}]-RANTES that impairs essential chemokine binding and oligomerization by glycosylaminoglycans, reduces the progression of established atherosclerosis in LDLR$^{-/-}$ mice [105, 106]. Both treatments result in reduction of lesion size and a more stable plaque phenotype via direct inhibition of leucocyte infiltration and subsequent reduction of MMP-9. Furthermore, the increased SMC content is associated with higher amounts of intersti-

tial collagen within lesions. Given the complexity of the C-C chemokine system, blocking one chemokine/chemokine receptor pair might affect other pairs. For example, oligomerization of RANTES on glycosylaminoglycans is required for its *in vivo* function, but also for CCR1-mediated leucocyte adhesion and activation [108]. Although CCL5/RANTES binds to CCR5, which is expressed at high levels on T cells, Met-RANTES binds to CCR5 and CCR1, the latter being strongly expressed on macrophages [105].

Clinical studies In patients with increased cardiovascular risk a phase II clinical trial with MLN1202, a human monoclonal antibody towards CCR2, provides evidence that CCR2 antagonism reduces C-reactive protein concentrations, a biomarker of inflammation associated with coronary artery disease. However, MLN1202 administration results in an increase in serum MCP-1 concentrations and a decrease in circulating monocytes due to a decreased clearance by CCR2 and decreased emigration of inflammatory monocytes from the bone marrow by CCR2, respectively [109, 110]. Consequently, reduced plaque progression by CCR2 inhibition is partly attributed to direct inhibition of monocyte recruitment and partly to decreased blood monocyte count, which might increase infection risk.

Decreased monocyte recruitment at the level of the macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) is a cytokine with potent inflammatory functions and is highly upregulated in atherosclerotic plaques, especially at shoulder regions where most macrophages and foam cells reside.

Experimental studies MIF affects monocyte adhesion, migration and activation by upregulating adhesion molecules VCAM-1 and ICAM-1 [111], influencing chemotaxis via binding with its receptors CXCR2, CXCR4 and CD74 [112], and regulating innate immune responses via Toll-like receptor (TLR) 4 [113]. This evidence supporting the role of MIF in atherosclerosis development has encouraged the exploration of MIF inhibition as a potential therapeutic strategy. MIF-deficient mice or treatment with MIF-antibody foster changes in plaque biology towards stabilized atherosclerotic plaques by reducing macrophage and foam cell number and by increasing SMC and collagen content [114–116].

Inhibited monocyte activation by platelets

Activated platelets release chemokines, such as RANTES and platelet factor 4, and interact with monocytes through ligation of various adhesion molecules, including P-selectin, CD40L and triggering receptor expressed on myeloid cells 1 (TREM-1) ligand [117–121]. Platelet-monocyte interaction induces activation of monocytes resulting in an increase in monocyte adhesive capacity by upregulation of β-integrins and secretion of pro-inflammatory cytokines, chemokines and tissue factor [122–124]. Furthermore, platelet-monocyte complexes tether and roll on endothelial cells with a very high avidity thereby enhancing endothelial activation and further monocyte recruitment [125, 126]. Accordingly, inhibition of platelet activation might reduce the number of plaque macrophages.

Experimental studies

Administration of the antiplatelet drug aspirin to hypercholesterolaemic mice reduces the development and progression of atherosclerotic plaques with an increase in SMCs and a decrease in macrophages [127–129]. The ADP $P2Y_{12}$ receptor antagonist, clopidrogrel, inhibits platelet activation and aggregation, and significantly reduces plaque size and induces a stable plaque phenotype in atherosclerotic mice [130]. Moreover, clopidogrel decreases the expression of P-selectin and CD40L on platelets and, therefore, reduces platelet-leucocyte interaction and platelet-dependent leucocyte activation [131–133].

Inhibited macrophage activation by T lymphocytes

Inhibited macrophage activation at the level of interferon-γ

The most important signal for macrophage activation is IFN-γ, a cytokine with predominantly pro-atherogenic properties, which is highly expressed in T lymphocytes of atherosclerotic plaques. IFN-γ mediates differentiation of monocytes into macrophages, pro-inflammatory cytokine release from macrophages and T cells, recruitment of leucocytes through up-regulation of chemokines and adhesion molecules, and foam cell formation through up-regulation of scavenger receptors.

Experimental studies Exogenously administered IFN-γ increases the number of T cells and MHC II-positive cells, including macrophages and dendritic cells, in the significantly expanded lesions [134]. In contrast, genetic ablation of IFN-γ or its receptor in atherosclerotic mice reduces lesions together with cellularity but increases extracellular collagen content [135]. Inhibition of IFN-γ function by gene transfer of a soluble mutant IFN-γ receptor not only prevents plaque progression but also stabilizes advanced plaques by interfering with plaque composition and inflammation [136]. The phenotypic shift towards stabilization involves decreased expression of cytokines, chemokines, adhesion molecules, MMPs and CD40. Moreover, statins and agonists of nuclear receptors, PPAR and LXR, partly exert their anti-inflammatory effects by limiting production of pro-inflammatory cytokines,

such as IFN-γ. These studies provide evidence that IFN-γ is a potential therapeutic target involved in early and late development of atherosclerosis as well as in plaque destabilization, but systemically inhibiting IFN-γ limits its clinical use due to impaired immune surveillance and function.

Inhibited macrophage activation at the level of the CD40 ligand

Another signal for macrophage activation is the CD40 ligand (CD40L) expressed on the majority of immune cells, including lymphocytes, monocytes, dendritic cells, neutrophils and mast cells, which binds CD40 on the macrophage cell surface, but it is also present on endothelial cells and SMCs. Activation of CD40-CD40L signalling in atherosclerosis mediates various functions including expression of the pro-inflammatory cytokines IL-6 and TNF-α, the pro-atherogenic chemokines CCL2, CCL5 and IL-8, MMPs, procoagulants, and the adhesion molecules VCAM-1, ICAM-1 and E-selectin. These downstream effects are associated with monocyte recruitment, inflammation, expansion of the lipid core, degradation of the fibrous cap and risk for thrombosis. Accordingly, interruption of CD40-CD40L signalling might reduce atherosclerosis and its complications.

Experimental studies Atherosclerotic plaques of $CD40L^{-/-}$ $apoE^{-/-}$ mice show a decrease in plaque area, lipids, macrophages and T lymphocytes and an increase in collagen content [137]. Administration of anti-CD40L antibody in $apoE^{-/-}$ and $LDLR^{-/-}$ mice changes plaque phenotype into a lipid- and macrophage-poor collagen-rich stable plaque. Most profound effects are present in advanced lesions in the delayed treatment group indicating the important role of the CD40-CD40L pathway in late atherosclerotic plaques [138, 139]. Furthermore, deficiency of CD40-tumour necrosis factor receptor-associated factor 6 (CD40-TRAF6) signalling in atherosclerotic mice abolishes atherosclerosis, impairs recruitment of monocytes to the arterial wall and polarizes macrophages towards an anti-inflammatory phenotype [140]. However, systemic modulation of the immune system compromises host defence. Therefore, cell-type specific targeting of the CD40-CD40L pathway is under extensive investigation to overcome this adverse effect.

Selective induction of macrophage death in atherosclerotic plaques

Pharmacological depletion of macrophages via selective induction of cell death (apoptosis, autophagy) may be a promising alternative strategy to alter plaque composition in order to increase plaque stability [141]. The main challenges of this approach are (i) to initiate macrophage death in a selective way, without inducing inflammation and affecting other cell types in the plaque, in particular SMCs and endothelial cells and (ii) local application of the drug to avoid systemic loss of macrophages. Moreover, the drug-induced type of cell death might determine possible complications. Apoptosis is a type of cell death characterized by caspase activation leading to chromatin condensation, nucleosomal DNA degradation, budding of apoptotic bodies and exposure of signalling molecules targeting for its phagocytosis. Engulfment of apoptotic bodies by phagocytic cells has been considered immunologically silent, but inefficient removal of apoptotic cells may lead to induction of postapoptotic necrosis and inflammatory responses [142]. Therefore, the complications of macrophage apoptosis depend on efficiency of apoptotic cell phagocytosis, which is defective in advanced atherosclerotic lesions further promoting plaque instability [143]. Autophagy features sequestration of cytoplasmic material within double-membrane vacuoles, called autophagosomes, that mature into degrading autolysosomes after fusion with lysosomes. During autophagy, cells progressively digest the cytoplasmic content so that activation of (post-)inflammatory responses is believed to be limited. Moreover, autophagy in macrophage foam cells may enhance cholesterol efflux by delivering lipid droplets to the lysosomes where free cholesterol is generated for ABCA1-dependent-efflux [144, 145]. Therefore, induction of macrophage autophagy may have several beneficial effects towards plaque stabilization [146].

Experimental studies

By using drug-containing liposomes, cytotoxic drugs such as clodronate can be intracellularly delivered into macrophages via phagocytosis, and these liposomes are not cytotoxic to nonphagocytic cells [147]. The intracellularly metabolized clodronate inhibits mitochondrial oxygen consumption leading to apoptosis (Figure 2 (A)). This macrophage 'suicide' approach has been successful in reducing neointimal formation by reducing macrophages in vascular injury models in mice, rats and rabbits [148, 149]. However, the systemic administration of clodronate-containing liposomes also reduces blood monocytes, which increases the risk of immunosuppression and infection. Interestingly, this adverse effect is not observed when recombinant tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is systemically used in diabetic $apoE^{-/-}$ mice. TRAIL induces apoptosis in infiltrating macrophages in atherosclerotic plaques, but not in circulating macrophages, and markedly attenuates atherosclerosis development (Figure 2 (C)) [150]. The stabilizing effects of TRAIL are also attributed to preservation of endothelial cell coverage and the enrichment of SMCs in the fibrous cap within atherosclerotic plaques. Therefore, TRAIL might represent a promising therapeutic in atherosclerosis treatment. Moreover, serum concentrations of soluble TRAIL are significantly lower in patients with cardiovascular disease, further supporting a protective role of TRAIL in atherosclerosis [151].

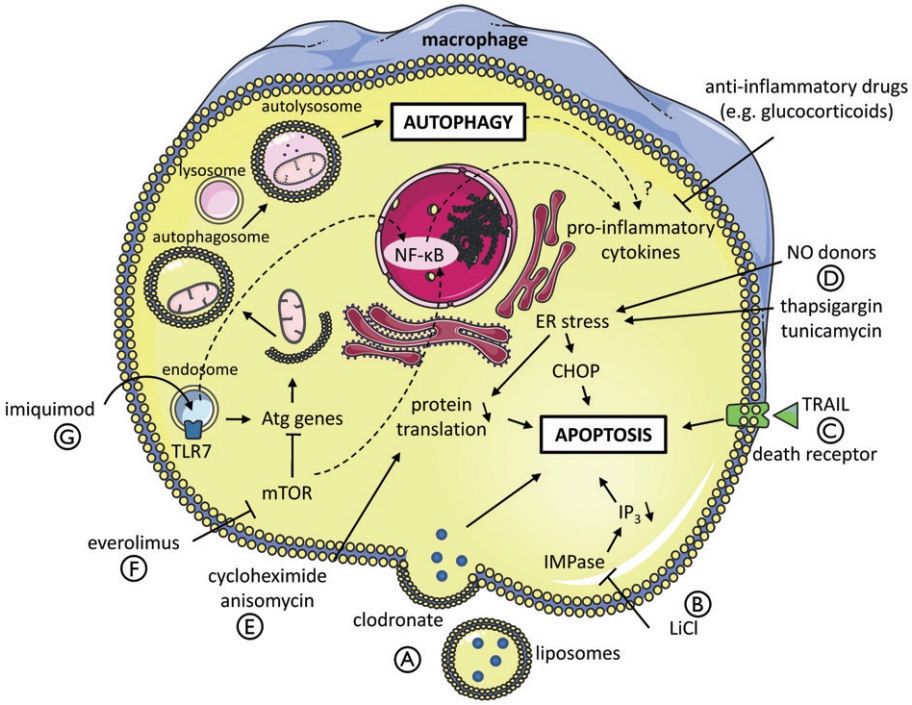

Figure 2

Schematic overview of targets to induce selective macrophage death. Clodronate-containing liposomes are ingested by phagocytes and the released clodronate induces apoptosis (A). Interfering with phosphatidylinositol cell signalling through inhibition of IMPase by lithium chloride leads to decreased free inositol. The subsequent decrease in IP_3 concentrations triggers apoptotic cell death (B). Systemic administration of recombinant TRAIL induces apoptosis via its death receptor in infiltrating monocytes in atherosclerotic plaques but not in circulating monocytes (C). Treatment of atherosclerotic plaques with an NO donor clears macrophages via apoptosis. Induction of macrophage apoptosis is based on inhibition of *de novo* protein synthesis and induction of ER stress (CHOP expression). ER stress inducers, thapsigargin and tunicamycin, also initiate selective macrophage apoptosis (D). Protein translation inhibitors, cycloheximide and anisomycin, selectively clear macrophages from atherosclerotic plaques via apoptosis (E). mTOR inhibitor, everolimus, induces autophagic cell death in macrophages via activation of Atg genes that attribute to autophagosome and ultimately autolysosome formation (F). Stimulation of the endosomal receptor TLR7 with imiquimod initiates autophagy exclusively in macrophages (G). TLR7 stimulation by imiquimod, as well as mTOR inhibition by everolimus, leads to the release of pro-inflammatory cytokines that can be abolished by adding anti-inflammatory drugs, such as glucocorticoids. CHOP, CCAAT/-enhancer-binding protein homologous protein; ER, endoplasmic reticulum; IMPase, inositol monophosphatase; IP_3, inositol triphosphate; mTOR, mammalian target of rapamycin; NO, nitric oxide; TLR, Toll-like receptor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand

Selective targeting of macrophages in atherosclerotic plaques is also achieved in molsidomine-treated hypercholesterolaemic rabbits. The NO donor molsidomine preferentially kills activated macrophages, e.g. inducible NO synthase (iNOS)-expressing macrophages in atherosclerotic plaques, without influencing circulating monocytes/macrophages. Indeed, systemic treatment with molsidomine during lipid lowering decreases the ratio between macrophages and SMCs favouring plaque stability [152]. In an *in vitro* follow-up study, it was shown that other NO donors, spermineNONOate or S-nitroso-N-acetylpenicillamine, induce endoplasmic reticulum (ER) stress, as confirmed by upregulation of CCAAT/-enhancer-binding protein homologous protein (CHOP) expression and inhibition of *de novo* protein synthesis, leading to macrophage apoptosis, which is likely to be independent of iNOS expression (Figure 2 (D)) [153]. Since CHOP expression was increased in molsdomine-treated atherosclerotic plaques and ER stress inducers thapsigargin and tunicamycin also initiated selective macrophage death, these data confirm the finding that induction of ER stress during treatment with an NO donor initiates selective macrophage apoptosis (Figure 2 (D)). Although ER stress is present in both macrophages and SMCs upon NO donor treatment, only macrophages undergo apoptosis. This finding might be explained by the initial presence of ER stress in intimal macrophages that is balanced by compensatory cell survival pathways. Activation of an additional mediator and/or pathway, such as ER stress by NO donors, provokes macrophage apoptosis.

On the other hand, local administration of the protein synthesis inhibitors, cycloheximide or anisomycin, to atherosclerotic plaques via osmotic minipumps selectively induces apoptosis in macrophages without inducing ER stress and without affecting SMCs and endothelial cells (Figure 2 (E)) [154, 155]. The selectivity is most likely due to the high metabolic activity of macrophages, which are therefore more sensitive to protein synthesis inhibitors than SMCs, and to

modulation of SMCs towards a quiescent contractile phenotype upon protein translation inhibition, which renders these cells less sensitive to cell death. However, protein synthesis inhibition does not always induce apoptosis. Stent-based delivery of everolimus in atherosclerotic plaques of cholesterol-fed rabbits leads to a marked reduction of macrophages via autophagic cell death without altering the SMC content [156]. Everolimus-induced autophagy is mediated by inhibition of mammalian target of rapamycin (mTOR), an important serine/threonine protein kinase that is involved in cell survival (Figure 2 (F)). Besides mTOR inhibitors, a novel pathway for pharmacological modulation of autophagy has been described [157]. Lithium, an inhibitor of inositol monophosphatase, induces autophagy in nonneuronal and precursor neuronal cells by depleting inositol and consequent inositol triphosphate (IP_3) concentrations. Recent experiments in our laboratory show that local treatment of atherosclerotic plaques with lithium triggers selective macrophage apoptosis, and not autophagy, by depletion of IP_3 in macrophages only (Figure 2 (B)) [158]. Another example of selective induction of macrophage autophagy is based on TLR7 expression, since it is expressed on macrophages but not on SMCs and endothelial cells (Figure 2 (G)). Stimulation of TLR7 by imidazoquinoline compound imiquimod induces autophagic cell death in macrophages without affecting other cells, at least *in vitro* (De Meyer, unpublished data, 2011). *In vivo*, however, imiquimod-induced macrophage autophagy in atherosclerotic plaques stimulates plaque progression by cytokine release and enhances infiltration of inflammatory cells by increased VCAM-1 expression. These adverse pro-inflammatory effects could be abolished by adding dexamethasone. Recent *in vitro* results show that everolimus-induced autophagy in macrophages may also stimulate cytokine secretion [159]. These findings corroborate that the consequences of induction of selective macrophage autophagy in atherosclerotic plaques have to be taken into account, and that additional anti-inflammatory drugs are recommended to prevent inflammation and infiltration of new monocytes.

Targeting plaque macrophages

After identifying therapeutic targets to eliminate macrophages from atherosclerotic plaques, the use of drugs has been stymied by adverse effects, including impaired host defence, due to unselective drug availability. Therefore, local delivery systems to target plaque macrophages are being developed to overcome this therapeutic drawback.

Experimental studies

As previously mentioned, liposomes are effective drug carriers targeting macrophages via phagocytosis. Interestingly, target specificity can be augmented by adding ligands, such as peptides, antibodies and lectins, on the surface of liposomes. Cyclopentanone prostaglandins (CP-PGs)-containing liposomes coated with anti-VCAM-1 antibodies specifically target VCAM-1, which is highly expressed on endothelial and foam cells at atherosclerotic sites, and deliver the anti-inflammatory CP-PGs [160]. Administration of these endothelium-directed CP-PGs-containing liposomes reverses atherosclerotic lesions via anti-inflammatory, antiproliferative, anticholesterologenic and cytoprotective effects [160]. Besides active targeting of lesional macrophages, liposomal ligands compete with the natural ligands making them viable anti-atherosclerotic agents. For example, Kelly *et al*. suggested that hexarelin containing-liposomes directed against scavenger receptor CD36 on macrophages in atherosclerosis [161] can deliver therapeutic agents to the lesions and block LDL uptake at the same time [162].

Besides liposomes, nanoparticles are also phagocytozed by macrophages and these polymer-based nanostructures can be designed to allow controlled drug or gene delivery [163, 164]. Furthermore, they can be functionalized by ligands for active targeting towards atherosclerosis sites upon systemic administration [164, 165].

Clinical studies

Drug-eluting stents have proved to be a useful strategy for local drug delivery and prevention of in-stent restenosis [166, 167], but are compromised by complications, such as late stent thrombosis. The impaired healing of the arterial wall that is responsible for late stent thrombosis is predominantly attributed to delayed restoration of endothelial coverage and function in combination with persistent inflammation around stent struts. These adverse effects have led to the development of vascular restoration therapy by bioresorbable drug-eluting scaffolds. By degradation of the polymers and release of everolimus, the vessel lumen is significantly enlarged after 2 years and the vessel wall is characterized by infiltration of functional SMCs, absence of remodelling, restoration of endothelial cell structure and function, and reduction in plaque/media [168]. It has been hypothesized that selective induction of macrophage autophagy by everolimus may contribute to the reduction in plaque size [156, 168].

Conclusion

Macrophages play a key role in the initiation and progression of atherosclerosis as well as in plaque destabilization. Continuous infiltration of monocytes into atherosclerotic plaques and macrophage survival drives disease progression and, therefore, therapies that affect overall macrophage accumulation would likely improve plaque stabilization. Despite massive use of effective drugs such as statins, the risk for cardiovascular events remains substantial. The plaque stabilizing effects of statins are attributed

to their lipid lowering properties, and partly to their anti-inflammatory effects. Therefore, additional anti-inflammatory therapeutic interventions that result in reduced plaque macrophage content are of great interest. New LDL lowering, HDL raising drugs, compounds that target adhesion molecules or chemokines or drugs that directly induce macrophage death tackle vascular inflammation by reducing the inflammatory stimulus in atherosclerotic plaques, promoting cholesterol efflux, inhibiting monocyte recruitment and reducing the number of plaque macrophages, respectively, and may offer new perspectives. Even though results with these new drugs are promising in animal studies, clinical trials are so far disappointing due to side effects or the lack of additional benefits. Targeting plaque macrophages has been the focus of recent research, not only for cell-specific delivery of drugs, but also for the detection and imaging of vulnerable plaques. Recent progress in macrophage-targeted nanostructures and bioresorbable drug-eluting scaffolds may facilitate therapeutic atherosclerosis treatment with minimal adverse effects.

Competing Interests

There are no competing interests to declare.

This work was supported by the Agency for Innovation by Science and Technology in Flanders (IWT), the Fund for Scientific Research (FWO)-Flanders and the University of Antwerp (TOP-BOF). Illustrations are adapted from Servier Medical Art. The authors are indebted to Hans De Loof for fruitful discussions.

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