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Review

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The local effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the inflammatory atheroma: beyond LDL cholesterol lowering

Ting Xiong[1,2], Alexander S. Qian[1,2], Bernardo L. Trigatti[1,2]

[1]Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences and McMaster University, Hamilton L8S 4K1, Canada.
[2]Department of Biochemistry and Biomedical Sciences, McMaster University Hamilton, Ontario L8S 4K1, Canada.

Correspondence to: Dr Bernardo L. Trigatti, Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences and McMaster University and Department of Biochemistry and Biomedical Sciences McMaster University, 1280 Main St W, Hamilton L8S 4K1, Canada. E-mail: trigatt@mcmaster.ca

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has gained extensive attention since the discovery of its role in mediating hepatic low-density lipoprotein (LDL)-receptor (LDLR) degradation, and therefore regulating plasma LDL cholesterol clearance. However, emerging studies have indicated that PCSK9 may have pleiotropic effects on the development of atherosclerosis and eventually cardiac dysfunction by mechanisms independent of the regulation of LDL cholesterol levels. In this review, we will discuss recent findings of how PCSK9 affects cellular responses and the pro-inflammatory microenvironment within the local atherosclerotic vascular wall, which are critical for atherosclerotic plaque progression. We will also discuss recent research on the potential direct effects of PCSK9 on cardiomyocyte viability and function. These pathways may be critical for understanding the mechanisms of PCSK9's cardioprotective effects beyond LDL cholesterol lowering.

Keywords: Atherosclerosis, inflammation, myocardial infarction, PCSK9

INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death worldwide[1,2]. A major underlying mechanism of CVD is the coronary atherosclerotic plaque induced ischemia and the subsequent myocardial infarction (MI). Atherosclerosis develops within the artery wall at preferred sites, such as artery bifurcations, where blood flow is non-laminar and disturbed and therefore have lower shear stress[3]. Atherosclerosis is a chronic inflammatory disease that is initiated by the retention of low-density lipoproteins (LDLs) in the intima of the artery wall[4]. The continued retention of LDLs persistently drives monocyte infiltration into the subendothelial layer[4]. Those monocytes differentiate into macrophages, take up modified (mainly oxidized) LDLs (Ox-LDLs), and become lipid-laden foam cells[4]. Vascular smooth muscle cells (VSMCs) can also contribute to the foam cell formation by uptake of Ox-LDLs[5,6]. In the advanced atherosclerotic plaque, apoptotic and/or necrotic foam cells, cell debris, extracellular lipids, and inflammatory cytokines accumulate to form a large necrotic core. Plaques with large necrotic cores are prone to rupture, exposing the contents of the plaque to the blood, activating platelets and the coagulation cascade, and resulting in thrombus generation[7]. The so-called atherothrombosis in coronary arteries reduces or even blocks the supply of blood to the myocardium, leading to MI.

Atherosclerosis is the combinational outcome of long-lasting hypercholesterolemia and chronic inflammation. Statin therapy is effective across large populations at reducing cholesterol levels and therefore reducing the development of atherosclerotic CVD[8]. However, there are still significant residual risks of CVD even in individuals with low cholesterol levels[9], likely due to high levels of inflammation. The clinical trial of the interleukin (IL)-1β inhibitor canakinumab has shown that inhibiting inflammation alone is enough to lower the rate of recurrent cardiovascular events (CVEs) without altering lipid levels, indicating targeting inflammation as a potential therapy against CVD[10].

The proprotein convertase subtilisin/kexin type 9 (PCSK9) diverts the low-density lipoprotein (LDL)-receptor (LDLR) from the cell surface to the lysosome for degradation in liver cells[11,12]. It has been shown to be an attractive target for lowering LDL cholesterol in the blood[13,14]. Inhibiting PCSK9, provides a chance to decrease cholesterol levels for people who are intolerant to or have major side effects from statin treatment. Indeed, two monoclonal antibodies against PCSK9 (evolocumab and alirocumab), have shown potent cholesterol lowering effects and reduced CVEs in large clinical studies[13,14]. Interestingly, a meta-analysis revealed that by achieving the same levels of LDL cholesterol lowering, the relative risk reduction for CVEs tended to be greater with PCSK9 inhibitors compared with statins, although the apparent lower risk was not statistically significant[15]. More importantly, it has been shown that circulating PCSK9 levels predicted future CVEs, independently of established risk factors, including LDL cholesterol[16]. This suggests that in addition to exerting cardioprotective effects through lowering LDL cholesterol, inhibition of PCSK9 may have cardioprotective effects that are independent of LDL cholesterol lowering. Accumulating research has suggested that PCSK9 may exert effects on atherogenesis within the artery wall beyond its influences on LDL cholesterol levels[17-22]. Studies in model organisms have shown that overexpressing PCSK9 elevated the plaque size at the aortic sinus and root in apolipoprotein E (ApoE) knockout (KO) mice, without altering plasma cholesterol levels[20,21]. On the other hand, silencing PCSK9 through shRNA mediated PCSK9 knockdown, decreased atherosclerosis development in the aortic sinus in ApoE KO mice, independently of plasma cholesterol levels[18]. Atherothrombosis development in coronary arteries involves a variety of different cell types, including but not limited to VSMCs, endothelial cells (ECs), macrophages, T cells, dendritic cells (DCs)[23], and platelets. In the following review, we discuss evidence related to how PCSK9 modulates cellular responses and the pro-inflammatory microenvironment in the artery wall, and how those processes may affect atherothrombosis progression locally. We also discuss the potential direct role of PCSK9 on the myocardium, which is critical for heart function after ischemia and post-MI remodeling [Figure 1].

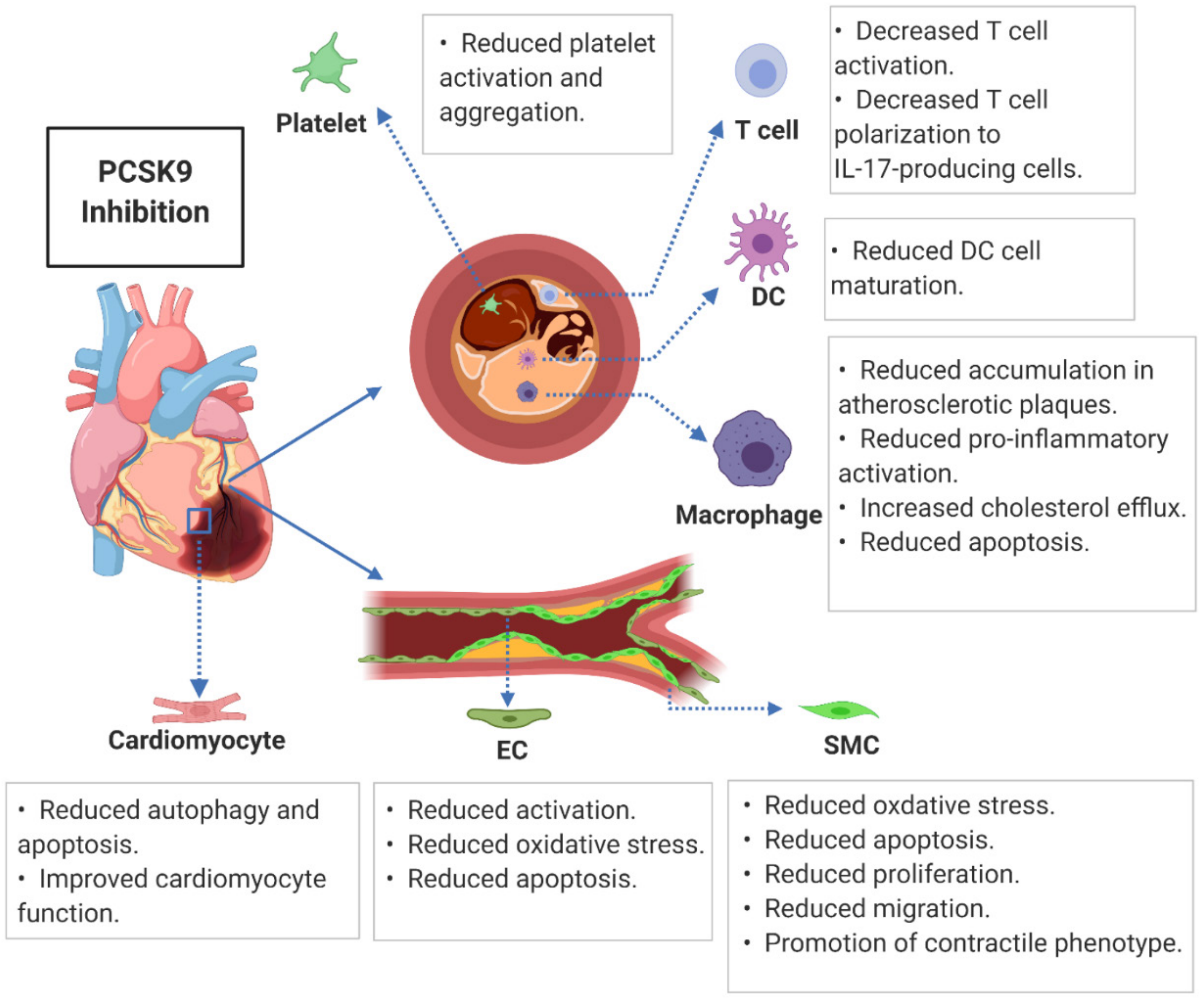

Figure 1. Potential mechanisms by which PCSK9 inhibition protects against CVD independently of LDL cholesterol lowering. Inhibition of PCSK9 affects cellular responses in bone marrow derived cells (monocytes/macrophages, DCs, T cells and platelets), ECs and VSMCs in the artery wall, and cardiomyocytes, which might all contribute to cardioprotective effects mediated by inhibition of PCSK9. Figure created with BioRender.com. CVD: Cardiovascular disease; LDL: low-density lipoprotein; DCs: dendritic cells; ECs: endothelial cells; VSMCs: vascular smooth muscle cells

VSMCS AND ECS IN THE VASCULAR WALL

In arteries, regions of curvature and bifurcations exhibiting turbulent blood flow and low shear stress are favored sites for atherosclerosis development[24]. These hemodynamic properties induce EC dysfunction[24]. Dysfunctional ECs become pro-inflammatory and pro-thrombotic, and exhibit a disrupted barrier function[25]. The pro-inflammatory ECs express vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which contribute to leukocyte recruitment[26,27]. When the barrier function of ECs is impaired, low shear stress may alter VSMC function[3,28]. VSMCs can be either beneficial or detrimental to the stability of atherosclerotic plaques, depending on phenotypic modulation[5,29,30]. VSMCs in the medial layer of the artery wall are differentiated and at a mature contractile state, expressing alpha-smooth muscle actin (SM α-actin), SM22α, calponin, and myosin heavy chain (MHC)[30]. However, mature contractile VSMCs remain remarkably plastic[30]. They can de-differentiate, proliferate, and migrate, contributing to the formation of a neo-intimal layer in the artery wall and the growth of the atherosclerotic lesion[30,31]. There, they can acquire either a synthetic or a macrophage-like phenotype[32-34]. The synthetic VSMCs migrate to the surface of the atherosclerotic plaque, below the endothelial cells and secret collagen rich extracellular matrix, which forms a thick fibrous cap, stabilizing the plaque against rupture[29,31,35]. On the other hand, VSMCs can lose their contractile properties, including contractile markers such as α-actin, and express macrophage markers, such

as CD68 and Lgals3, and therefore are referred to as macrophage-like smooth muscle cells[5,6,36]. They take up Ox-LDL through scavenger receptors, including SR-A, CD36, and LOX-1[37], become cholesterol-laden foam cells, and secret inflammatory cytokines, which contribute to plaque destabilization[5,35].

Although circulating PCSK9 is mainly derived from hepatocytes, PCSK9 is also found to be expressed in a variety of tissues[38]. Interestingly, it was reported to be expressed in VSMCs, and, to a lesser extent, in ECs and macrophages, and PCSK9 protein has been detected in human atherosclerotic plaques[39,40]. This raises the possibility that PCSK9 derived locally from the artery wall might have an involvement in plaque development. *In vitro* studies have shown that PCSK9 secreted by cultured VSMCs can decrease LDLR protein levels in macrophages[40,41] [Figure 2]. Several studies suggested that different factors regulate PCSK9 expression levels in VSMCs and ECs, including shear stress, reactive oxygen species (ROS), and pro-inflammatory stimuli such as Ox-LDL, toll-like receptor 4 ligands [e.g., lipopolysaccharide (LPS)], and pro-inflammatory cytokines[39,41-43]. Ding *et al.*[39] found that in mouse aortas, at arterial branching points and at the bifurcation of the iliac arteries, expression levels of PCSK9 were elevated along with expression levels of VCAM-1, known to be elevated at sites of EC activation[26] [Figure 1]. Inhibition of PCSK9 by alirocumab treatment reduced levels of EC expression of ICAM-1, another marker of EC activation[26,44]. The transcriptional regulator, NF-κB, is known to be a key mediator of EC activation in regions of the mouse aorta exposed to hemodynamic factors that predispose to atherosclerotic plaque development[45]. Hemodynamic forces appear to regulate PCSK9 expression along the mouse aorta partly through a TLR4-MyD88-NF-κB pathway[43]. Moreover, VSMCs and ECs exposed to low shear stress in culture exhibit increased PCSK9 expression compared to that in cells exposed to high shear stress[39]. This pattern of PCSK9 expression parallels patterns of oxidative stress and EC activation[39] [Figure 1]. There appears to be a positive feedback relationship between PCSK9 expression and ROS levels[39]. ROS at moderate levels generated by normal physiological processes appear to serve as signaling molecules[46]; however, excessive ROS generation in the vascular wall is pathological and promotes atherosclerosis, by contributing to lipoprotein oxidation, endothelial dysfunction, leukocyte migration and activation, VSMC proliferation, DNA damage, and matrix metalloproteinases-induced collagen degradation[47,48]. Ox-LDLs, generated by ROS mediated oxidation of LDLs, lead to further ROS production via a pathway mediated by the Ox-LDL receptor, LOX-1[49,50]. Ding *et al.*[39,42] also found that there is a bidirectional interplay between PCSK9 and LOX-1 expression in VSMCs and ECs. siRNA mediated LOX-1 knockdown reduced LPS-induced PCSK9 expression in VSMCs and ECs[39,42]. On the other hand, recombinant human PCSK9 (hPCSK9) was reported to increase LOX-1 protein levels, while siRNA mediated PCSK9 knockdown decreased LPS-induced LOX-1 levels in VSMCs and ECs[39,42]. Conversely, Sun *et al.*[51] reported that PCSK9 associated with LDL particles, but not either PCSK9 or LDL alone, increased gene expression of LOX-1 and ICAM-1 along with CCL-2, CCL-7, IL-1β, and IL-6 in cultured ECs, suggesting that PCSK9 affects EC gene expression by modulating the properties of LDL particles. The reciprocal regulation between PCSK9 and LOX-1 expression may be mediated through mitochondrial ROS (mtROS), as it affected both PCSK9 and LOX-1 expression levels[42]. Excessive mtROS production leads to mtDNA damage, which can induce autophagy, inflammation, or apoptosis[52-54]. It is reported that induced mtROS production by antimycin A, rotenone, and thenoyltrifluoroacetone, increased PCSK9 expression and mtDNA damage in VSMCs[55]. It has also been shown that there is a positive feedback interaction between PCSK9 expression levels and mtDNA damage in VSMCs, partially mediated by mtROS[55]. Enhancement of mtDNA damage induced by autophagy inhibitors increased and inhibition of mtDNA damage via an autophagy inducer (rapamycin) decreased PCSK9 expression in VSMCs[55]. The effect of antimycin induced mtDNA damage on PCSK9 protein levels was mediated by the activation of p38 mitogen-activated protein kinase (MAPK)[55], which has been shown to respond to environmental stress, cytokines, and DNA damage[56,57]. Conversely, recombinant hPCSK9 increased and siRNA mediated PCSK9 knockdown decreased phosphorylation of mammalian target of rapamycin (mTOR) and LPS-induced mtDNA damage in VSMCs[55]. This result suggests that PCSK9 might regulate mtDNA damage via mTOR activation, as mTOR inhibition by rapamycin decreased mtDNA damage[55]. However, the importance of PCSK9, ROS, and LOX-1 interaction in VSMCs and ECs on atherosclerosis development *in vivo* has not been elucidated yet.

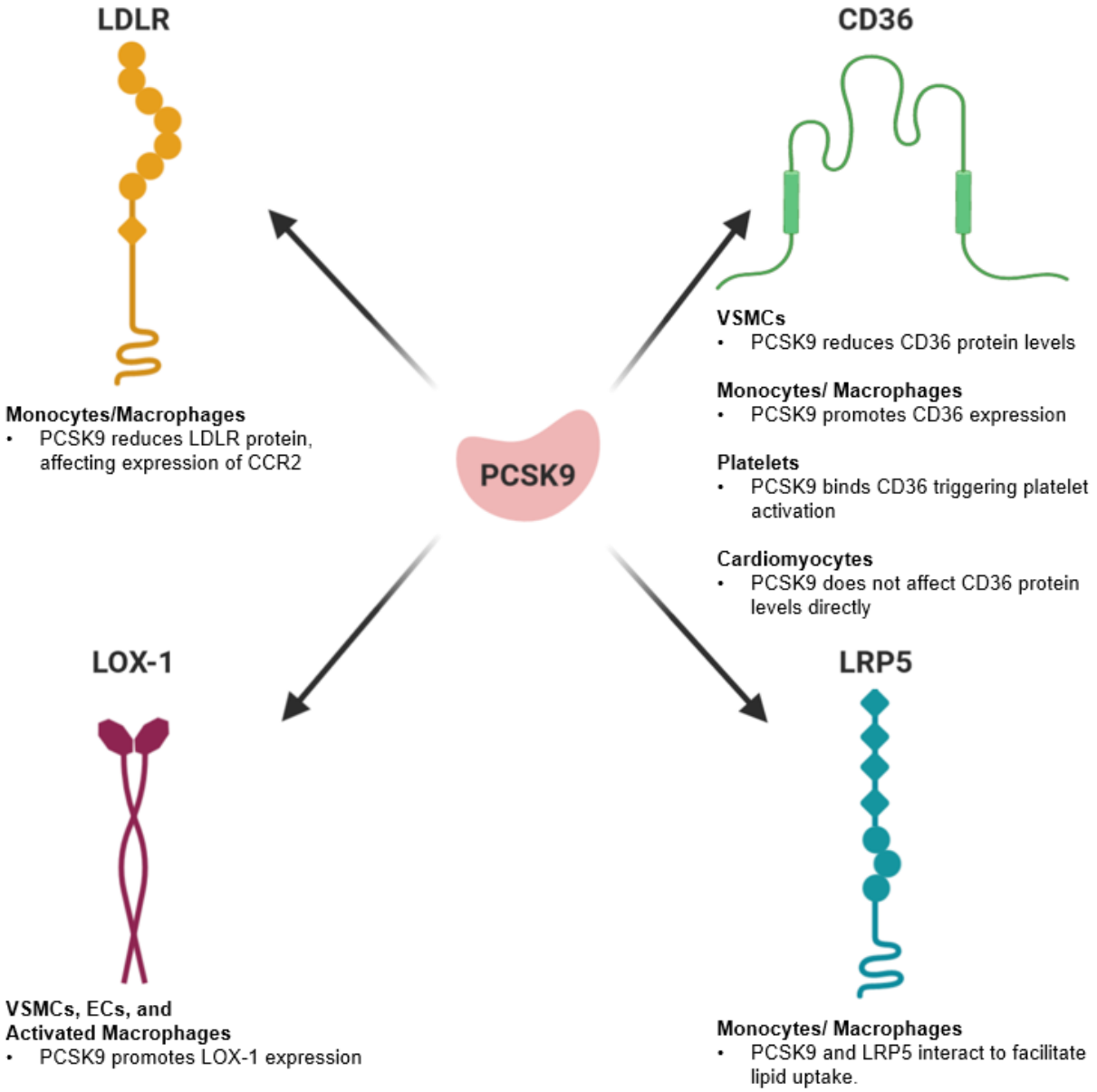

Figure 2. PCSK9-dependent effects on receptors expressed in cells in atherosclerotic plaques and the heart. PCSK9's interactions with or effects on the LDL receptor, CD36, LRP-1, and LOX-1 in endothelial cells, vascular smooth muscle cells, monocytes/macrophages, platelets, and cardiomyocytes are summarized with effects that may alter downstream cell signalling mediating cell activation, cell migration, inflammation, and lipid uptake. References are provided in the text. Figure created with BioRender.com. LDL: low-density lipoprotein

Apoptosis is a significant feature of atherosclerotic plaque development. EC apoptosis compromises the barrier function of the EC layer, contributing to increased lipid deposition and plaque vulnerability[58]. Apoptosis in SMCs may result in reduced collagen production and decreased plaque stability[59]. *In vitro* studies have shown that PCSK9 inhibition reduced apoptosis levels in both in human umbilical vein endothelial cells (HUVECs) and VSMCs[55,60,61] [Figure 1]. The Bcl/Bax-caspase9-caspase3 pathway is involved in the protective effects of PCSK9 inhibition on EC and VSMC apoptosis[55,60,61]. However, the link between PCSK9 and the Bcl/Bax pathway is unclear. Studies have shown that c-Jun N-terminal kinases (JNK) and p38 MAPK mediated the phosphorylation and mitochondrial translocation of Bax, and led to apoptosis in human hepatoma HepG2 cells[62]. It was also shown that JNK and p38 MAPK mediated the phosphorylation of Bcl-2, which can enhance Bcl-2 degradation[63,64]. Interestingly, knockdown of PCSK9 by

shRNA decreased phosphorylation of JNK and p38 MAPK in HUVECs[61], suggesting PCSK9 might trigger apoptosis through the JNK/p38 MAPK-Bcl-2/Bax signaling pathway. Yet, interventional studies should be conducted to test if PCSK9 mediated apoptosis is affected by inhibiting JNK/p38 MAPK activation. Whether PCSK9 induces apoptosis of ECs and VSMCs during atherosclerotic plaque development also needs to be investigated *in vivo*.

The dramatically increased proliferation capability of neo-intimal compared to medial VSMCs contributes to both neo-intimal hyperplasia and atherosclerosis progression. Importantly, Ferri *et al.*[22] have shown that knocking out the PCSK9 gene mitigated neo-intimal formation and VSMC accumulation within the neo-intima in mice subjected to a non-occlusive collar placement around carotid arteries. Furthermore, aortic VSMCs isolated from PCSK9 deficient mice have been shown to exhibit reduced proliferation rates and migration levels when compared to those from PCSK9 expressing mice[22] [Figure 1]. PCSK9 deficient VSMCs reportedly exhibited a more spindle and elongated shape and increased expression of SM α-actin and MHC-II compared to VSMCs from PCSK9 expressing mice, suggesting that inactivation of the PCSK9 gene might modulate VSMC phenotypes[22] [Figure 1]. Knock out of the PCSK9 gene in mice has been reported to increase CD36 protein levels in VSMC[22] and CD36 is considered as an important Ox-LDL receptor involved in macrophage foam cell formation and activation in atherosclerotic plaques[65-68] [Figure 2]. VSMCs in the neo-intima are thought to adopt a macrophage-like phenotype including the ability to take up Ox-LDL and accumulate lipid droplets and a foam cell-like morphology in atherosclerotic plaques[36]. However, whether PCSK9 inhibition affects the ability of VSMC to adopt a macrophage-like phenotype has not yet been reported.

MONOCYTES/MACROPHAGES

Monocytes are recruited to the vessel wall at sites of atherosclerotic lesion development and infiltrate through the EC layer into the tunica intima layer of the artery where they mature into macrophages that can take up modified lipoproteins[4]. PCSK9 may promote monocyte recruitment into atherosclerotic plaques at multiple steps including, as indicated above, through activation of ECs (VCAM-1 and ICAM-1 expression)[39,44,69]. Once in the artery wall, macrophages become foam cells as they accumulate lipids through scavenger receptor-mediated uptake of Ox-LDL particles. Recent studies suggest that PCSK9 may play an important role in foam cell formation by increasing lipid accumulation and limiting lipid efflux in macrophages[70,71]. Macrophage CD36 has been reported to play important roles in atherosclerosis development by virtue of its involvement in Ox-LDL uptake, pro-inflammatory responses, engulfment of apoptotic cells, and macrophage emigration[72]. In culture, the exposure of TNFα primed mouse peritoneal macrophages to exogenous PCSK9 resulted in increased Ox-LDL uptake and this correlated with an upregulation of gene expression and protein levels of scavenger receptors CD36, SR-A, and LOX-1[70] [Figure 2]. Furthermore, PCSK9 gene deletion or siRNA-mediated silencing in macrophages has been reported to reduce SR-A, LOX-1, and CD36 expression[70]. However, another study has shown that conditioned media containing PCSK9 did not change CD36 protein levels in PMA-induced THP-1 macrophages compared with control media which is deficient in PCSK9[73]. On the other hand, PCSK9 plays a well-known role in receptor internalization and degradation. In addition to PCSK9 directed degradation of LDLR and its close family members VLDLR and apolipoprotein E receptor 2 (ApoER2)[74], PCSK9 has also been reported to mediate internalization and degradation of CD36 in cultured adipocytes and hepatocytes, limiting fatty acid uptake and triglyceride accumulation[73]. Therefore, the reports that PCSK9 inactivation in macrophages reduces CD36 expression is unexpected. However, the reported effects of PCSK9 on macrophage CD36 levels await confirmation from *in vivo* pre-clinical or clinical studies.

In human macrophages derived from monocytes isolated from healthy blood donors, PCSK9 and LDLR-related protein 5 (LRP5) have been reported to interact to control lipid uptake from aggregated LDL[75] [Figure 2]. LRP5 appears to mediate macrophage uptake of aggregated LDL and PCSK9 has been reported to

form an intracellular complex with LRP5 and assist in its trafficking to the cell surface[75]. Silencing of either LRP5 or PCSK9 results in reduced uptake of aggregated LDL by macrophages[75]. On the other hand, the treatment of mouse peritoneal macrophages with purified hPCSK9 has been reported to result in reduced gene expression and protein levels of the ATP binding cassette transporter, ABCA1[71]. Consequently, PCSK9 treatment of macrophages reduced ABCA1 dependent cholesterol efflux to apolipoprotein A1[71] [Figure 1]. ABCA1 mediated cholesterol efflux from macrophages to apolipoprotein A1 is believed to play a critical atheroprotective role in the removal of excess cholesterol from macrophages via a process called reverse cholesterol transport[76]. Taken together, it appears that PCSK9 derived from macrophages themselves or extracellular PCSK9 encountered by macrophages influence macrophage cholesterol homeostasis to promote macrophage foam cell formation, a process generally considered to contribute to atherosclerotic plaque formation.

Studies have reported that LDL levels appear to regulate the level of C-C chemokine receptor type 2 (CCR2) expression on monocytes[41,77]. CCR2 on monocytes appears to play a critical role in regulating circulating monocyte counts and monocyte migration across the intact EC barrier of arteries in response to its ligand CCL2, a key step in monocyte accumulation in the atherosclerotic artery wall[78]. One study reported that monocyte CCR2 expression correlated with LDL-cholesterol levels in patients with familial hypercholesterolemia, who display elevated LDL cholesterol due to impaired LDLR mediated LDL clearance[77]. Administration of anti-PCSK9 monoclonal antibody treatment to familial hypercholesterolemia patients, which reduced LDL cholesterol, coordinately reduced lipid accumulation and CCR2 expression in circulating monocytes, as well as the capacity of those monocytes to migrate through cultured EC monolayers in response to added CCL2[77]. In contrast, another study reported that LDL dependent upregulation of monocyte CCR2 expression was dependent on LDLR expression in monocytes[41]. In that study, PCSK9 secreted from VSMCs reduced CCR2 expression on monocytes by virtue of reducing monocyte LDLR levels and LDLR mediated LDL uptake by monocytes[41]. This study suggests that PCSK9, secreted locally in the environment of the atherosclerotic plaque, may actually limit monocyte or macrophage chemotaxis within the plaque itself. Whether this also impacts monocyte recruitment to the plaque has not been tested. It is also unclear whether the effects of PCSK9 on LDL-cholesterol levels or on monocyte LDL-cholesterol uptake predominates in regulating monocyte CCR2 levels.

Emerging research reveals the potential role of PCSK9 in modulating monocyte and macrophage inflammation in the progression of atherosclerosis. The global overexpression of mouse or human PCSK9 in ApoE KO or LDLR KO mice did not alter plasma cholesterol levels due to the absence of the key components of the ApoE-LDLR axis for LDL clearance targeted by PCSK9[20,21]. However, despite the lack of changes in plasma lipids, PCSK9 overexpression in ApoE KO mice increased aortic lesion size and the abundance of pro-inflammatory Ly6Chi monocytes in atherosclerotic plaques[20,21] [Figure 1]. Human PCSK9 overexpression in ApoE KO mice was also accompanied by increased levels of PCSK9 protein in atherosclerotic plaques[21]. Interestingly, the increase in Ly6Chi monocytes and PCSK9 expression in plaque were not evident in LDLR KO mice[21], suggesting that PCSK9 exerts pro-inflammatory effects in an LDLR dependent manner. The silencing of PCSK9 in ApoE KO mice by lentivirus-mediated shRNA reduced atherosclerosis development and reduced the abundance of macrophages and expression of inflammatory markers within atherosclerotic plaques[18]. Exogenous PCSK9 can induce a pro-inflammatory response in human macrophages by upregulating the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (CCL2, CXCL2)[79] [Figure 1]. The release of pro-inflammatory cytokines such as TNF-α and IL-1β by PCSK9 seems to involve TLR4-NF-κB signaling in a manner similar to that described for EC activation[18,19,43,69,75,80,81].

Human and murine primary monocytes and macrophages express intracellular and secreted PCSK9 in culture[75,80]. Although a majority of circulating PCSK9 is derived from the liver, endogenous expression of PCSK9 in monocytes and macrophages may affect local environments such as within the atherosclerotic

lesion. When human PCSK9 was overexpressed selectively in bone marrow-derived cells transplanted into ApoE KO mice, atherosclerotic lesion sizes were not increased compared to ApoE KO mice transplanted with bone marrow from non-transgenic controls[19]. However, selective PCSK9 overexpression in bone marrow-derived cells was sufficient to result in increased levels of Ly6Chi monocytes in atherosclerotic plaques of ApoE KO mice but not in those of LDLR KO mice[19]. This suggests that PCSK9 expression in bone marrow-derived cells, such as monocytes, may play a role in altering monocyte inflammatory profiles and circulating monocyte counts independently of PCSK9 expression from other sources, such as the liver. In support of this, Giunzioni *et al.*[19] reported that overexpression of human PCSK9 in macrophages increased LPS stimulated induction of mRNA levels of the pro-inflammatory markers TNF-α and IL-1β and suppression of the anti-inflammatory markers IL10 and Arg1 [Figure 1]. Conversely, others have reported that PCSK9 expression is upregulated in cultured mouse peritoneal macrophages exposed to LPS, TNF-α, or Ox-LDL[70]. Similar findings have been reported in the liver *in vivo* and in cultured VSMCs[42,82]. It has also been recently demonstrated that activation of the NLRP3 inflammasome upregulated intracellular PCSK9 expression and PCSK9 secretion from murine peritoneal macrophages, suggesting the possibility of a positive feedback loop of PCSK9 expression and macrophage activation[81]. The secretion of PCSK9 was dependent on IL-1β and MAPK pathway signalling and IL-1β deficient mice fed a high-fat diet had reduced serum PCSK9 levels[81]. Determining the mechanistic link between PCSK9, TLR4/NF-κB signalling, and NLRP3 inflammasome activation represents an interesting area of future research.

Macrophage cell death is a key driver in atherosclerosis progression and the formation of necrotic cores within lesions. As described earlier, PCSK9 seems to influence apoptosis of ECs and VSMCs[55,60,61]. Similarly, several lines of evidence suggest it may also influence apoptosis of macrophages. Partial knockdown of PCSK9 by siRNA in THP-1 macrophages seems to reduce cell death induced by Ox-LDL[83] [Figure 1]. The mechanism of this effect will need to be explored further, however some studies suggest PCSK9 may influence cell death by mediating the degradation of ApoER2, a LDLR family member which binds to ApoE and exerts anti-inflammatory and anti-apoptotic effects[84]. In neurons, PCSK9 was shown to increase apoptosis by the degradation of ApoER2[85]. ApoER2 is also expressed in macrophages[86]. The knockdown of ApoER2 in RAW 264.7 macrophages increased Ox-LDL induced cell death, inhibited pro-survival Akt signalling, and increased peroxisome proliferator-activated receptor γ (PPARγ) expression[86]. This deficiency of ApoER2 in LDLR KO mice translated to larger and more advanced atherosclerotic lesions with large necrotic cores and plaque apoptosis[86]. Of course, whether PCSK9 controls macrophage apoptosis in a manner similar to neurons, ECs, or VSMCs remains to be determined.

DENDRITIC CELLS AND T CELLS

Along with monocytes and macrophages, activated T cells[87] and antigen-presenting DCs[88] are commonly found in atherosclerotic lesions. Kühnast *et al.*[44] observed that inhibition of PCSK9 with alirocumab reduced T cell abundance within aortic lesions. However, it is not clear if this was a consequence of the inactivation of circulating PCSK9, derived largely from the liver, or of PCSK9 locally in the atherosclerotic plaque. More recently, it has been discovered that PCSK9 is involved in DC maturation and T cell activation and polarization to T helper-1 (Th1) and T helper-17 (Th17) subsets[89] [Figure 1]. PCSK9 inhibition in DCs prevented T-cell activation induced by Ox-LDL and promoted T regulatory cell populations through increased DC secretion of TGF-β and IL-10[89] [Figure 1]. Furthermore, PCSK9 gene inactivation in mice deficient in both LDLR and Apobec1 resulted in a reduction of levels of IL-17 in the plasma and Th17 cells in atherosclerotic plaques[90] [Figure 1]. These results may suggest that PCSK9 is associated with T cell programming within atherosclerotic lesions. However, there remains debate over whether IL-17 and Th17 cells protect against or promote atherosclerosis development[91].

PLATELETS

Platelets contribute to atherothrombosis by accelerating both atherosclerosis progression[92] and thrombus generation. Activated platelets facilitate the binding of leukocytes to ECs on the atherosclerotic lesion sites and release pro-inflammatory cytokines, therefore promoting atherosclerosis[92,93]. Exposure of subendothelial collagen to circulating platelets when the plaque ruptures initiates primary hemostasis, which leads to thrombus generation. Platelet adhesion, activation and aggregation are important steps during the primary hemostasis. Multiple factors can activate platelets, including collagen, adenosine diphosphate (ADP), thrombin, and thromboxane A2 (T_X A_2). T_X A_2, generated from arachidonic acid through cyclo-oxygenase -1 (COX-1)/thromboxane synthase pathway in activated platelets, is unstable and rapidly hydrolyzed into T_X B_2[94]. T_X B_2 is further converted in the liver into 11-dehydro-thromboxane B2 (11-dh-TxB2), which is excreted in the urine[94]. An observational study has shown plasma PCSK9 levels are correlated with urinary levels of 11-dh-TxB2 in patients with atrial fibrillation[95]. However, T_X A_2 derives not only from the COX-1/thromboxane synthase pathway in activated platelets but also from the COX-2/thromboxane synthase pathway in leukocytes and endothelial cells exposed to inflammatory stimuli[94,96]. Therefore, the association between plasma PCSK9 levels and urinary 11-dh-TxB2 levels may alternatively reflect effects on inflammatory pathways. Plasma PCSK9 levels have also been observed to be correlated with levels of ADP-induced *ex vivo* platelet aggregation in patients with acute coronary syndrome[97], directly suggesting an association between plasma PCSK9 levels and platelet activation. Interestingly, anti-PCSK9 monoclonal antibody treatment in patients with hypercholesterolemia was associated with reduced levels of *ex vivo* ADP-induced platelet aggregation and reduced plasma markers of platelet activation, including soluble CD40 ligand, platelet factor-4, and soluble P-selectin[98]. In mice subjected to $FeCl_3$-induced carotid artery injury, PCSK9 deficiency was associated with reduced levels of activated GP IIb/IIIa, P-selectin and platelet–leukocyte aggregates, and thrombus formation at the injury site[99]. A factor which complicates the interpretation of the effects of PCSK9 inhibition on platelet activation and thrombus formation is the reduced LDL cholesterol levels resulting from anti-PCSK9 antibody treatment or PCSK9 gene inactivation, since LDL cholesterol levels have been reported to affect platelet activation[100]. Therefore, direct measurements of the effect of PCSK9 on platelet activation including studies in model organisms should be conducted. LDL cholesterol levels are not altered by plasma PCSK9 inhibition or overexpression in LDLR KO mice[20,21,101], which becomes an advantage for investigating the direct role of PCSK9 on platelets *in vivo*. In one such study in LDLR KO mice, evolocumab treatment reduced $FeCl_3$-induced thrombus formation in mesenteric arterioles[102] [Figure 1]. Moreover, evolocumab treatment also reduced *ex vivo* ADP-induced aggregation of platelets from LDLR KO mice[102] [Figure 1]. These results suggest that PCSK9 plays a direct role in platelet activation and thrombus generation.

CD36 on platelets can bind to Ox-LDL, which has been shown to promote platelet activation[103-105]. CD36 is also a major platelet receptor for thrombospondin-1, a platelet α-granule protein that is released upon platelet activation and promotes platelet mediated thrombus formation via interactions with CD36[106]. Multiple downstream signaling pathways initiated by CD36, including Src kinase, extracellular signal-regulated kinase 5 (ERK5), JNK, and p38MAPK pathways have been shown to be involved in platelet activation[103,104,107,108]. *In vitro* studies have shown that co-incubating platelets with PCSK9 increased collagen, thrombin and ADP stimulated platelet aggregation and oxidative stress[102,109]. Such an effect on collagen-induced platelet aggregation was further amplified by the presence of LDL, which could be oxidized by PCSK9-induced oxidative stress[109]. Interestingly, inhibition of CD36 significantly decreased PCSK9 induced platelet aggregation with or without the presence of LDL[102,109], suggesting PCSK9 induced platelet aggregation was dependent on CD36 signaling. PCSK9 appeared able to activate several signaling pathways via CD36, including Src kinase, ERK5, JNK, and p38MAPK pathways[102]. As indicated earlier, in some cell types (e.g., hepatocytes and adipocytes) but not others (such as macrophages), PCSK9 has been shown to target CD36 for degradation[70,73]. It was reported that PCSK9 did not affect CD36 protein levels in human megakaryocytic cells[102], suggesting that PCSK9 affects platelet activation via CD36 signaling, rather than by affecting CD36

protein levels. To examine the *in vivo* consequences of PCSK9 mediated platelet activation via CD36, Qi *et al.*[102] carried out studies in which mice were depleted of endogenous platelets and then reconstituted with platelets pretreated with PCSK9 before left coronary artery ligation (LCAL) surgery to induce experimental MI. They reported that reconstituting mice with PCSK9-pretreated platelets significantly increased levels of microthrombi and development of MI compared with platelets that were not pretreated[102]. They also demonstrated that this effect was reduced when platelets were from CD36 deficient donor mice[102]. These results suggest that PCSK9 mediated platelet activation via CD36 has an influence on the expansion of MI.

CARDIOMYOCYTES

Acute cardiac ischemia resulting from occlusive coronary artery atherothrombosis triggers MI. Early responses in the myocardium after MI include cardiomyocyte death and inflammation in the infarct border zone[110]. Macrophages and neutrophils are recruited to and accumulate in the infarct zone where they play major roles in the clearance of apoptotic cells[111]. However, the resolution of the inflammatory response is a prerequisite for the transition from the inflammatory to the reparative phase[111]. Persistent accumulation of macrophages and pro-inflammatory cytokines, however, ultimately acts to impair cardiac remodelling and left ventricle (LV) function[111]. An observational study has reported that circulating PCSK9 levels were associated with reduced LV ejection fraction 6 months after a first ST-elevation myocardial infarction (STEMI)[112], and that this association was independent of serum LDL cholesterol levels and statin use. This study suggested that PCSK9 might contribute to adverse outcomes of cardiac remodelling post-MI.

Schlüter *et al.*[113] first reported that PCSK9 is expressed by terminally differentiated cardiomyocytes. Ox-LDL not only impaired the load-free cell shortening (an indicator of cardiomyocyte contractile function) but also increased PCSK9 expression in cardiomyocytes[113]. Inhibiting PCSK9 by siRNA reversed the effects of Ox-LDL on cell shortening of cardiomyocytes[113] [Figure 1]. Exposure of cultured cardiomyocytes to hypoxia, followed by reoxygenation (H/R), culture conditions that are meant to mimic ischemia/reperfusion (I/R) *in vivo*, also induced PCSK9 expression in murine primary cardiomyocytes[114]. Inhibiting hypoxia inducible factor-1 α (HIF-1α) attenuated hypoxia induced PCSK9 expression and autophagy in primary cardiomyocytes[115]. AMP-activated protein kinase (AMPK) is an energy sensor and maintains energy homeostasis. It promotes autophagy when phosphorylated/activated under many conditions, including ischemia, hypoxia, and glucose starvation[116-118]. Ding *et al.*[115] have reported that PCSK9 is involved in hypoxia induced autophagy in cardiomyocytes by bridging ROS generation and activating AMPK signalling [Figure 1]. Whether PCSK9 induced autophagy of cardiomyocytes is cardio-protective or deleterious during I/R remains unclear. Nevertheless, PCSK9 expression enhanced inflammatory cytokine release in a co-culture system of cardiomyocytes and macrophages under H/R conditions[114]. Furthermore, the media from the co-culture system reduced the viability and increased the apoptosis of cardiomyocytes in a manner that was reversed by siRNA mediated knockdown of PCSK9 [Figure 1] or chemical inhibition of NF-κB[114]. These *in vitro* studies suggest a direct role for PCSK9 in the viability and function of cardiomyocytes. However, the effects of PCSK9 mediated inflammatory cytokine release on cardiac remodelling still needs to be elucidated *in vivo*.

Ding *et al.*[115] extended their findings in the *in vivo* preclinical mouse LCAL surgical model of MI. They reported a dramatic increase in PCSK9 protein levels and autophagy markers in the infarct border zone[115]. Importantly, they reported that inhibiting PCSK9 gene expression (PCSK9 knockout) or function, using peptide based inhibitors, reduced cardiomyocyte autophagy and infarct sizes, and improved LV fractional shortening in mice that had undergone LCAL, without altering plasma LDL cholesterol levels[115]. These results suggest that PCSK9 may exert direct effects on cardiomyocyte survival and cardiac function, although the contribution of altered PCSK9 mediated platelet activation described above, cannot be ruled out. One potential mechanism of the PCSK9 inhibition mediated protection on cardiac dysfunction is via modulating CD36 levels. CD36 mediates fatty acid uptake in cardiomyocytes[72]. It has been suggested that inhibition of

CD36 in cardiomyocytes reduced lipid accumulation and contractile dysfunction[119]. However, *in vivo* studies have reported controversial results regarding the role of cardiomyocyte CD36 in cardiac function. On the one hand, cardiomyocyte-specific inhibition of CD36 improved functional recovery after I/R[120] and high fat diet induced cardiac hypertrophy and dysfunction[121]. On the other hand, inactivation of CD36 selectively in cardiomyocytes accelerated the progression from cardiac hypertrophy to heart failure, which was associated with energetic stress, as indicated by AMPK activation[122]. As described earlier, PCSK9 has been shown to exert different effects on CD36 levels in different cell types[70,73]. It would be interesting to investigate if PCSK9 mediates CD36 degradation or alters CD36 gene expression in cardiomyocytes. One study, however, has actually found that PCSK9 did not change CD36 protein levels in both HL-1 cardiomyocytes and heart tissues[73], suggesting the protection against cardiac dysfunction in LCAL mouse model by inhibition of PCSK9 may occur independently of alterations in CD36 levels in cardiomyocytes.

CONCLUSIONS

The PCSK9 era started in 2003 when Abifadel *et al.*[123] reported that mutations in the PCSK9 gene are associated with autosomal dominant hypercholesterolemia. Since then, this field has moved forward rapidly. Two FDA approved monoclonal antibodies against PCSK9 are available for clinical use[13,14,124,125]. While most of the focus on the role of PCSK9 inhibition in CVD has been on LDL cholesterol lowering, emerging basic studies utilizing either mouse models or cell lines have increasingly suggested that PCSK9 may also act via mechanisms that are independent of LDL cholesterol lowering. These mechanisms include influences on a variety of cell types and cellular pathways relevant to CVD. Whether these effects are the result of actions of PCSK9 derived locally in the cell types/tissues affected or due to circulating PCSK9 derived from the liver are not entirely clear, and further research will be needed to clarify this. Nevertheless, they raise the possibility that targeting PCSK9 may be beneficial even in individuals with low LDL cholesterol or who have already achieved LDL cholesterol lowering targets by other means.

DECLARATIONS

Authors' contributions

Article conception and design: Xiong T, Qian AS, Trigatti BL
Acquisition and analysis of bibliographic information: Xiong T, Qian AS, Trigatti BL
Drafting of the manuscript: Xiong T, Qian AS
Critical revision and final approval of the manuscript: Xiong T, Qian AS, Trigatti BL

Availability of data and materials

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Conflicts of interest

Xiong T and Qian AS declared that they have no conflicts of interest. Trigatti BL declare that he is bound by confidentiality agreements that prevent him from disclosing his conflicts of interest in this work.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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