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CONTEMPORARY REVIEW

Effects of PCSK9 Targeting: Alleviating Oxidation, Inflammation, and Atherosclerosis

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ABSTRACT: Characterized as a chronic inflammatory disease of the large arteries, atherosclerosis is the primary cause of cardiovascular disease, the leading contributor of morbidity and mortality worldwide. Elevated plasma cholesterol levels and chronic inflammation within the arterial plaque are major mediators of plaque initiation, progression, and instability. In 2003, the protein PCSK9 (proprotein convertase subtilisin/kexin 9) was discovered to play a critical role in cholesterol regulation, thus becoming a key player in the mechanisms behind atherosclerotic plaque development. Emerging evidence suggests that PCSK9 could potentially have effects on atherosclerosis that are independent of cholesterol levels. The objective of this review was to discuss the role on PCSK9 in oxidation, inflammation, and atherosclerosis. This function activates proinflammatory cytokine production and affects oxidative modifications within atherosclerotic lesions, revealing its more significant role in atherosclerosis. Although a variety of evidence demonstrates that PCSK9 plays a role in atherosclerotic inflammation, the direct mechanism of involvement is still unknown, driving a gap in knowledge to such a predominant player in cardiovascular disease. Investigation of proteins structurally related to PCSK9 may interestingly be the link in unveiling the mechanistic role of this protein's involvement in oxidation and inflammation. Importantly, the unique structure of PCSK9 bears structural homology to a one-of-a-kind domain found in the metabolic protein resistin, which is responsible for many of the same inflammatory outcomes as PCSK9. Closing this gap in knowledge of PCSK9`s role in atherosclerotic oxidation and inflammation will provide fundamental information for understanding, preventing, and treating cardiovascular disease.

Key Words: atherosclerosis ■ inflammation ■ oxidation ■ PCSK9 ■ resistin

Cardiovascular diseases are the leading causes of morbidity and mortality worldwide.[1] Atherosclerosis as the main underlying cause involves the deposition and accumulation of lipids and fibrous materials beneath endothelial cells lining the large arteries. A variety of genetic and environmental factors influence atherosclerotic plaque development and progression, one of the most prominent causes being elevated blood cholesterol levels followed by chronic inflammation within the arterial wall.[2] Regardless of extensive past and present investigation of atherosclerotic plaque pathophysiology, the complexity of the chronic inflammatory state of the arteries during atherosclerotic plaque advancement and progression has presented more questions with every answer. In 2003, the protein PCSK9 (proprotein convertase subtilisin/kexin 9) was discovered to play a critical role in cholesterol regulation, thus becoming a key player in the mechanisms behind atherosclerotic plaque development (Figure 1).

PCSK9 inhibitors play an important role in the lipid management of patients with hypercholesterolemia, diabetes, and acute coronary syndrome.[3–8] New emerging evidence suggests that PCSK9 could have functions beyond cholesterol regulation by contributing to inflammation,[9] oxidation, and atherosclerosis. It is

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Nonstandard Abbreviations and Acronyms

CAP1	cell surface receptor adenylyl cyclase-associated protein 1
CRD	cysteine-rich domain
EGF-A	epidermal growth factor-like repeat-A
hQSOX1b	human quiescin sulfhydryl oxidase 1b
IL-1a	interleukin-1a
IκB	inhibitor of nuclear factor kappa B
IL-1β	interleukin-1β
LDL-R	low-density lipoprotein receptor
LOX-1	lectin-like oxidized low-density lipoprotein receptor-1
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-kappa B
MCP-1	monocyte chemoattractant protein-1
Ox-LDL	oxidized LDL
PCSK9	protein proprotein convertase subtilisin/kexin 9
PKA	protein kinase A
SREBP-2	sterol regulatory element-binding protein-2
THP-1	Tamm-Horsfall protein-1
TLR4	toll-like receptor 4
VSMC	vascular smooth muscle cells

thought to cause stimulation of the proinflammatory response within the plaque, being a potential direct link to atherosclerosis, yet the mechanisms remain unknown. Multiple recent studies have shown that there is a correlation between PCSK9 and inflammation, independent of cholesterol levels.[10–15] More specifically, PCSK9 has been shown to be associated to the TLR4 (toll-like receptor 4)/NF-κB (nuclear factor-kappa B) proinflammatory pathway. TLR4 is a transmembrane receptor involved in local or systemic immune responses, causing the activation and nuclear translocation of NF-κB, a transcription factor for a wide variety of inflammatory cytokines[13]; it is also a known mechanistic contributor of atherosclerotic inflammation.[16] It has been shown both in vivo and in vitro that overexpressing PCSK9 positively correlates to the upregulation of TLR4 and expression and activation of its downstream target NF-κB, and silencing PCSK9 has the reverse effects.

Supporting evidence has shown that PCSK9 has been found to have a unique C-terminal cysteine-rich domain (CRD) that is structurally homologous to another plasma protein, resistin.[17–19] This CRD of resistin is known to bind to and activate TLR4[20] and the cell surface receptor adenylyl CAP1 (cyclase-associated protein 1),[21,22] both triggering a proinflammatory response. PCSK9 has now also been shown to bind to CAP1 to modulate LDL-R (low-density lipoprotein receptor) degradation.

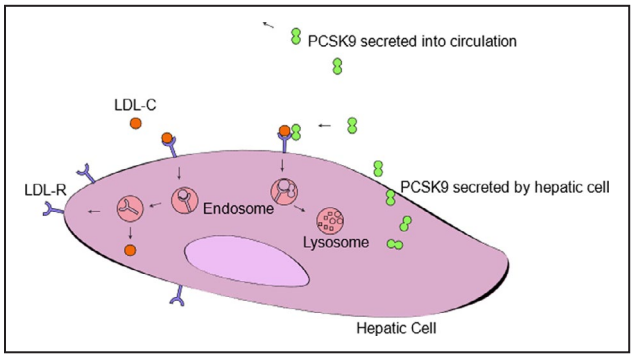

Figure 1. Secretion of PCSK9 from hepatocytes into the circulation.
PCSK9 is primarily expressed and secreted by hepatic cells, where it functions to degrade cell surface LDL-R, inhibiting cell surface receptor recycling and contributing to the decrease of plasma cholesterol clearance. LDL-C indicates low-density lipoprotein cholesterol; LDL-R, low-density lipoprotein receptor; and PCSK9, protein proprotein convertase subtilisin/kexin 9.

The goals of this review were to emphasize the undeniable evidence that PCSK9 stimulates a proinflammatory response within atherosclerotic plaque, and that this mechanism may be better understood with the investigation of other structurally related proteins that trigger similar pathways. It could also bring new knowledge to the broader field of immunology, because PCSK9 expression has been reported in some other inflammation-induced disorders in addition to atherosclerosis.[23–25]

ATHEROSCLEROTIC PLAQUE INITIATION AND PROGRESSION AND ROLE OF OXIDATIVE MODIFICATIONS

Damage to endothelial cells that line the vessel wall begins the initiation of plaque development. This endothelial damage is triggered by a wide variety of modulators inducing oxidative stress such as excess LDL-C (low-density lipoprotein cholesterol),[26] cigarette smoke toxins,[27] and high blood pressure.[28] Injury of the vessel barrier promotes deposition of circulating LDL-C (especially when modified and in excess) in endothelial cells of the arterial tunica intima, followed by a layer of vascular smooth muscle cells (VSMCs).[2,29] Accumulation of LDL-C in the intima causes a response of spontaneous oxidation, leading to the production of oxidized LDL (Ox-LDL).[30,31] The chemical modification of LDL-C causes the molecule to acquire oxidative modified specific epitopes known as damage-associated molecular patterns. These epitopes are then recognized by pattern recognition receptors such as CD36 (cluster of differentiation 36), TLR4, LOX-1 (lectin-like

oxidized low-density lipoprotein receptor-1), and CRP (C-reactive protein), stimulating a variety of immune responses such as proinflammatory cytokine production and internalization of the oxidative species by vascular and phagocytic cells.[32–35] On the other hand, low-dose reactive species like Nox4-generated hydrogen peroxide can mediate vasoprotective and antiatherosclerotic mechanisms.[36–39]

The proinflammatory response generated by Ox-LDL recruits circulating monocytes to enter the intima, where they differentiate into resident macrophages. The macrophages engulf Ox-LDL molecules, causing a phenotypical change into foam cells, ultimately resulting in cell death, proinflammatory cytokine release, and fatty lesion formation. This chronic inflammation in the arterial wall drives additional monocyte infiltration, foam cell development, plaque deposition, and eventually arterial occlusion and plaque formation. Instability and plaque injury causes myocardial infarction and thrombolytic stroke.[40] VSMCs are well-known for playing a role in atherosclerotic progression and inflammation modulation. When fatty depositions accumulate in the plaque, VMSCs migrate and phenotypically transform into resident macrophages to engulf the oxidized species, further contributing to atherosclerotic lesion development and progression.[41,42] As demonstrated here, oxidation and chronic inflammation are important driving forces of atherosclerosis, and thus atherosclerotic cardiovascular disease (CVD) (Figure 2).

The role of oxidation in atherogenesis is complex and has many contributing factors. First, LDL (low-density lipoprotein) molecules within the artery wall can be oxidized to different levels, contributing to atherosclerosis development. Minimally oxidized LDLs have a low affinity to macrophage scavenger receptors that are contributing to foam cell production. Minimally oxidized LDL is known to stimulate adhesion molecules, chemokines, and cytokines, leading to extravasation of cells into the arterial wall and further LDL oxidation.[43–46] Furthermore, minimally oxidized LDL has also been found to induce tissue factor expression in endothelial cells. Extensively oxidized LDL stimulates the proliferation of VSMCs and is recognized by macrophage scavenger receptors, leading to engulfment and formation of foam cells, directly contributing to fatty plaque accumulation.[47,48]

The oxidation of LDL is multifactorial, including many contributing factors from various cells. Lipoxygenase is an intracellular enzyme that has been found to contribute to the direct enzymatic oxidation of LDL in macrophages within the arterial walls. These enzymes can also contribute to nonenzymatic oxidation by the byproduct production of radical oxidants.[49–51] Myeloperoxidase is another common oxidizing enzyme that contributes to arteriosclerosis. Myeloperoxidase is expressed in activated neutrophils and monocytes as well as resident macrophages. This enzyme uses hydrogen peroxide and chloride to produce the cytotoxic reactive species hypochlorous acid. As with the lipoxygenase enzyme, byproducts of myeloperoxidase can generate the production of radical oxidants, further contributing to an increased oxidative state within the artery wall.[52]

Another important aspect of the role of oxidation in arteriosclerosis is its impact on HDL (high-density lipoprotein). HDL is known to be a cardioprotective molecule, because it mediates antioxidant effects to LDL and promotes reverse cholesterol transport from foam cells. The oxidative modifications of HDL cause some loss of its protective function. Various oxidants such as peroxyl and hydroxyl radicals as well as myeloperoxidase-generated oxidative species can cause HDL oxidation.[53] Free radical–mediated lipid oxidation events can generate biological aldehydes, such as malondialdehyde, 4-hydroxynonenal, and acrolein.[54,55] Malondialdehyde and 4-hydroxynonenal aldehydes are known to oxidatively modify amino acid side chains found on HDL, which in turn impairs HDL antiatherogenic functions. HDL modification by acrolein aldehydes inhibits the cholesterol transport function of HDL.[56] Thus, oxidative events aiding in atherogenesis are not only associated with LDL oxidation. These events are multifactorial, involving many cells and molecules via diverse biochemical and physiological pathways.

PCSK9s involvement in the complex oxidation process during atherogenesis is understudied. PCSK9 is known to cause the stimulation of a set of chemokines and cytokines, specifically from macrophages. This stimulation results in an increased infiltration and activation of monocytes. In addition, VSMCs are activated by PCSK9, stimulating the conversion to activated macrophages. These cellular processes can further be aggravated by oxidative stress in the arterial site by production of previously discussed oxidative enzymes. Interestingly, it has recently been shown that evolocumab (a PCSK9 inhibitor) significantly prevents cytotoxicity induced by hydrogen peroxide and reduces hydroperoxides and malondialdehyde levels in human umbilical vein endothelial cells.[57] Further investigation of PCSK9's direct involvement in the regulation of oxidative stress would bring great advancement to the field.

PCSK9 DISCOVERY AND INVOLVEMENT IN CHOLESTEROL REGULATION

PCSK9 is the ninth family member of the class of proteins known as proprotein convertases, most predominantly expressed and secreted in hepatic cells. In

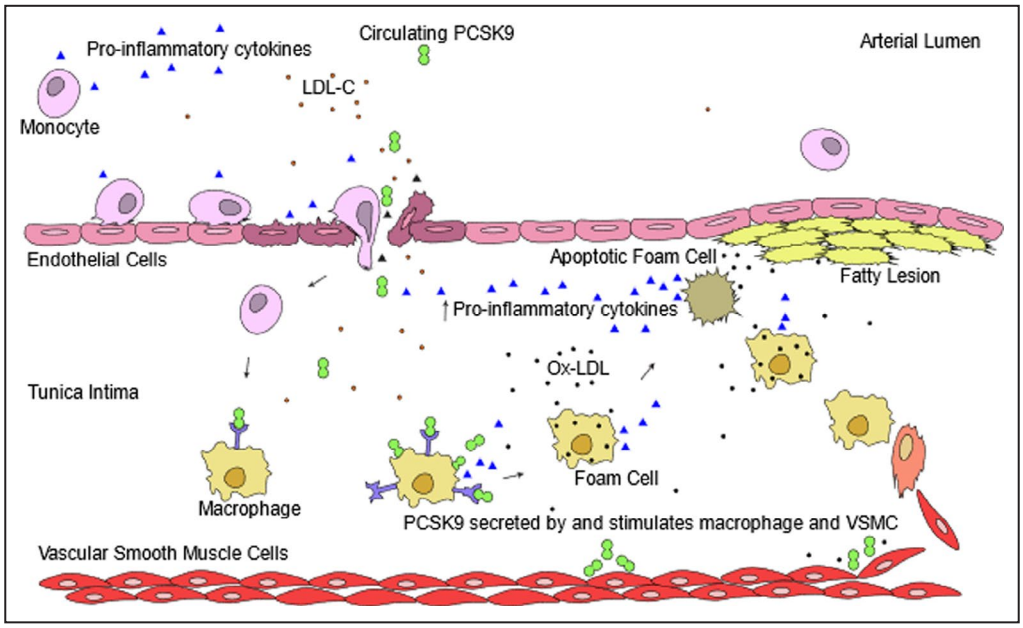

Figure 2. Schematic representation of PCSK9s involvement in atherosclerotic plaque development.

Endothelial cell damage allows infiltration of circulating LDL-C and to the intimal space. Oxidation of LDL-C in the intimal space stimulates proinflammatory activation and phagocytosis of macrophages. Engulfment of excess Ox-LDL by macrophages generates foam cells, which contributes to fatty deposits in the arterial wall, proinflammatory cytokine release, and apoptosis. Circulating PCSK9 secreted from hepatic cells also enters the intimal space, where it stimulates macrophage cells to produce proinflammatory cytokines and VSMC migration and transformation to macrophages, which in turn become apoptotic foam cells. Atherosclerotic macrophages and VSMCs also secrete PCSK9 within the plaque, contributing to increased inflammation and fatty deposition. Collectively, the proinflammatory stimulation within the plaque drives recruitment and infiltration of more circulating monocytes, feeding the cycle. LDL-C indicates low-density lipoprotein cholesterol; Ox-LDL, oxidized low-density lipoprotein; PCSK9, protein proprotein convertase subtilisin/kexin 9; and VSMC, vascular smooth muscle cells.

2003, the idea that PCSK9 is linked to cholesterol metabolism was introduced when Abifadel et al identified a novel genetic mutation found in patients with severe hypercholesterolemia. The missense mutation was found on chromosome 1 in the *PCSK9* gene, causing gain-of-function of PCSK9 and autosomal dominant familial hypercholesterolemia.[58] Further research revealed that gain-of-function mutations causing hypercholesterolemia are associated with the highest risk for CVD, whereas loss-of-function mutations are associated with hypocholesterolemia, significantly reducing the risk of CVD.[59] PCSK9 expression was then shown to be primarily regulated by the transcription factor SREBP-2 (sterol regulatory element-binding protein-2), when intracellular cholesterol levels caused changes in PCSK9 messenger RNA levels via SREBP-2.[60,61]

PCSK9's molecular mechanism on cholesterol metabolism was quickly uncovered, relieving posttranslational regulation of hepatic LDL-Rs by the binding of PCSK9 to the receptor. The colocalization of the 2 proteins causes degradation of the receptor, rather than recycling of the receptor to the cell surface, where it is used for cholesterol clearance. Cholesterol is an organic sterol lipid that must be transported in the aqueous blood stream by LDL as a polar transporter molecule. The presence of PCSK9, especially in excess, causes decreased levels of LDL-R, and thus elevates circulating levels of LDL-C, which in turn results in greater amounts deposited and oxidized in the intima space, leading to enhanced plaque development.[62,63] The specific binding site on LDL-R for PCSK9 was initially localized to the EGF-A (epidermal growth factor-like repeat-A) domain of the receptor.[64] More recently, CAP1 was identified as a new binding partner of PCSK9 and a key mediator of caveolae-dependent endocytosis and lysosomal degradation of LDL-R,[21] both resulting in inhibition of LDL-R cell surface recycling when PCSK9 is bound. After this mechanistic discovery, PCSK9 was quickly targeted for therapeutic monoclonal antibody design to reduce blood cholesterol levels in patients with elevated cholesterol levels, thus reducing the risk of atherosclerotic plaque progression.[62,65–67] In addition, novel strategies to target PCSK9 are currently being developed.[68,69] However, because of the quick chase of effective therapeutics to lower cholesterol, PCSK9 biology and its possible

influence on other physiological processes are not generally well understood.

PCSK9 has also been shown to be associated with binding to other receptors of the LDL-R family, including very low-density lipoprotein receptor (VLDL-R), CD36, apolipoprotein E receptor 2 (ApoER2), and low-density lipoprotein receptor-related protein 1 (LRP1).[70–72] Interestingly, PCSK9 was later found to be expressed in VSMCs, having a direct effect for reduction of LDL-R on macrophage cells within the arterial plaque,[73] as well as modulating phenotype, proliferation, and migration of VSMCs.[74] This led to investigations of PCSK9 within an atherosclerotic plaque and association with immune-related cells, and a novel function of its involvement in atherosclerotic inflammation.

PCSK9 AND INFLAMMATION

Recently, PCSK9 has been gaining recognition as having more physiological roles in atherosclerotic plaque development in addition to cholesterol regulation. Many studies do demonstrate that PCSK9 enhances atherosclerosis in an LDL-R–dependent manner. LDL-R–deficient mice expressing no PCSK9 or high levels of PCSK9 exhibited similar levels of plasma cholesterol and aortic cholesteryl esters accumulation to wild-type mice.[75] A limitation of this study is lack of inflammatory marker assessment in this model to differentiate the relationship of PCSK9 to cholesterol and inflammation.

Other studies show PCSK9 has a role in inflammation. It was found to be a biomarker for illness severity in patients suffering from multiple traumatic injuries (Table 1).[76–81] It has also been shown to be positively correlated to levels of circulating CRP,[25] an acute biomarker of inflammation that has interestingly been demonstrated to increase the uptake of LDL-C into arterial macrophages and act as a stronger predictor of cardiovascular disease than LDL-C levels.[82,83]

Both experimental and clinical data support the concept that systematic inflammation causes an increase of PCSK9 expression.[10] New evidence on PCSK9's alternate role in atherosclerosis points to it being linked to the chronic inflammatory state of the atherosclerotic plaque, driving plaque progression and instability. This new evidence has demonstrated that *PCSK9* is positively correlated with a various range of proinflammatory genes that drive plaque development and progression[12,13,84]; thus, it plays a role in the chronic inflammatory state of atherosclerotic plaque. Ricci et al excitingly demonstrated that PCSK9 promotes proinflammatory effects on macrophages. Incubation of THP-1 (Tamm-Horsfall protein-1)–derived macrophages as well as human primary macrophages with human recombinant PCSK9-stimulated expression of IL-1β (interleukin-1β), IL-6 (interleukin-6), TNF-α (tumor necrosis factor-α), CXCL2 (CXC motif chemokine ligand 2), and MCP-1 (monocyte chemoattractant protein-1) messenger RNA in both cell lines. In addition, THP-1 macrophages cocultured with hepatocellular carcinoma cell line G2 (HepG2) overexpressing human recombinant PCSK9 caused increased expression of TNF-α and IL-1β messenger RNA in the THP-1 macrophages. Ricci also revealed a positive correlation between PCSK9 and TNF-α plasma levels in adults.[85] These results suggest that the proinflammatory response of PCSK9 on macrophages is mainly, but not

Table 1. Clinical Research Associated With PCSK9 and Inflammation

Reference	Model type	Findings
Polisecki et al[81]	PROSPER subjects	Genetic variations of *PCSK9* caused decrease in circulating LDL-C concentration but not in CHD risk.
Le Bras et al[76]	HYPOLYTE subjects*	Illness severity in patients with severe multiple traumas is predicted by use of PCSK9 as a late biomarker.
Cheng et al [12]	Human tissue—coronary artery	Serum PCSK9 levels positively correlates to quantity of necrotic core tissue, independent of LDL-C.
Fang et al[25]	Healthy patients and patients with SLE	Patients with SLE have significantly higher circulating PCSK9 levels compared to controls. PCSK9 was found to be positively correlated to CRP concentrations.
Zhang et al[80]	Patients with CAD	PCSK9 concentration in patients with CAD was positively correlated to CRP concentrations.
Zanni et al[79]	Patients infected and noninfected with HIV	HIV-infected subjects have a significant increase of PCSK9 levels, which positively correlates with markers of systemic monocytes compared with noninfected subjects.
Li et al[78]	Patients with CAD	Plasma PCSK9 concentration is associated with WBCC.
Li et al[77]	Patients with and without CAD	Elevated PCSK9 levels are associated with elevated WBCC and CRP in patients with CAD. Severity of CAD is positively associated with PCSK9 concentration.

Composition of human clinical research indicating PCSK9's involvement in inflammation. Author, reference, model type, and key findings of each investigation are displayed. CAD indicates coronary artery disease; CHD, coronary heart disease; CRP, C-reactive protein; HYPOLYTE, hydrocortisone polytraumatise; LDL-C, low-density lipoprotein cholesterol; PCSK9, protein proprotein convertase subtilisin/kexin 9; PROSPER, Prospective Study of Pravastatin in the Elderly at Risk; SLE, systemic lupus erythematosus; and WBCC, white blood cell count.

*HYPOLYTE subjects' information can be found at ClinicalTrials.gov no. NCT00563303.

exclusively, dependent on the presence of the LDL-R. Bone marrow–derived macrophages from LDL-R$^{+/+}$ C57BL/6 mice were stimulated by human recombinant PCSK9, increasing expression of TNF-α (31.1±6.1–fold). Other in vitro studies of Ricci could be strengthened by including an LDL-R knockout in these models.

In 2012, Tang et al demonstrated that PCSK9 small interfering RNA suppresses the Ox-LDL–induced upregulation of proinflammatory cytokine expression (IL-1α, IL-6, and TNF-α) in THP-1–derived macrophages. This group also revealed that the exposure of THP-1–derived macrophages to Ox-LDL caused significant degradation of IκB-α (inhibitor of nuclear factor kappa B-α) and increased expression and nuclear translocation of NF-κB p65, which was significantly attenuated by the use of PCSK9 small interfering RNA. In combination, Tang et al demonstrated that PCSK9 small interfering RNA protects against inflammation via the inhibition of NF-κB activation in Ox-LDL–stimulated THP-1–derived macrophages.[84] In this study, Tang et al used Ox-LDL to induce proinflammatory cytokine expression. Using a stimulant that does not function via LDL-R or eliminating LDL-R from the models might give further insight into the pathway of inflammatory stimulation of PCSK9.

In 2017, Tang et al then investigated the in vivo effect of PCSK9 on TLR4 expression and NF-κB expression and nuclear translocation in atherosclerotic aortas with PCSK9 silencing (lentivirus [LV]-PCSK9 short hairpin [sh] RNA) in apolipoprotein E knockout mice. TLR4 and NF-κB expression in the aortas of the LV-PCSK9 shRNA group showed a significant downregulation compared to the control. Using immunostaining, the group was able to detect TLR4 and NF-κB in atherosclerotic lesions of the aorta sinus plaques of the LV-PCSK9 shRNA group, demonstrating a significant decrease in both proteins compared to the control (Table 2).[13,86,87] This group also showed that PCSK9 potentially regulates inflammatory cytokine secretion (TNF-α, IL-1β, and MCP1) through the activation of the toll-like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway in Raschke W et al., murine macrophage cell line 264.7 transformed by Abelson Leukemia virus (RAW264.7) macrophages. When stimulated with Ox-LDL, as expected, there was a significant increased gene expression of inflammatory cytokines as modulators of TLR4/NF-κB activation. With the addition of LV-PCSK9, the expression of these cytokines significantly increased compared to the Ox-LDL–stimulated cells. When LV-PCSK9 shRNA was introduced to the cells, there was significant decrease of production of these cytokines compared to the other 2 experimental groups. In addition, Tang et al also demonstrated that the LV-PCSK9–treated macrophages upregulated TLR4 expression and promoted the expression of p-IκBa (phosphorylated IκBa), the degradation of IκBa, and NF-κB nuclear translocation. This group then showed that LV-PCSK9 shRNA significantly attenuated all these effects.[13] The discoveries of Tang et al demonstrate that the TLR4/NF-κB pathway may be one of the major mechanisms that links PCSK9 to atherosclerotic inflammation. Further examination of the direct binding of PCSK9 to TLR4, thus activating NF-κB, must be done to confirm that this is the primary mechanism. Table 1 compiles some of the scientific research indicating PCSK9's clear involvement in inflammation (Figure 3).

Although these studies have some limitations, it does not mean that LDL-R is the only receptor mediating inflammatory stimulation for PCSK9, because other studies showed that PCSK9 causes upregulation of TLR4 (implied receptor stimulation), resulting in increased cytokine expression. It is also important to note that other studies discuss the inflammatory actions of PCSK9.[88–92] The role of PCSK9 inflammation is controversial, because clinical studies failed to demonstrate the effect of PCSK9 inhibitors on the markers of inflammation. There are also conflicting results on the independent association between PCSK9 levels and atherosclerosis in the general population. In one study, plasma PCSK9 was associated with the progression of carotid atherosclerosis,[93] whereas another study showed no correlation between PCSK9 concentration and carotid intima media thickness.[94] Intravascular imaging studies of coronary artery plaques also yielded conflicting observations. One study tantalizingly detected a correlation between plasma PCSK9 and plaque necrotic core independent of LDL-C,[12] but another study showed no effect of PCSK9 inhibitor evolocumab on plaque composition.[95] The function of PCSK9 in inflammation might be tissue specific. Lower levels of circulating PCSK9 in normolipidemic subjects are associated with adipose tissue inflammation,[96] whereas PCSK9 inhibition reduces inflammation in the liver.[97] Additional studies will clearly define the role of PCSK9 as a link between cholesterol and inflammation and how much of the inflammatory response is cholesterol independent.

ROLE OF TLR4 IN CHRONIC ARTERIAL INFLAMMATION

TLR4 is part of the toll-like receptor family of proteins, a family of membrane-spanning proteins displayed on innate immune cells such as macrophages that recognize certain pathogen-associated patterns such as lipopolysaccharide found on gram-negative bacterial cell walls. Binding of a ligand to the toll-like receptors results in the recruitment of the connecter molecule MyD88 (myeloid differentiation factor 88) to the Toll/IL-1 receptor domain of the receptor. This triggers a

Table 2. Murine Research Associated to PCSK9 and Inflammation

Reference	Model type	Findings
Feingold et al[10]	C57BL/6 mice	Inflammatory stimulants (lipopolysaccharide, zymosan, and turpentine) cause increased expression of PCSK9 mRNA in hepatic tissue.
Tang et al[13]	Apolipoprotein E knockout mice	LV-PCSK9 shRNA treatment of mice causes significantly less atherosclerotic plaque development, decreased number of macrophages, and decreased expression of vascular proinflammatory proteins (TNF-α, IL-1β, MCP-1, TLR4, and NF-κB) compared with untreated mice.
Landlinger et al[87]	APOE*3Leiden.CETP mice	AT04A anti-PCSK9 vaccine causes significant reduction of various plasma proinflammatory markers SAA, MIP-1β/CCL4, MDC/CCL22, SCF, and VEGF-A compared with control. Additionally, AT04A vaccine causes significant decrease in atherosclerotic lesion area, total lesions in the aorta, and aortic inflammation compared with control.
Kühnast et al[86]	APOE*3Leiden.CETP mice	Monocyte recruitment to atherosclerotic plaques is reduced with alirocumab treatment. Total macrophage and necrotic core content was also reduced with treatment.

Composition of murine experimental research indicating PCSK9's involvement in inflammation. Author, reference, model type, and key findings of each investigation are displayed. APOE*3Leiden indicates apolipoprotein E3-Leiden; AT04A, affitope-based vaccine; CCL4, C-C motif chemokine ligand 4; CCL22, C-C motif chemokine ligand 22; CETP indicates cholesteryl ester transfer protein; IL-1β, interleukin-1β; LV-PCSK9, lentivirus PCSK9; MCP-1, monocyte chemoattractant protein-1; MDC, macrophage-derived chemokine; MIP-1β, macrophage inflammatory protein-1β; mRNA, messenger RNA; NF-κB, nuclear factor-κB; PCSK9, protein proprotein convertase subtilisin/kexin 9; SAA, serum amyloid A; SCF, cytokine stem cell factor; shRNA, short hairpin RNA; TNF-α, tumor necrosis factor-α; TLR4, toll-like receptor 4; and VEGF-A, vascular endothelial growth factor-A.

cascade of intracellular signal transductions, leading to the activation and nuclear translocation of the transcription factor NF-κB, and the induction of a variety of proinflammatory cytokines including, IL-1, IL-6, IL-12, TNF-α, IL-1β, and MCP-1.[98] NF-κB is typically sequestered in the cytoplasm by its inhibitory protein, IκB, and prevented from interaction with nuclear localization signals. When IκB becomes p-IκB, it degrades, allowing NF-κB to receive signals from the nuclear localization signals and to be translocated to the nucleus (Table 3). The TLR4/NF-κB proinflammatory signaling pathway plays a significant role in the chronic inflammatory state of the atherosclerotic plaque, because human and murine atherosclerotic plaques express TLR4, and TLR4 expression in macrophages is upregulated in the presence of Ox-LDL.[16,99,100] Additionally, Ox-LDL sequestered in the intimal space of arterial lesions directly induces TLR4/NF-κB signaling pathways in resident macrophages, which is especially enhanced though macrophage scavenger receptor CD36.[101–105] In regard to arterial endothelial cells, TLR activation promotes lipid and white blood cell accumulation within the arterial plaque.[102]

Interestingly, in 2004, Michelsen et al discovered that the deletion of TLR4 on apoE$^{-/-}$ mice caused a significant decrease in aortic atherosclerosis, aortic sinus lipid accumulation, and macrophage infiltration.[16] TLR4's ability to facilitate such significant effects on plaque formation and progression in the absence of any pathogen-associated patterns suggest that the driving force of plaque inflammation is via endogenous ligands.

It is undeniably evident that PCSK9 plays a prominent role in the stimulation of inflammation within the atherosclerotic plaque. PCSK9 is shown to have a positive correlation to proinflammatory cytokine expression and NF-κB upregulation, which is a major mechanistic driving force of plaque inflammation. Additionally, the findings of its homologous CRD to resistin's, which is the domain responsible for the interaction between resistin and inflammatory initiation via TLR4 and CAP1, provide support for the novel function of PCSK9.

PCSK9 STRUCTURE AND STRUCTURAL HOMOLOGY TO RESISTIN

Supporting evidence linking PCSK9 to inflammation was found by the discovery of a rather unique CDR domain on PCSK9, that is structurally homologous to the protein resistin's one-of-a-kind CDR,[18,19] which is responsible for proinflammatory stimulation similar to PCSK9 function. The structural homology of the C-terminal cysteine-rich domain from PCSK9 and the resistin homotrimer is shown in Figure 4. Imaging and superposition of the molecules have been realized with PyMOL.[106] PCSK9 belongs to the proprotein convertase family, where all zymogen forms consist of an N-terminal pro-domain, a subtilisin-like catalytic domain capable of protease function, and a C-terminal domain.[107] The pro-domain of proprotein convertases functions as an intramolecular chaperone in the endoplasmic reticulum, assisting in proper folding of the catalytic domain before secretion of the polypeptide. After proper folding of the catalytic domain, the

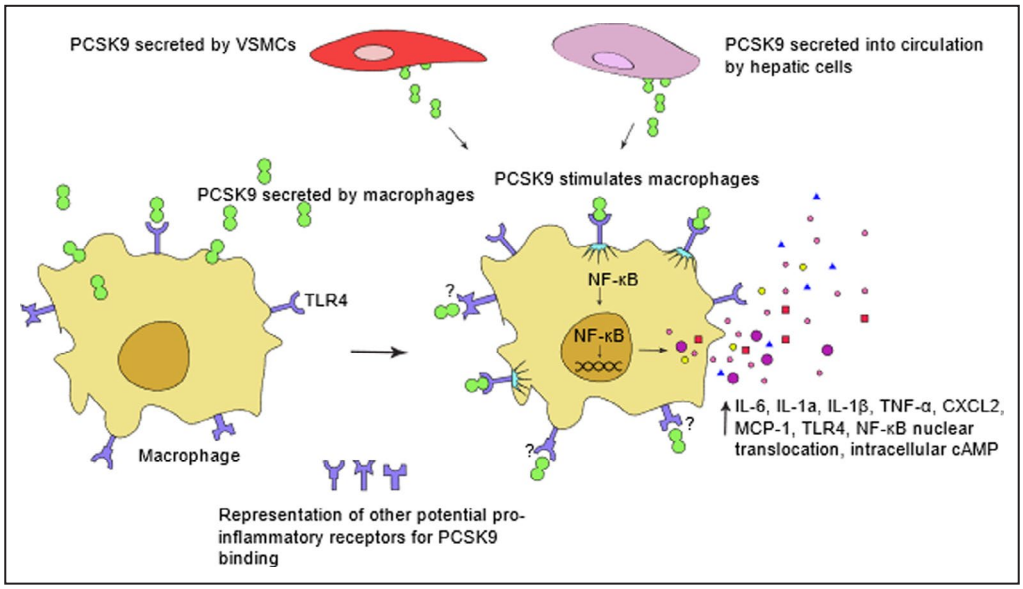

Figure 3. Activation of macrophages within atherosclerotic plaque by interaction with PCSK9. PCSK9 is now known to stimulate proinflammatory effects within an atherosclerotic plaque. PCSK9 is expressed and secreted by hepatic cells, macrophages, and vascular smooth muscle cells. PCSK9 activates macrophage proinflammatory effects by cell surface receptor binding of TLR4 and potentially several other inflammatory stimulating receptors. This graphic represents the binding of PCSK9 to and activation of TLR4, triggering NF-κB activation and nuclear translocation. Nuclear translocation of the transcription factor NF-κB stimulates the expression and secretion of a variety of proinflammatory cytokines, contributing to increased monocyte infiltration and plaque deposition. Other receptors on the macrophage represent additional proinflammatory-stimulating binding partners for PCSK9 within cells in the atherosclerotic plaque. CXCL2 indicates CXC motif chemokine ligand 2; IL-1a, interleukin-1a; IL-1β, interleukin-1β; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-kappa B; PCSK9, protein proprotein convertase subtilisin/kexin 9; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; and VSMCs, vascular smooth muscle cells.

pro-domain undergoes autocatalysis, breaking the covalent bond anchoring the catalytic and pro-domains. The protein then undergoes a second catalytic cleavage in the pro-domain, allowing full access, thus function, of the protease catalytic active site. PCSK9 is a unique proprotein convertase molecule, because it does not undergo the second cleavage event, allowing the pro-domain to remain noncovalently associated with the catalytic active site. This blocking of the catalytic triad interestingly results in the protein being

Table 3. In Vitro Cell Culture Research on PCSK9 and Inflammation

Reference	Model type	Findings
Tang et al[84]	RAW264.7 cell line	PCSK9 overexpression in cultured Ox-LDL–induced macrophages caused increase expression of proinflammatory cytokines, TLR4, and IκBα, IκBα degradation, and nuclear translocation of NF-κB.
Ricci et al[85]	THP-1–derived macrophages and human primary macrophages	Incubation of THP-1–derived macrophages and human primary macrophages with PCSK9 causes a significant increased expression of IL-1β, IL-6, TNF-α, CXCL2, and MCP-1 mRNA. THP-1–derived macrophages cocultured with HepG2s overexpressing hPCSK9 causes an increased expression of TNF-α and IL-1β mRNA in macrophages.
Tang et al[84]	THP-1–derived macrophages	Ox-LDL–stimulated macrophages express PCSK9 in a dose-dependent manner and cause nuclear translocation of NF-κB. PCSK9 small interfering RNA in Ox-LDL stimulated THP-1–derived macrophages, suppressed expression of inflammatory cytokines, and attenuated NF-κB nuclear translocation.

Composition of some in vitro cell culture experimental research indicating PCSK9's involvement in inflammation. Author, reference, model type, and key findings of each investigation are displayed. CXCL2 indicates CXC motif chemokine ligand 2; IκBα, inhibitor of nuclear factor kappa Bα; IL-1β, interleukin-1β; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; mRNA, messenger RNA; NF-κB, nuclear factor-kappaB; Ox-LDL, oxidized low-density lipoprotein; PCSK9, protein proprotein convertase subtilisin/kexin 9; THP-1, Tamm-Horsfall protein-1; TLR4, toll-like receptor 4; and TNF-α, tumor necrosis factor-α.

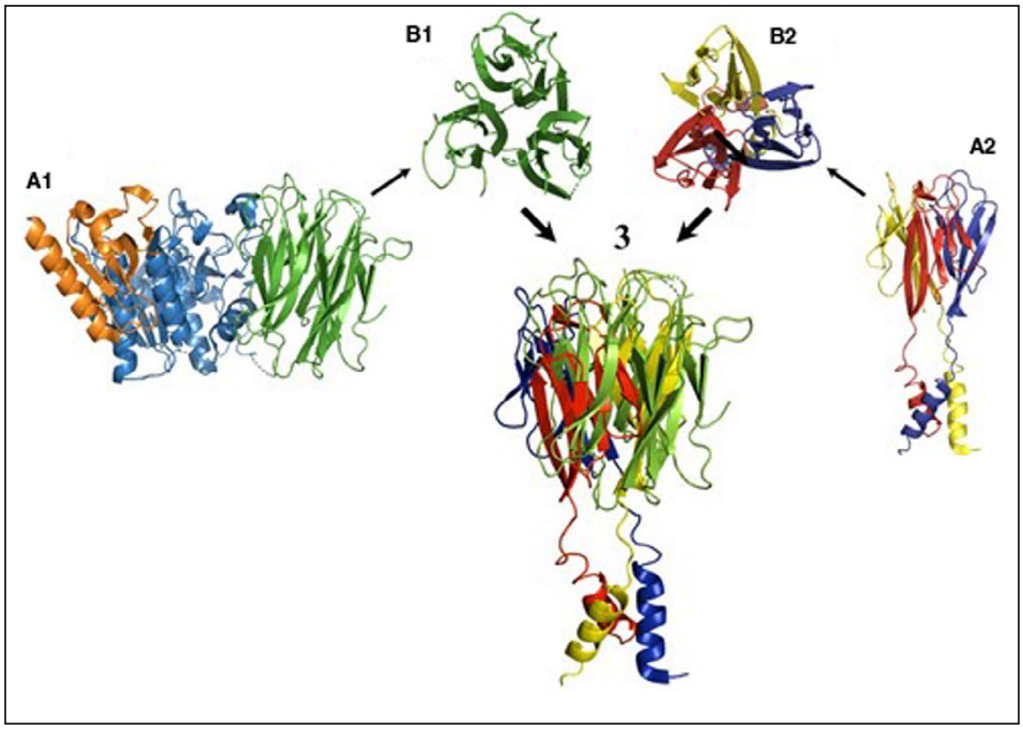

Figure 4. Structural homology of the C-terminal cysteine-rich domain from PCSK9 and the resistin homotrimer.
The crystal structure of PCSK9 (**1A**) contains an inhibitory prodomain (orange), a serine protease domain (light blue), and a C-terminal CRD (green). The resistin (**2A**) homotrimer (domains are colored red, blue, and yellow) forms a 3-stranded α-helical coiled-coil under a 6-stranded β-strand jelly-roll structure. The structural homology of the CRD region of PCSK9 and the resistin homotrimer are shown from a top view (**1B** and **2B**). Superposition of the CRD of PCSK9 and the resistin homotrimer (**3**, colors of structures according to **1A** and **1B**) leads to rotation of the nonconserved region of resistin.[18] For a better visualization, the inhibitory prodomain and the serine protease domain have been excluded from the superposition. Structures are shown in a cartoon representation. Imaging and superposition of the molecules have been realized with PyMOL.[106] CRD indicates cysteine-rich domain; and PCSK9, protein proprotein convertase subtilisin/kexin 9.

prohibited from using its protease functioning abilities. Another unique structural aspect of PCSK9 is that its C-terminal domain is unlike the other proprotein convertase proteins. PCSK9's C-terminal consists of an extremely distinctive CRD. The unique C-terminal domain consists of 3 subdomains or modules (M1–M3) that are densely packed and are associated via a pseudo 3-fold axis, with a 6-stranded, 2-sheet β-sandwich formed by each subdomain.[17] The cysteines found in each module bind in a 1 to 6, 2 to 5, 3 to 4 pattern between β-sheets β1 to β6, β2 to β6, and β3 to β5, respectively.[18] This noteworthy 3-jelly-roll structure of the CRD of PCSK9 has only been recognized in 1 other molecule, the metabolic protein resistin.[18,108] The resistin molecule contains a 3-stranded, α-helical, coiled-coil just under a 3-stranded, β-strand jelly-roll structure, the only structural difference in C-terminal domains of the 2 molecules being the coiled-coil component of resistin, where this is lacking in PCSK9.[18] This exciting evidence, displaying PCSK9's CRD exclusive structural homology to resistin, is a critical link in understanding the physiology of PCSK9s involvement in atherosclerotic proinflammation, because the homologous domain of resistin is associated with proinflammatory stimulation within atherosclerotic plaques.

REDOX MODIFICATIONS—EFFECTS ON RESISTIN AND PCSK9

Because resistin's inflammatory stimulating function takes place via its CRD, which is also speculated for PCSK9, it is important to discuss how redox modifications of these cysteines modulate function. Disulfide bonds are formed during posttranslational modification within the endoplasmic reticulum, via oxidation of sulfhydryl groups between 2 cysteines. Disulfide bond modifications promote proper protein folding and enhanced protein stability.[109] Disulfide bonds can be modulated once the protein is in its functional location because of the presence of enzymes or the

surrounding environment. Highly oxidative environments favor disulfide bond formation.

Resistin is part of the *found in inflammatory zone* (*FIZZ*) family of genes, which play a role in inflammation. The oxidative state of some of these FIZZ proteins has been studied, and it is seen that a highly oxidative environment is favorable for protein function. In the endoplasmic reticulum, sulfhydryl oxidases catalyze disulfide bond formation by reduction of molecular oxygen to hydrogen peroxide. hQSOX1b (human quiescin sulfhydryl oxidase 1b) presence was found to be critical to biological function of the resistin-like protein mFIZZ1 expressed in the wheat germ embryo. In the absence of this enzyme, biological function of murine found in inflammatory zone 1 (mFIZZ1) was lost.[110]

Because resistin has been linked to insulin resistance, interestingly, it has been demonstrated that oxidative stress may enhance insulin resistance and have impact on endogenous expression of resistin in the adipocyte.[111] Also, it has been shown that serum resistin can be significantly reduced after short-term antioxidant treatment with ascorbic acid, further indicating that importance of oxidative state of this cysteine rich protein.[112]

Little is known about the redox state of PCSK9 and how this affects its function. It can be speculated that the oxidative state plays a role on PCSK9 function, because the CRD of this protein is so structurally homologous to resistin's active CRD. If this is assumed, the atherosclerotic environment has high oxidative stress, which would lead to retention/formation of disulfide bonds in PCSK9. Because PCSK9 is known to be functional in the atherosclerotic environment, it can be speculated that disulfide bonds of the CRD could aid in biological function.

RESISTIN AND PCSK9—RELATION TO INFLAMMATION

Resistin was initially studied in mice, where it was found to be an adipokine associated with obesity and insulin resistance.[113] More recent research has unveiled that resistin in humans is a potential key player in atherosclerotic proinflammatory stimulation and lipid accumulation. It was demonstrated that macrophages within atherosclerotic lesions secrete resistin, directly effecting endothelial function and VSMC migration to the lesion.[114,115] Cho et al then demonstrated that resistin is expressed in atherosclerotic lesions and causes stimulation of monocytes, endothelial cells, and VSMCs, inducing inflammation within the arterial plaque.[116] Resistin has also been found to promote lipid internalization in human macrophages via CD36 upregulation as well and acts as a regulator of macrophage phenotypic transformation to foam cells within the intima space.[117,118] Resistin was also shown to be a potent proinflammatory cytokine inducer (IL-6, IL-12, and TNF-α) via NF-κB regulation.[119,120] Recently, signal transducer and activator of transcription 3 (STAT3) was identified a regulator of leptin- and resistin-mediated transcriptional induction of PCSK9.[121] Furthermore, interferon-γ and the suppressor of cytokine signaling 3 (SOCS3) pathways are involved in the PCSK9 activation.[122,123] Additionally, resistin was found to be positively correlated to the inflammatory biomarker CRP, just as PCSK9 is.[124] Potential similarities of biological effects of PCSK9 and resistin might involve proinflammatory effects. The one-of-a-kind CRD domain of resistin that can trigger these immense proinflammatory effects strongly suggests that PCSK9 may function in the same manner, by use of the homologous CRD domain, because PCSK9 seems to trigger many of the same inflammatory responses as resistin. Investigation of the mechanism of resistin's distinctive CRD domains role in inflammation stimulation revealed that the CRD domain can bind to and trigger inflammation through CAP1 and TLR4. CAP1 is a cell surface receptor that mediates inflammatory response for human monocytes. The direct binding of human resistin via the CRD domain to CAP1 in monocytes specifically upregulates cAMP, PKA (protein kinase A) activity, and NF-κB–related transcription of inflammatory cytokines.[22] It was recently speculated and confirmed that PCSK9 also binds to CAP1 in the same manner as resistin, via the homologous CRD domain. Jang et al demonstrated with a variety of molecular and biophysical techniques that PCSK9 binds CAP1 in human liver and kidney cells via the CRD, specifically to facilitate endocytosis and lysosomal degradation of LDL-R.[21] Although PCSK9 have been shown to now bind CAP1 via its CRD domain in liver and kidney cells, this research excludes examination of this interaction in regard to proinflammatory stimulation, specifically in mononucleated immune cells.

Because resistin has been shown to bind CAP1, further in vitro binding assays were performed between CAP1 mutants and rhResistin to gain insights of where this binding occurs on the receptor. The 293A cells of various CAP1 histidine (His)-tagged mutants were treated with recombinant human resistin (rhResistin), followed by immunoprecipitation with anti-His antibodies. Western blotting was performed with both anti-resistin and anti-His antibodies. Results demonstrated that human resistin binds to CAP1 via the src-homology 3 (SH3) binding domain. Various molecular modeling schemes supported these findings.[22]

As this C-terminal domain of resistin, which binds SH3 of CAP1, has structural homology with the CRD of PCSK9, further investigation was done to see if PCSK9 binds this same domain on CAP1. Coimmunoprecipitation of human PCSK9-flag™

segment A (hPCSK9-FlagA) on a variety of CAP1 mutants were tested in human embryonic kidney (HEK) 293 cells. PCSK9 interacted with all mutants and wild-type CAP1 except for the mutant with a deletion of src homology 3 binding domain (SH3BD), suggesting that CAP1 binds PCSK9 via this domain as resistin does.[21]

Importantly, resistin was also found to cause LDL-R degradation, in the absence of PSCK9. HepG2 cells incubated with PCSK9 small interfering RNA caused a significant increase of LDL-R. When these cells included the addition of resistin, significant LDL increase was ablated. This further supports the speculation that resistin and PCSK9 may have the same physiological mechanisms.[125]

The proinflammatory effects of resistin in regard to TLR4 activation were also investigated, demonstrating that resistin binds to TLR4 specifically with the CDR domain, triggering NF-κB nuclear translocation and proinflammatory response.[20,126] Future studies should be conducted to investigate PCSK9's likely direct binding to TLR4 via the CRD domain, to bring insight on its role in atherosclerotic inflammation.

CONCLUSIONS

CVD is the leading cause of death in the world. Initiation and advancement of CVD is caused by the chronic inflammatory disease of the large arteries, atherosclerosis. Atherosclerosis is the development of fatty lesions within arterial walls, driven by chronic inflammation, leading to arterial obstruction or thrombolytic rupture of unstable plaque. It is well known that oxidative stress and the chronic inflammatory state of the plaque is responsible for the cycle of mononuclear cell infiltration, fatty sheath formation, and inflammatory cytokine release. However, not all players and mechanistic principles in this cycle are known or fully understood.

Exciting novel findings suggest that the proprotein PCSK9 is one of the unknown major players in atherosclerotic inflammation. Although initially found to regulate cholesterol metabolism, accumulating evidence demonstrates that PCSK9 is expressed within the plaque and is involved in the modulation of gene expression of a variety of proinflammatory proteins, as summarized in Table 1.

This evidence linking PCSK9 and inflammation is supported though the discovery that PCSK9s rare C-terminal CRD has incredible structural homology to a domain on resistin that is responsible for proinflammatory stimulation, similar to PCSK9's effect. This homologous domain on resistin is known to bind and activate both CAP1 and TLR4 (pattern recognition receptors found on most cell surfaces). CAP1 and TLR4 are both stimulated by the binding resistin's CRD, ultimately causing activation and nuclear translocation of NF-κB, a transcription factor for an assortment of proinflammatory cytokines. Notably, PCSK9 was found to bind to CAP1 in human liver and kidney cells though the CRD, to eliminate cell surface recycling of and to facilitate degradation of LDL-R.[21] This evidence may further contribute to the potential mechanisms of PCSK9 and inflammation, but investigation of PCSK9's CRD binding to and activation of CAP1 in inflammatory-related cells is nonexistent. Even more so, the investigation of TLR4 and PCSK9 in atherosclerotic plaque progression is needed. Although resistin's C-terminal CRD is known to bind and stimulate TLR4, the homologous CRD of PCSK9 and its potential direct interaction with TLR4 has, to the best of our knowledge, yet to be explored.

Because protein folding and structure directly dictate protein function, it is not an implausible idea to examine structural characteristics of PCSK9 to understand mechanistic aspects of its involvement in atherosclerotic inflammation. It is now undeniable that PCSK9 plays a role in the cyclic chronic inflammatory state of a fatty lesion, but can we link structural domains/motifs of this protein to others that are known to directly cause inflammation? It is especially important to note that unlike all other convertases, interestingly, PCSK9's catalytic active site is sterically hindered by the prodomain through noncovalent interactions. Being the only convertase enzyme that primarily causes its effects without use of the catalytic domain, and with multiple different binding domains recognized, raises interest and speculation on how this molecule is involved with atherosclerotic chronic inflammation. Insight into the direct mechanism of PCSK9, oxidation, and inflammation will help fill in some of the gaps in knowledge of full comprehension of the chronic inflammatory state of atherosclerosis. Bringing deeper knowledge to this subject can immensely contribute to the health and well-being of many individuals now and especially in the future, because this intricate and complicated chronic oxidation and inflammation are the major contributors to CVD, and thus morbidity and mortality worldwide. Targeting the alleviated oxidation and inflammation might contribute to the beneficial effects of PCSK9 inhibition in CVDs.

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None.

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