Cardiovascular diseases (CVD) represent the most important health challenges for modern aging societies. Approximately 17 million people die of CVD each year, which represents ≈30% of all deaths. Atherosclerosis, the underlying pathology of CVD, is increasing in prevalence worldwide because of the adoption of Western life-styles.1 Treatment for atherosclerosis includes medical therapy, the mechanical reversal of arterial stenosis, or artery bypass graft surgery. Preventive measures to reduce cardiovascular risk factors and cholesterol-lowering therapies are effective in delaying atherosclerotic disease;2 yet a considerable residual risk for cardiovascular events remains. A better understanding of the pathogenic mechanisms that lead to atherosclerosis will be a key to identify novel therapeutic targets that can be clinically tested.

Atherosclerotic lesions contain large amounts of cholesterol, cholesteryl esters, and cholesterol crystals. It is well established that high blood cholesterol levels are causally linked to the pathogenesis of atherosclerosis and hyperlipoproteinemia because of genetic defects is known to cause accelerated atherosclerosis. Furthermore, atherosclerosis-like vascular lesions can be experimentally induced in animals fed a high-cholesterol diet.3 On the contrary, lowering levels of serum cholesterol slows down atherogenesis, which can cause plaque regression and reduces the overall risk of cardiovascular events.4

In addition to excessive amounts of lipids, atherosclerotic lesions harbor all classes of immune cells and serum levels of acute-phase reactants and inflammatory mediators are linked to the risk of coronary heart disease.5 Atherosclerosis

Danger Signaling in Atherosclerosis

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Abstract: All aspects of the pathogenesis of atherosclerosis are critically influenced by the inflammatory response in vascular plaques. Research in the field of innate immunity from the past 2 decades has uncovered many novel mechanisms elucidating how immune cells sense microbes, tissue damage, and metabolic derangements. Here, we summarize which triggers of innate immunity appear during atherogenesis and by which pathways they can contribute to inflammation in atherosclerotic plaques. The increased understanding gained from studies assessing how immune activation is associated with the pathogenesis of atherosclerosis has provided many novel targets for potential therapeutic intervention. Excitingly, the concept that inflammation may be the core of cardiovascular disease is currently being clinically evaluated and will probably encourage further studies in this area. (Circ Res. 2015;116:323-340. DOI: 10.1161/CIRCRESAHA.116.301135.)

Key Words: atherosclerosis ■ cardiovascular diseases ■ inflammation ■ innate immunity ■ pattern recognition receptors

Cardiovascular diseases (CVD) represent the most important health challenges for modern aging societies. Approximately 17 million people die of CVD each year, which represents ≈30% of all deaths. Atherosclerosis, the underlying pathology of CVD, is increasing in prevalence worldwide because of the adoption of Western life-styles.1 Treatment for atherosclerosis includes medical therapy, the mechanical reversal of arterial stenosis, or artery bypass graft surgery. Preventive measures to reduce cardiovascular risk factors and cholesterol-lowering therapies are effective in delaying atherosclerotic disease;2 yet a considerable residual risk for cardiovascular events remains. A better understanding of the pathogenic mechanisms that lead to atherosclerosis will be a key to identify novel therapeutic targets that can be clinically tested.

Atherosclerotic lesions contain large amounts of cholesterol, cholesteryl esters, and cholesterol crystals. It is well established that high blood cholesterol levels are causally linked to the pathogenesis of atherosclerosis and hyperlipoproteinemia because of genetic defects is known to cause accelerated atherosclerosis. Furthermore, atherosclerosis-like vascular lesions can be experimentally induced in animals fed a high-cholesterol diet.3 On the contrary, lowering levels of serum cholesterol slows down atherogenesis, which can cause plaque regression and reduces the overall risk of cardiovascular events.4

In addition to excessive amounts of lipids, atherosclerotic lesions harbor all classes of immune cells and serum levels of acute-phase reactants and inflammatory mediators are linked to the risk of coronary heart disease.5 Atherosclerosis
Multicellular organisms are constantly exposed to microbial molecules that share specific biochemical features and usually represent molecular motifs or patterns of microbial signature molecules, such as lipopolysaccharides, bacterial flagellin, lipoproteins, peptidoglycans, or nucleic acid variants. Atherosclerotic lesions exhibit features of tissue infections and were, therefore, even described with the word abscess by Leary in 1935. Not surprisingly, it has long been debated whether bacterial products or infections are causally linked to the pathogenesis of atherosclerosis.

Indeed, both viral and bacterial infections have been implicated in the pathogenesis of atherosclerosis for both the induction and perpetuation of inflammatory responses in atherosclerotic lesions. Epidemiological data suggest that pathogenic infections can promote atherosclerotic events, and both myocardial infarction and stroke are increased during acute infections. In addition, many studies have documented that the risk of developing atherosclerosis is correlated with chronic infections by various pathogens, including bacteria such as Chlamydia pneumoniae, Helicobacter pylori, and Porphyromonas gingivalis and viruses such as cytomegalovirus, HIV, and influenza A virus (Figure 1). Here, we will highlight the pathogens whose link to CVD has been well studied.

**Bacteria**

*Chlamydia pneumoniae* is an obligate intracellular Gram-negative bacterium that infects epithelial cells and macrophages. It was the first infectious organism detected within cells of human atherosclerotic plaques and is one of the few pathogens from which viable organisms have been isolated from vessel lesions. Although clinical trials targeting *C pneumoniae* have...
failed to demonstrate an atheroprotective effect,12–15 many studies using experimental animal models have shown an increase of aortic cytokines and acute-phase proteins, as well as accelerated atherogenesis after respiratory infection with *C. pneumoniae*.16–18 This increase of atherosclerotic lesion development in response to *C. pneumoniae* is abolished in the absence of toll-like receptors (TLRs) 2 and 4, myeloid differentiation primary response protein 88 (MyD88),20 the tumor necrosis factor (TNF)-α-p55 receptor,21 as well as interleukin (IL)-17A22 in mice. Furthermore, *C. pneumoniae* infection was found to reduce the anti-inflammatory properties of high-density lipoprotein (HDL).23 Interestingly, T cells reactive to both human heat shock protein (HSP) 60 and *C. pneumoniae* 60-kDa HSP have been isolated from human plaques, suggesting that *C. pneumoniae* may also contribute to atherogenesis via molecular mimicry between bacterial and self-antigens, such as HSPs.24,25

Periodontal disease is an important risk factor for total cerebrovascular disease and, in particular, nonhemorrhagic stroke.26 Many periodontal organisms associated with CVD have been found in human atherosclerotic lesions by immunocytochemistry and polymerase chain reaction, including *P. gingivalis*, *Streptococcus sanguis*, *Fusobacterium nucleatum*, *Tannerella forsythia*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Bacteroides forsythus*.27 Despite the failure to isolate viable organisms from human plaques, both the clinical and experimental data support that periodontal organisms have significant effects on the development of atherosclerosis. Furthermore, periodontal disease is associated with an increase in systemic cytokines and acute-phase proteins and could, therefore, also have indirect effects on atherogenesis.28 For example, C-reactive protein levels are significantly elevated in patients with both periodontal disease and CVD,29 and treatment of periodontal disease reduces plasma haptoglobin, IL-18, and interferon (IFN)-γ levels.30 Interestingly, oral challenge of apolipoprotein E (ApoE)−/− mice with *P. gingivalis*, in a manner that elicits periodontal disease, accelerates atheroma formation.31,32 Of note, *P. gingivalis* has also been reported to accelerate lesion formation via a TLR2-dependent mechanism.33

Besides, its well-established role as a cause of gastritis, *H. pylori* is also considered to be a contributor to atherosclerosis. Although *H. pylori* DNA has been identified in atherosclerotic plaques,34,35 viable organisms could not be isolated. Pharmacological eradication of *H. pylori*, however, reduces systemic cytokines and significantly reduced restenosis in patients after percutaneous transluminal coronary angioplasty.36 In addition, *H. pylori* eradication is associated with a reduction of secondary coronary events in patients with CVD.37 In the South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA) trial, a significant reduction in C-reactive protein and improvement in event-free survival was reported with combined treatment for both *C. pneumoniae* and *H. pylori*.38 However, data from experimental mouse models with C57Bl/6, LDL receptor (LDLR)+/+ or ApoE−/− and LDLR−/− mice are controversial and inconclusive.39 Overall, the strength of the data linking *H. pylori* to atherogenesis is modest, especially in comparison with what has been reported for *C. pneumoniae* and *P. gingivalis*.

Although products of other bacteria, including *Streptococcus pneumoniae*, *Enterobacter hormaechei*, *Borrelia burgdorferi*, and *Mycoplasma pneumoniae*, have been detected in atherosclerotic plaques,25 there is no associative data linking them to atherogenesis.

**Viruses**

Viral nucleic acids and envelope proteins can also act as danger signals in atherosclerosis. Infection with influenza virus, for instance, is associated with acute coronary syndrome and induction of fatal myocardial infarctions.40 Large studies have also shown that influenza vaccination has a marked protective effect against primary and secondary cardiovascular events.41–43 Despite these clear protective properties of influenza vaccination, the data supporting a direct effect of influenza virus on atherosclerosis is weak. To date, the presence of influenza viral DNA or protein within human atherosclerotic plaques has not been reported. Only short-term studies of the effects of influenza A infection on recruitment of proinflammatory cells to vascular plaques in ApoE−/− and LDLR−/− mice have shown increases in both macrophages and lymphocytes. This suggests that influenza A infection accelerates the development of early atherosclerotic lesions.44,45 Recruitment of inflammatory cells is associated with influenza virus DNA in the aorta and with increased plasma IL-6 levels.43 Interestingly, influenza A infection in mice alters the relative levels of paraoxonase, platelet-activating factor acetylhydrolase, andapolipoprotein J/clusterin in HDL, causing HDL to lose its anti-inflammatory properties.46

Cytomegalovirus is a herpes virus with a high prevalence in the general population. Cytomegalovirus is, therefore, often detected in human plaques,47–49 but only 1 study has shown a direct association between CVD mortality and cytomegalovirus antibody levels.50 However, in experimental studies, murine cytomegalovirus infection in ApoE−/− mice was associated with accelerated lesion development and elevated plasma cytokines, such as IFN-γ and TNF-α.51–53

Although several studies have reported a positive association between HIV infection and the extent of atherosclerosis in coronary arteries,54 it is difficult to discern direct from indirect mechanisms. Antiretroviral therapy has also been shown to have independent effects on lesion development55 and high activity antiretroviral therapy is associated with the hallmarks of atherosclerosis, including lipodystrophy, central adiposity, hyperlipidemia, insulin resistance, and endothelial dysfunction.56,57 Moreover, HIV infection is potentially associated with profound changes in lipid and lipoprotein metabolism in the liver and because HIV infection of macrophages impairs reverse cholesterol transport and facilitates foam cell formation by inhibiting ATP-binding cassette transporter A1–mediated cholesterol efflux.58 Evaluations of atherosclerotic plaques from HIV-infected individuals at autopsy revealed accelerated disease, extensive lipid deposition, and calcification, as well as prominent inflammatory infiltration.59,60

Viral infections have also been used in animal models to induce atherosclerotic plaques that closely resemble those found in human arterial lesions.61 Of note, atherosclerosis even
develops under normocholesterolemic conditions in chickens on infection with Marek disease herpesvirus.62

Even though the evidence for pathogens as danger signals and inducers of atherosclerosis is profound and consistently increasing, several questions remain to be answered. First, it needs to be established if the suggested pathogens have a direct causative effect within the lesion or if the effect on lesion inflammation is mediated through a systemic proinflammatory response. Second, the time of infection and its relation to atherogenesis has not yet been well studied. Acute or recurring infections may affect existing vulnerable plaques and thereby contribute to cardiovascular events without being involved in plaque development in the first place. Third, the direct link between pathogen, danger signal, and host response needs to be identified. Although it is conceivable that infections can directly or indirectly contribute to atherogenesis, studies in germ-free animals have provided evidence that microorganisms are not required in Western diet–induced atherosclerosis in murine models.81

Our current concept dismisses a single pathogen to be responsible for the development of atherosclerotic lesions and rather suggests that recurring infections with multiple organisms or the total infectious burden with its direct and indirect mechanisms is of clinical significance. Koch postulate for a causative relationship between a microbe and a disease, such as atherosclerosis, cannot, therefore, be fulfilled easily. Modern deep sequencing techniques were applied to the atherosclerotic lesions of humans recently. These data reveal that bacterial DNA can indeed be identified in a large proportion of endarterectomy samples. Of note, most phylotypes identified are not cultivable to date, therefore, currently only association studies can be performed. Yet, these studies have revealed that certain microbiota found in atherosclerotic plaques correlated to those found in the oral cavity and the gut within the same individual, suggesting that bacteria in atherosclerotic lesions derive from the oral cavity, the gut, or even the skin.64–66 These exciting new data open the possibility that previously undetected microbes could indeed contribute to atherogenesis.

Another mechanism by which microbes could influence atherogenesis is their ability to alter the host metabolome. It was found that the gut microbiome in patients with symptomatic atherosclerotic diseases is altered when compared with healthy controls. Furthermore, microbial changes in symptomatic patients correlates to an increased inflammatory status of the host.67 Recent innovative studies have revealed a mechanistic link between diet, the gut microbiome, and an increased risk for the development of atherosclerosis. Trimethylamine-N-oxide is a metabolite of dietary choline, which can be found abundantly in eggs, liver, or red meats. Gut microbiota from omnivores, but not vegetarians, effectively convert choline to trimethylamine, which in turn, is converted by flavin monoxygenase enzymes in the liver to the proatherogenic metabolite trimethylamine-N-oxide. Chronic dietary supplementation of t-carnitine, an abundant nutrient in red meat, which contains a trimethylamine structure similar to that of choline, altered cecal microbial composition, markedly enhanced synthesis of TMA (trimethylamine) and trimethylamine-N-oxide, and increased atherosclerosis in mice. These effects were not detectable if intestinal microbiota was concurrently suppressed.68,69

Together, these studies suggest that microbes can influence atherogenesis by various direct or indirect means and, therefore, they should be taken into account as contributors to atherosclerosis progression.

**Sterile Triggers in Atherosclerosis**

Infectious organisms are mostly recognized by the immune system through the presence of molecules that are either not synthesized by the host or that differ enough in their molecular structure to be distinguished from self-molecules. The immune system is also able to detect altered self-molecules or the appearance of self-molecules that are spatially separated from the pattern recognition receptors (PRRs) in homeostatic conditions. These host-derived molecules are also called danger signals or danger-associated molecular patterns (DAMPs). DAMPs can result from tissue damage, metabolic dysfunction, or environmental stressors. In addition, DAMPs can arise during infections when microbes cause cellular and tissue damage.

Numerous danger signals can mediate a proinflammatory vascular response and have been linked to the development of atherosclerotic disease. For instance, crystalline cholesterol deposits, calcium precipitates, and debris from dead cells are readily found in the necrotic core and elsewhere in atherosclerotic lesions. Furthermore, recent studies have demonstrated that many vascular danger signals, such as extracellular matrix (ECM) components, lipids, and lipoproteins that are modified by oxidation or glycation. In this section, we will summarize the role of DAMPs in atherogenesis.

**Extracellular Matrix**

Degradation products of ECM macromolecules represent danger signals arising after tissue damage or during tissue remodeling. These components derived from the ECM can induce inflammatory immune responses in the vascular system. One such component is integrin-binding fibronectin, which is composed of 2 nearly identical splice products from a single gene that are linked by a pair of disulfide bonds. One of the spliced exons is the extra domain A that has been shown to signal via TLR4.70 Mice lacking extra domain A develop smaller atherosclerotic lesions when compared with EDA+/+ littermates.71,72 Of note, fibronectin also has protective properties and ApoE−/− mice deficient in hepatocyte-derived plasma fibronectin displayed smaller fibronectin depositions in atherosclerosis-prone areas, lacked vascular smooth muscle cells, and failed to develop a fibrous cap in vascular lesions.73 To what extent circulating fibronectin mediates activation of innate immune pathways is not known.

Another potential endogenous danger signal is hyaluronan. It is an abundant glycosaminoglycan of the ECM that accumulates in human atherosclerotic plaques and promotes vascular inflammation by activation of TLR2 and TLR4.74,75 Extravascular fibrinogen deposits are an early and persistent hallmark of atherosclerosis and can stimulate macrophage chemo- kine secretion by binding to TLR4.76 Furthermore, degradation products of heparan sulfate, a polysaccharide expressed
by all cells, are released on tissue damage and can activate dendritic cells by induction of TLR4 signaling. In addition to these characterized immunostimulatory matrix degradation products, there are probably other ECM components that can modulate the inflammatory response in atherosclerotic vessel walls. Recently, mass spectrometric analysis of secreted products from activated macrophages has identified a large number of proteases that are released from macrophages in a cell stimulation–specific manner. Hence, it seems that the activation status of macrophages can directly influence the digestive and thereby contribute not only to the tissue remodeling but also to the inflammatory response via the generation of immune-modulating ECM-degradation products.

Beyond ECM-degradation products, the intact ECM also has a critical function in atherogenesis in that it is required for the retention and thereby accumulation of other endogenous danger signals listed below.

**Modified Molecules**

Oxidation-specific epitopes can also activate innate immune responses and represent potent danger signals that contribute to atherosclerosis. A detailed overview has previously been published in *Circulation Research*. The best-investigated oxidized product in human atherosclerosis is oxidized LDL (oxLDL). Oxidation of LDL can be mediated by enzymatic mechanisms through 15- or 12/15-lipoxygenase, myeloperoxidase, or peroxidase-like activity of hemoglobin. Nonenzymatic oxidation is dependent on free radicals, such as superoxide, hydrogen peroxide, and nitric oxide. These products are generated by nicotinamide adenine dinucleotide phosphate oxidases, nitric oxide synthases can be produced by catalysis through transition metal ions (Cu\(^{2+}\) and Fe\(^{2+}\)) and hemin. Extensively oxidized LDL is internalized by macrophages via scavenger receptors (SRs), including cluster of differentiation (CD) 36, SR-A1 and SR-A2, SR-BI, PSOX, MARCO, and LOX-1. These receptors can recognize oxidation-specific epitopes and thereby ingest and clear these products. Specifically, oxidized phospholipids, 1-palmitoyl-2-(5′-oxovaleroyl)-sn-glycero-3-phosphocholine, or oxidized sn-2 fatty acids that terminate in γ-hydroxy (or oxo)-α,β-unsaturated carbonyl groups, for instance, mediate recognition and internalization. In contrast, SR do not recognize so-called minimally modified LDL, which contain relatively low concentrations of oxidation epitopes and differ from oxLDL in that they predominantly contain hydroxides, hydroperoxides, endoperoxides, and other early lipid peroxidation products of phospholipids and cholesterol esters. In a recent study, the cardiovascular effects mediated by oxidized phospholipids were dependent on single-nucleotide polymorphisms in the IL-1 gene cluster that had previously been associated with elevated levels of proinflammatory cytokines. These findings directly link individual genetic predisposition to the atherogenicity of oxidized phospholipids. Of note, it has also been shown that the enhanced uptake of oxLDL via CD36 results in the formation of cholesterol crystals, which in turn, contribute to an inflammatory response. Thus, the phase transition of cholesterol from soluble to crystalline can occur after oxLDL has been internalized in a receptor-specific manner. It is possible that interindividual differences in oxLDL uptake behavior could, therefore, result in different production of inflammatory mediators.

Advanced glycation end-products are modified proteins, lipids, and nucleic acids that become irreversibly cross-linked with reducing aldose sugars. These compounds have been shown to contribute to the atherosclerotic process directly and via a receptor-mediated mechanisms, for example, via activation of the receptor for advanced glycation end-products. It has also been shown that in smokers, advanced glycation end-products are drastically increased in various tissues, including the vascular. Hence, it is possible that the immune recognition of advanced glycation end-products is an important determinant for the increased risk of atherosclerosis seen in cigarette smokers. Interestingly, some cell death–associated danger signals, such as high-mobility group box 1 (HMGB1) and S100 calcium-binding proteins also bind receptor for advanced glycation end-products and mediate proinflammatory and atherogenic pathways. However, it should also be noted that the efficacy of some ligands is controversial and contamination of endogenous products with microbial-derived triggers could lead to misleading results.

**Cell Death**

In general, one can differentiate between programmed and nonprogrammed cell death. Necrosis or nonprogrammed cell death is the traumatic result of external injury to a cell by physical or chemical stress. Apoptosis, however, is a form of programmed cell death initiated by nonfatal stressors and cellular signaling pathways that end in either TNF or FAS cascades. Pyroptosis is an another type of programmed cell death, which is mediated by caspase-1 in response to proinflammatory danger signals and which is dependent on inflammasome activation. Apoptosis is generally considered silent and compromised cells are quickly and efficiently removed without causing any inflammation. Apoptotic cells are rather known to mediate anti-inflammatory effects. In contrast, necrosis and caspase-1–mediated pyroptosis activate innate immune responses. Necrotic or pyroptotic cell death is thought to occur in all stages of atherogenesis and could, therefore, represent important contributors to disease progression.

The danger signals that arise from these inflammatory forms of cell death and thought to contribute to vascular disease are discussed below. HSPs are usually expressed at low levels, but their expression is upregulated in response to a wide variety of stress stimuli, including some danger signals. On cell death, they are released into the extracellular milieu where they themselves act as danger signals. HSPs induce the production of proinflammatory cytokines, such as IL-1 in a TLR2- and TLR4-dependent pathways. HSP60, the most extensively studied HSP, is expressed in endothelial cells of arteries prone to atherosclerotic plaque formation because of enhanced shear stress. Although controversial, HSP60 has been associated with atherogenesis in both the experimental and clinical studies.
HMGB1 is an intracellular DNA-binding protein that functions as a proinflammatory cytokine after being actively secreted by stimulated macrophages. Once secreted, HMGB1 is detected by TLRs 2 and 4.\textsuperscript{106-108} Of note, HMGB1 release is dependent on the activation of the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (NLRP) 3 inflammasome, suggesting that NLRP3 activation can modulate TLR responses by increasing the DAMP HMGB1.\textsuperscript{109} Extracellular levels of HMGB1 are increased in atherosclerotic lesions,\textsuperscript{110} and elevated serum levels are associated with coronary artery disease\textsuperscript{111} and are furthermore reported to be a prognostic indicator for future cardiovascular events in patients with acute coronary syndrome.\textsuperscript{112}

Another DAMP is the nucleotide triphosphate ATP. Extracellular levels of ATP are kept at a low level through the activity of extracellular ATPases. Intracellular ATP expelled from dying cells acutely increases extracellular ATP levels, which stimulates the P2X7 channel. P2X7 activation results in K\textsuperscript{+} release from intracellular pools, which in turn activates the NLRP3 inflammasome, thus functioning as a potential danger signal.\textsuperscript{113} Recent studies have also demonstrated that ATP enhances macrophage lipid deposition and migration\textsuperscript{114} and contributes to atherogenesis via purinergic receptors by inducing leukocyte recruitment in mice.\textsuperscript{115}

Serum uric acid levels have been shown to predict the severity and morphology of coronary atherosclerosis,\textsuperscript{116} as well as the progression of subclinical coronary artery disease.\textsuperscript{117} Similar to ATP, the acute release of uric acid after cell death can lead to an inflammatory response in surrounding immune cells. The solubility of uric acid is much greater in the intracellular environment than in the extracellular environment. Thus, it is thought that the release of uric acid from intracellular stores leads to the formation of uric acid crystals in the microenvironment of the dying cells leading to immune activation.\textsuperscript{118} Uric acid crystals represent a major culprit in gout where they form in the joints and activate the NLRP3 inflammasome.\textsuperscript{119} However, uric acid could also represent a more general danger signal associated with tissue damage. Indeed, experimental reduction of uric acid levels using uricase transgenic mice reduces the cell death–induced inflammatory response to uric acid in vivo.\textsuperscript{120} Although treatment with the xanthine oxidase inhibitor allopurinol in gout patients did not have a beneficial effect on cardiovascular outcome,\textsuperscript{121} treatment of patients with stable coronary artery disease with low-dose colchicine was effective in preventing cardiovascular events.\textsuperscript{122} The efficacy of a therapeutic decrease of uric acid levels on atherosclerosis development has not yet been tested. Endogenous nucleic acids may also functions as danger signals that are released from dying cells. mRNA for instance is recognized by TLR3.\textsuperscript{123} RNA/DNA-containing immune complexes bind to TLR7 and TLR9 and stimulate immune responses through synergistic stimulation of either Fc-receptors or the B-cell receptor.\textsuperscript{124-126} Unmethylated CpG motifs, present in mammalian DNA promoter elements, are ligands of TLR9 and modulate inflammatory responses.\textsuperscript{127}

### Metabolic Products

Fatty acids can also act as danger signals and have sometimes\textsuperscript{128} been proposed to be natural ligands of TLR4.\textsuperscript{129} Fatty acids selectively stimulate the release of IL-1\alpha by uncoupling mitochondrial respiration and are hence potent inducers of IL-1\alpha–driven vascular inflammation.\textsuperscript{130}

Cholesterol has low solubility in aqueous solutions and cholesterol crystals can be detected by standard histology as so-called cholesterol crystal clefts in advanced atherosclerotic lesions. Recently, using laser reflection microscopy has been demonstrated that, in addition to the large crystals that cause crystal clefts in the tissue, an abundance of smaller cholesterol crystals is present in the extracellular space, as well as inside immune cells in atherosclerotic lesions. Of note, several studies have documented that crystalline cholesterol represents a danger signal that may be important during atherogenesis. Phagocytosis of cholesterol crystals by murine and human macrophages results in NLRP3 inflammasome activation and subsequent secretion of large amounts of IL-1\beta.\textsuperscript{87,131,132} Notably, atherogenesis was decreased in LDLR\textsuperscript{−/−} mice transplanted with bone marrow deficient in NLRP3, apoptosis-associated speck–like protein containing a caspace recruitment domains (CARD), or IL-1\alpha/\beta.\textsuperscript{87} Together, these studies show that cholesterol in a crystalline state represents a danger signal that is recognized by innate immune cells leading to the induction of inflammatory responses. Another potential mechanism by which cholesterol crystallization can contribute to the clinical outcomes of atherosclerosis is the volume expansion of cholesterol during the crystallization process, which could result in the perforation of the fibrous cap covering the atherosclerotic lesion. This physical disruption of the fibrous cap is suggested to contribute to plaque instability and to favor lesion rupture.\textsuperscript{133}

### Tissue Environment

Beyond specific epitopes, chemical alterations in the vessel microenvironment may also function as danger signals. Tissue ischemia and inflammation cause an extracellular drop in pH.\textsuperscript{134} Moreover, disruption of the lysosomal membrane integrity induces an intracellular acidosis. A recent study suggests that acidosis triggers NLRP3 inflammasome activation and increases the production of the proinflammatory cytokines: IL-1\beta, IL-1\alpha, TNF-\alpha, and IL-6, which are associated with atherogenesis.\textsuperscript{135} Of note, the NLRP3 inflammasome has also been proposed as a sensor for metabolic stress, such as in hyperglycemia, although a direct link to atherosclerosis has not been investigated.\textsuperscript{136}

### Innate Immune Signaling Pathways

As outlined above, PRRs sense microbial molecular patterns and altered or mislocalized self-molecules. This family of germline-encoded receptors consists of soluble, membrane-bound, and cytoplasmic receptors. Furthermore, PRRs include endocytic receptors responsible for the uptake of microbes or danger signals, and signaling receptors that mediate cellular activation. Some endocytic receptors, such as SRs, do not necessarily lead to cellular activation, but rather function in the clearance of apoptotic cell fragments, pathogens, and
modified lipoproteins. For instance, SR-A and CD36 are involved in the clearance of oxLDL and thereby participate in the formation of foam cells.

Signaling PRRs include toll-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain–like receptors (NLRs). Stimulation of TLRs and RLRs results in the induction of proinflammatory gene expression (Figure 2), whereas NLR stimulation can also lead to the induction of proteolytic pathways that activate the highly proinflammatory cytokines of the IL-1 cytokine family, including IL-1β and IL-18. There is an extensive crosstalk between individual PRRs, and simultaneous or sequential triggering of different PRRs activates distinct transcriptional programs. The specific immune response within an atherosclerotic lesion is, therefore, dependent on the presence of particular sets of PRR ligands and on the complex interaction between different activated signaling pathways. In the following sections, we will summarize the current knowledge on the involvement of PRRs in the development of atherosclerotic disease. Most of these data derive from mouse models of atherosclerosis and the relevance of these pathways for human atherosclerosis needs to be further elucidated.

**Toll-Like Receptors**

TLRs are the best characterized signaling PRRs of the innate immune system. To date, 13 mouse and 10 human TLRs have been identified, which are capable of recognizing a broad spectrum of microbial and host-derived structures. In atherosclerotic lesions, both immune cells and many stromal cells, such as endothelial cells, smooth muscle cells, and fibroblasts, express certain sets of TLRs. Stimulation of TLRs initiates acute inflammatory responses through the induction of transcriptional programs that regulate the expression of cytokines, chemokines, costimulatory molecules, and many other factors. Intriguingly, and highly relevant for the inflammatory response in atherosclerotic lesions, TLR stimulation can also regulate the specific secretion of preformed molecules in a transcription-independent manner.79

**Figure 2.** Transcription-modifying pattern recognition receptors. Stimulation of membrane-bound toll-like receptors (TLRs) and cytoplasmic RIG-I–like receptors (RLRs) induces a complex intracellular signaling cascade that results in the production of proinflammatory cytokines and type I interferons (IFN) by activation and nuclear translocation of specific transcription factors. 3pRNA indicates 5′ triphosphate double-stranded RNA; CpG-ODN, oligodeoxynucleotides containing cytosine-guanine motifs; DAI, DNA-dependent activator of IFN-regulatory factors; dsDNA, double-stranded DNA, dsRNA, double-stranded RNA, IKK, inhibitor of kappa B kinase; IPS, IFN-β promoter stimulator; IRAK, IL-1 receptor–associated kinase; IFN, IFN response factor; LPS, lipopolysaccharide; MDA5, melanoma differentiation–associated protein 5, MyD88, myeloid differentiation primary response protein 88, NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells, PGN, polyacylated cysteine-containing lipopeptides, RIG-I, retinoic acid-inducible gene I, ssRNA, single-stranded RNA; TAK, transforming growth factor-β-activated kinase-1; TBK, TANK-binding kinase; TRAF, tumor necrosis factor receptor–associated factor; and TRIF, toll/IL-1 receptor homology domain–containing adapter inducing IFN-β.
where ligand binding results in a distinct inflammatory response compared with TLR4 engagement from the plasma membrane. Each TLR can be stimulated by specific sets of pathogen-associated molecular patterns or DAMPs.

On ligand binding and receptor activation, the signal is transmitted via 5 different adapter molecules: MyD88, toll/IL-1 receptor homology domain–containing adapter inducing IFN-β (TRIF), MyD88-adaptor–like (Mal)/toll/IL-1 receptor homology domain–containing adapter protein, TRIF-related adapter molecule, and sterile-α and armadillo motif–containing protein. With the exception of TLR3 and endosomal TLR4, which selectively use TRIF, all other TLRs require MyD88, which induces a signaling cascade ultimately leading to nuclear translocation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and IFN response factor transcription factors. MyD88 consists of a toll/IL-1 receptor homology domain and a death domain and acts as a proximal adapter molecule for TLRs. After ligand binding of TLRs, the MyD88 death domain assembles in a helical signaling oligomer consisting of 6 MyD88, 4 IL-1 receptor–associated kinase (IRAK) 4, and 4 IRAK2 death domains. This signaling complex, the so-called myddosome, assembles in a hierarchical manner, because MyD88 self-assembly leads to recruitment of IRAK4 and the MyD88-IRAK4 complex, in turn, recruits the IRAK4 substrates IRAK2 or the related IRAK1. IRAKs associate with tumor necrosis factor receptor–associated factor 6 (TRAF) 6, an E3 ligase that catalyzes the synthesis of polyubiquitin linked to Lysine 63 (K63) on target proteins. The resulting polyubiquitin chains enable the recruitment of transforming growth factor–β–activated kinase (TAK)–1–binding protein-2 and -3 (TAK–1–binding proteins), which in turn, activate TAK-1. TAK-1 activates the inhibitor of kappa B kinase (IKK) complex, which enables NF-κB to translocate into the nucleus where it regulates gene transcription. TAK-1 also mediates activation of mitogen-activated protein kinases, such as extracellular signal–regulated kinase 1, extracellular signal–regulated kinase 2, protein 38, and c-Jun N-terminal kinase, leading to the activation of activating protein-1. TLR3 and TLR4 are the only TLRs that can signal in an MyD88–independent manner via engagement of TRIF. TRIF binds to TRAF3 and TRAF6, which then associate with the IKK-related kinases, TANK-binding kinase-1 and IKKe. This, in turn, facilitates the nuclear translocation of IFN response factor-3 and induces the production of type 1 IFNs. However, TRIF also binds TRAF6 and activates TAK-1, leading to the phosphorylation of inhibitor of kappa B–α by IKKα and IKKβ and driving NF-κB activation.

Most TLRs are expressed in healthy human vessels, but specific regional expression patterns can be observed. In atherosclerotic lesions, the expression of several TLRs is increased. The first study directly linking the TLR/IL-1 receptor superfamily to inflammatory responses in atherosclerotic lesions demonstrated that MyD88 deficiency reduces both the atherosclerotic lesion development and macrophage accumulation in ApoE-/- mice. This study suggested that either TLR activation or IL-1 receptor family activation (or both) play pathogenic roles during atherogenesis. In the following, we summarize studies that followed this initial observation and substantiated that TLR and IL1R family members are triggered during atherogenesis causing mainly proatherogenic immune responses.

TLR2 is activated by multiple microbial-derived signals ranging from bacterial lipoproteins to fungal cell wall components to viral products. TLR2 increases its specificity for ligands through heterodimerization with either TLR1 or TLR6, allowing it to discriminate between subtle differences in the ligands. TLR2 is expressed on various cell types in atherosclerotic lesions, including macrophages, smooth muscle cells, endothelial cells, and fibroblasts. Of note, TLR2 expression is drastically increased on endothelial cells in vessels with disturbed blood flow that are prone to atherosclerosis in LDLR-/- mice. Importantly, diet-induced (high-fat diet), pathogen-associated (P. gingivalis challenge), and specific TLR2–stimulated atherosclerosis is decreased in TLR2-/- mice. As mentioned before, TLR2 heterodimerizes with TLR1 and TLR6, respectively. Although treatment of high-fat diet-fed LDLR-/- mice with the exogenous TLR2/TLR1 ligand Pam3CysK4 and exogenous TLR2/TLR6 ligand MALP-2 increased atherosclerotic lesion development in a TLR1- and TLR6–dependent manner, respectively; no difference in only high-fat diet–induced lesion size between LDLR-/- TLR1-/- mice and LDLR-/- TLR6-/- mice versus LDLR-/- mice was observed. This suggests that engagement of TLR1 and TLR6 is either redundant or, as opposed to TLR2, does not play a significant role in the context of diet-induced murine atherosclerosis.

Furthermore, data using cells isolated from endarterectomy specimens of patients with symptomatic carotid disease indicate that TLR2 signaling via MyD88 plays an important role in inflammation and matrix degeneration. Adenoviral transfer of a dominant-negative form of MyD88 and TLR2-blocking antibodies led to decreased production of proinflammatory factors, chemokines and various proteases in atheroma cell cultures, whereas IL-1 receptor antagonist (IL-1Ra), TLR4-blocking antibodies, or overexpression of a dominant-negative form of TRIF did not affect the production of the mediators studied. Local arterial TLR2 stimulation induced neointima and atherosclerotic plaque formation in mouse femoral arteries. Moreover, HDL from patients with chronic kidney disease as opposed to HDL from healthy volunteers is modified by an endogenous proinflammatory trigger, symmetrical dimethylarginine, that can induce proatherogenic pathways via TLR2. Of particular interest, both dysfunctional HDL from patients or symmetrical dimethylarginine–promoted signaling via TLR2 without engagement of TLRs 1 or 6. This resulted in noncanonical TLR2 signaling with endothelial superoxide production and reduction of nitric oxide bioavailability in the absence of NF-κB activation. Whether HDL isolated from patients with other diseases trigger a similar pathway and whether other TLRs can induce this noncanonical TLR2 signaling remains to be elucidated.

Studies investigating TLR3 in atherosclerosis are controversial. Endothelial function and regeneration are greatly impaired in wild-type but not in TLR3-/- mice on exogenous stimulation with the TLR3 agonist polyIC (polyinosinic:polycytidylic
Acid), a synthetic mimic for double-stranded viral RNA.\textsuperscript{168} Furthermore, polyIC treatment significantly increased atherosclerotic plaque development in \textit{ApoE}−/− mice. Similarly, TLR3−/− myeloid chimeric LDLR−/− mice had significantly reduced aortic inflammation, as well as atherosclerotic burden\textsuperscript{152}\textsuperscript{152}\textsuperscript{152}\textsuperscript{152} and the induction of a TRIF\textsuperscript{−/−} lack-of-function mutation in LDLR−/− mice conveyed atheroprotective effects associated with a reduced cytokine production from peritoneal macrophages in response to hyperlipidemia.\textsuperscript{151,169} In contrast, Cole et al\textsuperscript{170} have found increased atherosclerosis in \textit{ApoE}−/− TLR3−/− versus \textit{ApoE}−/− mice. One explanation for these discrepancies could be differences in treatment regimes resulting in specific cellular effects on macrophages, endothelial cells, or myeloid cells.

TLR4 is expressed by plaque macrophages in human and murine atherosclerotic lesions.\textsuperscript{158} It is the primary receptor for the Gram-negative cell wall component lipopolysaccharide. However, other bacterial toxins, HSPs, and viral envelope glycoproteins can also trigger TLR4 activation. For lipopolysaccharide recognition, TLR4 requires several accessory proteins. Lipopolysaccharide micelles bind the serum protein lipopolysaccharide-binding protein, which delivers monomeric lipopolysaccharide to CD14. In addition, the secreted molecule MD2 associates with the extracellular portion of TLR4 and specifically interacts with the acylated lipid A core of lipopolysaccharide to mediate its responsiveness.\textsuperscript{171} Of note, saturated fatty acids can also mediate TLR4 signaling and induce inflammatory gene expression, whereas unsaturated fatty acids block the activation of TLR4.\textsuperscript{172} Macrophages also generate reactive oxygen species in response to minimally modified LDL-induced TLR4 activation.\textsuperscript{173} TLR4 has a clear pathogenetic role in atherogenesis as clinical studies investigating TLR4 hypomorphic polymorphisms with impaired TLR4 signaling have shown an association with plaque size,\textsuperscript{174} acute coronary events,\textsuperscript{175}\textsuperscript{175}\textsuperscript{175}\textsuperscript{175} and efficacy of statin therapy.\textsuperscript{176} Systemic administration of lipopolysaccharide in hypercholesteremic rabbits or \textit{ApoE}−/− mice demonstrated the proatherosclerotic role of TLR4 stimulation.\textsuperscript{177,178} In a mouse model of atherosclerosis, \textit{ApoE}−/− TLR4−/− mice had significantly reduced lesion sizes when compared with \textit{ApoE}−/− littermates.\textsuperscript{179} In addition, serum IL-12 and monocyte chemotactic protein-1, plaque lipid content, lesion macrophage numbers, and cyclooxygenase-2 immunoreactivity were also highly decreased in \textit{ApoE}−/− TLR4−/− mice.\textsuperscript{179}

TLR7 binds viral single-stranded RNA and self-RNA released from necrotic cells when complexed with antibodies forming immune complexes or cationic antimicrobial peptides, such as LL37 and α-defensins.\textsuperscript{180,181} In \textit{ApoE}−/− mice, functional inactivation of TLR7 resulted in an unexpected accelerated lesion development, increased stenosis, and enhanced plaque vulnerability.\textsuperscript{182} TLR7 was found to limit activation of macrophages in response to TLR2 and TLR4 agonist and thus, in its absence, an increased atherogenesis was observed.\textsuperscript{182} Similarly, \textit{ApoE}−/− TLR9−/− double-knockout mice showed aggravated atherosclerotic lesion development with an accumulation of inflammatory cells. Furthermore, CD4 positive T-cell depletion in these \textit{ApoE}−/− TLR9−/− mice or treatment of \textit{ApoE}−/− mice with a TLR9 agonist resulted in significantly reduced atherosclerotic lesion size.\textsuperscript{183} These data suggest that TLRs 7 and 9 exert regulatory roles in atherosclerosis development, which warrants further investigations in the mechanisms.

**RIG-I–Like Receptors**

The RNA helicases RIG-I and melanoma differentiation-associated gene 5 constitute a further PRR family called RIG–I-like receptors.\textsuperscript{184,185} Both RLRs are cytoplasmic receptors consisting of 2 N-terminal CARD, a DExD/box helicase domain, and a C-terminal repression domain. They are specialized in the recognition of particular nucleic acids. RIG-I is activated by 5′-triphosphate RNA (3pRNA),\textsuperscript{186} whereas melanoma differentiation-associated gene 5 responds to double-stranded RNA molecules.\textsuperscript{187} RIG-I is expressed by macrophages, endothelial cells, dendritic cells, and fibroblasts in human atherosclerotic lesions.\textsuperscript{188,189} RLR activation recruits the adapter molecule mitochondrial antiviral-signaling protein (also known as IFN-β promoter stimulator-1, VISA [virus-induced signaling adapter] and Cardif [CARD adapter inducing interferon beta]), ultimately leading to IFN response factor-3 and NF-kB activation followed by production of pro-inflammatory transcriptional responses.\textsuperscript{190} 3pRNA stimulation of endothelial cells further increased endothelial RIG-I expression, impaired endothelial-dependent vasodilation, and augmented the production of reactive oxygen species.\textsuperscript{191} Furthermore, 1 study suggests that RIG-I mediates 25-hydroxycholesterol–induced IL-8 production in atherosclerosis.\textsuperscript{192} melanoma differentiation-associated gene 5 is also expressed in vascular cells and is important for stimulation of vascular smooth muscle cells by self-RNA.\textsuperscript{193} In addition, melanoma differentiation-associated gene 5 recognizes the model double-stranded DNA polyIC, which can also trigger TLR3 activation. Direct effects of RLR signaling in atherogenesis have not yet been explored, but given the fact that experimental administration of polyIC can reportedly increase atherogenesis, this could be a fruitful area of investigation.

**Scavenger Receptors**

CD36 is an archetypal PRR that binds polyanionic ligands of both pathogen and self-origin. It is well established that CD36 plays a role in the endocytic uptake of altered self-components, including oxidized phospholipids, apoptotic cells, and amyloid proteins.\textsuperscript{196} Although important for the clearance of these host molecules from the circulation and tissues, CD36-dependent signaling has also been implicated in the proinflammatory effects of these modified endogenous ligands.\textsuperscript{195,196} Recognition of oxLDL and amyloid-β peptide by CD36 triggers the assembly of a heterotrimeric complex composed of CD36, TLR4, and TLR6 and promotes sterile inflammation in response to atherogenic lipids.\textsuperscript{197} Another function of CD36 is the coordinated endocytosis and subsequent intracellular conversion of soluble oxLDL, amyloid-β, and amylin peptides into crystals or aggregates, respectively, resulting in lysosomal disruption and activation of the NLRP3 inflammasome.\textsuperscript{184} Importantly, CD36-deficient \textit{ApoE}−/− mice had decreased IL-1β levels, smaller atherosclerotic plaques, and fewer plaque cholesterol crystals compared with control animals. CD36 is, therefore, a central regulator
of inflammasome activation and more than a metabolic mediator in atherogenesis.198

**NLRs and Inflammasomes**

The proinflammatory cytokines of the IL-1β family are crucial mediators of inflammation in the vessel wall. This is exemplified by the overt phenotype of mice with constitutively increased IL-1 signaling.199 Absence of IL-1Ra, which results in higher IL-1 receptor signaling leads to massive transmural lethal inflammation in vessel walls of unchallenged mice. The inflammation develops in arterial branch points and flexures of the aorta and its primary and secondary branches during aging.200 Furthermore, IL-1 signaling is known to contribute to the inflammatory response in atherosclerosis. IL-1β deficiency in ApoE−/− mice results in decreased atherosclerosis201 and IL-1Ra promoted atherosclerotic lesion development in the same model.202 Moreover, IL-1Ra overexpression or treatment with recombinant IL-1Ra results in reduced atherosclerotic lesions.199,203 IL-1α can also trigger the IL-1R and, thereby, contribute to atherogenesis. A recent study has demonstrated that fatty acids can trigger IL-1α production via mitochondrial uncoupling, thus promoting atherogenesis.130

The mechanisms how IL-1 family cytokines are activated have only more recently come to light. The NLR family members, NLRP1, NLRP3, NLRP6, NLRP12, and NLRC4 (also termed IPAF [ice protease-activating factor]) as well as the PYHIN family protein absence in melanoma 2, have been shown to form multimolecular signaling platforms, termed inflammasomes. To date, only the NLRP3 inflammasome has been studied in the context of atherogenesis. On engagement of inflammasome sensor molecules (ie, the NLR members or absence in melanoma 2) the adaptor molecule apoptosis-associated speck–like protein containing a CARD is recruited via pyrin domain/pyrin domain interactions and forms a large fibrillar helical assembly similar to myddosome formation on TLR stimulation.204 This is followed by the recruitment and fibrillar polymerization of procaspase-1 via CARD/CARD interactions.204 This large signaling platform, which is also termed apoptosis-associated speck–like protein containing a CARD speck,205 promotes autocatalytic activation of caspase-1. The resulting p10 and p20 caspase-1 subunits assemble to form active caspase-1 heterotetramers that convert inactive pro-IL-1β and pro-IL-18 into their bioactive and secreted forms.206 After activation of the NLRP3 inflammasome, the activated cells not only secrete large amounts of proinflammatory cytokines but also undergo a highly inflammatory form of caspase-1–induced cell death that is termed pyroptosis (Figure 3). Considering the drastic outcome for NLRP3-activated cells and the possible damage evoked in surrounding tissues, it is not surprising that NLRP3 inflammasome activation is highly regulated. An initial priming step (signal 1) is required for subsequent NLRP3 inflammasome formation by an activating signal (signal 2). The priming step can involve danger signals and can be mediated through PRRs, such as the TLRs, cytokine receptors, or other factors known to induce activation of NF-κB. Priming is crucial not only to produce enough pro-IL-1β substrate but also to increase NLRP3 expression to a functional level.207 In most cells, endogenous NLRP3 expression is insufficient for inflammasome activation and the resultant cleavage of caspase-1 and hence accidental activation of the inflammasome is suppressed in the absence of cellular priming. The NLRP3 inflammasome assemblies in response to a variety of exogenous and endogenous danger signals. These include various microbial signals (bacteria, fungi, and viruses), pore-forming toxins, crystalline substances, peptide aggregates, and extracellular ATP released from dying cells.208 To date, no direct interaction between NLRP3 and any of its diverse activators has been demonstrated although several potential mechanisms explaining the assembly of the NLRP3 inflammasome have been proposed. First, NLRP3 recognizes intracellular reactive oxygen species, which are commonly produced in response to many danger signals. Second, physical disruption of the lysosomal membrane integrity by

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**Figure 3. Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (NLRP) 3 inflammasome activation.**

Expression, assembly, and activation of the inflammasome subunits are required for caspase-1 to convert inactive pro-IL-1β and pro-IL-18 into their bioactive and secreted forms. Danger signals bind pattern recognition receptors, thus providing an initial priming step, resulting in the activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which increases NLRP3 expression and the production on pro-IL-1β substrate. In a subsequent activation step, danger signals from various pathogens, pore-forming toxins, crystalline substances, peptide aggregates, and extracellular ATP trigger the assembly of the NLRP3 inflammasome, which is activated by mechanisms, such as production of reactive oxygen species (ROS), physical disruption of the lysosomal membrane integrity and cellular potassium efflux. This enables caspase-1 to form fibrillar polymers and build active caspase-1 heterotetramers, which in turn, cleave pro-IL-1β and pro-IL-18. TLR indicates toll-like receptor.
danger signals, such as crystalline materials and peptide aggregates can mediate NLRP3 inflammasome activation by a yet undefined mechanism. Third, cellular potassium efflux, mainly via the P2X7 ATP-gated K+ channel, has been shown to be a requirement for inflammasome activation.206

Of particular importance for atherogenesis, the NLRP3 inflammasome can be activated by crystalline substances. In atherosclerotic lesions, abundant amounts of cholesterol crystals can be found at all stages of disease and in addition, human plaques contain crystalline calcifications.208,209 These substances are triggers of the NLRP3 inflammasome leading to caspase-1 activation and the production of bioactive IL-1β and IL-18.87,132 Of note, oxLDL can provide both signal 1 and signal 2 required for the activation of the NLRP3 inflammasome as oxLDL triggers PRR signaling and increased uptake of oxLDL leads to cholesterol crystal formation.87,88 Relative to other TLR4 ligands, such as lipopolysaccharide, oxLDL, however, is not as potent in priming and activation of NLRP3.87,88 Another study found that cholesterol crystals activate complement, which induces proinflammatory complement split products that, in turn, provide the priming step for NLRP3 inflammasome formation.210 These studies suggest that cholesterol crystals and oxLDL may be sufficient by themselves to activate the NLRP3 inflammasome, whereas other proinflammatory factors, such as cytokines or TLR triggers may further enhance the activation of the NLRP3 inflammasome in atherosclerotic lesions. LDLR−/− mice carrying bone marrow deficient in NLRP3 or apoptosis-associated speck-like protein containing a CARD have decreased lesion development in response to Western diet87 suggesting that inflammasome activation can contribute to atherogenesis. However, ApoE-deficient mice lacking NLRP3 inflammasome components did not reveal reduced lesion size. Because in this model a more severe cholesterol-rich diet was used, it is possible that other redundant mechanisms that contribute to atherosclerosis development obscured the NLRP3 phenotype.211

Notably, the danger signals that activate the inflammasome to elicit IL-1β release can also induce IL-1α secretion. Depending on the type of inflammasome activator, release of IL-1α was inflammasome-dependent or inflammasome-independent. Soluble agonists induce IL-1α formation in an inflammasome-dependent but caspase-1-independent pathway, whereas particulate agonist drive an inflammasome-independent pathway.212 If and how these findings are relevant to atherosclerosis, has not been evaluated.

**Therapeutic Implications of Danger Signaling Pathways**

Investigation of danger signals and their signaling cascades in atherosclerosis has provided many novel targets for therapeutic treatment approaches (Table). These novel treatment strategies include the inhibition of the proinflammatory cytokines induced on danger signaling. Canakinumab, a human monoclonal antibody that potently inhibits IL-1β, is approved for the treatment of the IL-1β–driven rare hereditary illnesses, such as cryopyrin-associated autoinflammatory syndromes and for the treatment of drug-resistant acute gouty arthritis. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), is designed to address whether IL-1β blockade can reduce recurrent vascular events.213

Anakinra, an IL-1Ra, blocks the biological activity of naturally occurring IL-1 and is approved for the treatment of rheumatoid arthritis. It has been shown to improve left ventricular function in patients with rheumatoid arthritis214 and to decrease IL-6 and C-reactive protein levels in patients with type 2 diabetes mellitus.215 Interestingly, analysis of IL-1 receptor type I-deficient ApoE−/− mice with total functional inactivation of IL-1 signaling unexpectedly exhibited multiple features of increased plaque instability through inhibition of outward vessel remodeling.216 Hence, it is possible that IL-1 signaling is part of a process that mediates potentially beneficial plaque remodeling.

Other drugs that target the NLRP3 inflammasome217 have also been evaluated in clinical trials. For instance, colchicine is generally effective for the treatment of gout, and a recent study demonstrated that colchicine also significantly decreased cardiovascular events in patients with stable coronary artery disease.212 This study supports efforts to investigate further NLRP3 inflammasome inhibitors for the treatment of atherosclerosis.

The Cardiovascular Inflammation Reduction Trial (CIRT) will randomly assign postmyocardial infarction patients with either type 2 diabetes mellitus or metabolic syndrome to low-dose methotrexate or placebo.218 The CIRT primary end point is a composite of nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death. Like canakinumab, methotrexate not only inhibits proinflammatory cytokines of the IL-1 family but also reduces other proinflammatory cytokines, including TNF-α and IL-6. Methotrexate has been shown to reduce atherogenesis in cholesterol-fed rabbits.219 Direct inhibition of IL-6 and TNF-α also has therapeutic potential for the treatment of atherosclerosis. The monoclonal antibodies

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### Table. Potential Therapeutic Strategies

<table>
<thead>
<tr>
<th>Target</th>
<th>Strategy</th>
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<tbody>
<tr>
<td>Danger signals</td>
<td>Antibiotics Vaccinations Neutralizing antibodies targeting DAMPs Inhibition of ECM degradation Increasing solubility of crystalline substances Antioxidants Reduction of physical and biochemical stress Alteration of microbiota and flora</td>
</tr>
<tr>
<td>PRR</td>
<td>Antagonists of proatherogenic receptors Agonists of antiatherosclerotic receptors Modulation of PRR expression Interruption of PRR-signaling Preventions of inflammasome activation</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Cytokine neutralizing therapies Cytokine receptor antagonists</td>
</tr>
</tbody>
</table>

Danger signals and the respective host response induced, provide potential therapeutic targets for the treatment of atherosclerosis. DAMPs indicates danger-associated molecular pattern; ECM, extracellular matrix; and PRR, pattern recognition receptor.
tocilizumab (anti-IL-6R antibody) as well as infliximab and adalimumab (both anti–TNF-α antibodies) are currently used in patients with arthritis. However, their main drawback is an adverse increase in LDL cholesterol, which could have detrimental effects in patients with coronary artery disease.\(^{220}\)

The clinical inhibition of proinflammatory cytokines induced by danger signals will have to be carefully assessed for potential safety risks. Danger signals play a crucial role in host defense mechanisms, and inhibition of their systemic signaling responses may cause an overall increased risk of infection. Targeting particular danger signaling of individual PRRs rather than common downstream cytokines may, therefore, be less invasive, but also not as effective because usually numerous danger signals contribute to inflammatory responses driving the pathogenesis of complex diseases, such as atherosclerosis.

About TLRs, TLR2 is the most promising and thus most investigated therapeutic target for vascular disease. Blocking TLR2 signaling reduces proinflammatory pathways in an in vitro model of human atherosclerosis.\(^{165}\) In addition, anti-TLR2 therapy with a specific monoclonal antibody just minutes before reperfusion decreases infarct size and preserves cardiac function by reducing leukocyte influx and cytokine production in mice\(^{222}\) and pigs.\(^{222}\) Furthermore, anti-TLR4 therapies are also being investigated. The TLR4 antagonist Rs-lipopolysaccharide has been shown to reduce atherogenesis in diabetic ApoE\(^{-/-}\) mice,\(^{223}\) and treatment with an anti-TLR4 antibody led to lower blood pressure in spontaneously hypertensive rats.\(^{224}\) The recent findings implicating a protective function of TLRs 7 and 9 during atherogenesis suggest that studies investigating whether selective activation of these receptors could have beneficial therapeutic effects should be performed.

Although 4 large clinical trials have failed to demonstrate a long-term beneficial effect of antibiotics in patients with coronary artery disease,\(^{12–15}\) these data do not exclude microbial infections as a contributor to atherogenesis nor rule out anti-infectious strategies for the treatment of CVD. The macrolides used in these trials for instance were likely insufficient to reduce Chlamydia pneumoniae infection accelerates hyperlipidemia induced atherosclerotic lesion development in C57BL/6J mice.\(^{200}\) 2001;\(^{134}\) Atherosclerosis\(^{164}\); 1999;\(^{103}\):747–753. doi: 10.1172/JCI4582.


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