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TLR Signaling: An Emerging Bridge from Innate Immunity to Atherogenesis \(^1\)

Kathrin S. Michelsen, * Terence M. Doherty, † Prediman K. Shah, † and Moshe Arditi \(^2\)*

Chronic inflammation and disordered lipid metabolism represent hallmarks of atherosclerosis. Considerable evidence suggests that innate immune defense mechanisms might interact with proinflammatory pathways and contribute to development of arterial plaques. The preponderance of such evidence has been indirect clinical and epidemiologic studies, with some support from experimental animal models of atherosclerosis. However, recent data now directly implicate signaling by TLR4 in the pathogenesis of atherosclerosis, establishing a key link between atherosclerosis and defense against both foreign pathogens and endogenously generated inflammatory ligands. In this study, we briefly review these and closely related studies, highlighting areas that should provide fertile ground for future studies aimed at a more comprehensive understanding of the interplay between innate immune defense mechanisms, atherosclerosis, and related vascular disorders. The Journal of Immunology, 2004, 173: 5901–5907.

Considering the sheer magnitude of the resultant human and financial devastation, one would be hard-pressed to name a more urgent public health care goal than developing effective treatments, vaccines, or even cures for cardiovascular diseases. Atherosclerosis underlies the vast majority of cardiovascular disease and causes more global death and disability than any other pathology except infection (1). Yet despite over a half-century of intensive investigation following the coining of the term “risk factor” and the identification of serum cholesterol as a prime offending agent by the seminal Framingham Heart Study, a comprehensive understanding of the pathogenesis of atherosclerosis in genetic and molecular detail has proven elusive, and besides notable exceptions such as statin therapy and the use of aspirin, precious few treatments have emerged that can boast thoroughly proven efficacy in a wide range of patients.

The contribution of Framingham to our understanding and treatment of risk factors was decisive, compelling, and far-reaching, but it is now clear that traditional risk factors can account for at most one-half of the observed prevalence of the disease (2). Atherosclerosis has a complex, multigenic basis that is also not completely understood. Thus far, studies examining the genetic determinants of disease manifestation have shown only moderate power to account for observed variance (3). In the past two decades, research interest has ranged well afield of the role of lipids and their oxidized derivatives into more general inquiry centered on how inflammation influences atherogenesis (and vice versa) (4). Deviating more substantially from the sharp focus on lipid metabolism engendered by insights from Framingham and subsequent studies, recent investigations have increasingly suggested that inflammatory and immune mechanisms, activated by noninfectious and possibly even infectious agents, might be important in the development and/or destabilization of atherosclerotic plaque. Supporting evidence for this has been largely indirect until very recently. Now, exciting discoveries related to signaling via TLRs have rekindled intense interest in the interweavings of immune defense mechanisms with established pathologic determinants of plaque development and destabilization. In this study, we briefly review the background leading to these discoveries, highlight emerging evidence, and present the outlook for the future in terms of both new research frontiers and the resultant potential for developing innovative treatments for atherosclerosis-based cardiovascular diseases.

**TLR signaling and the innate immune response**

The innate immune system is the first line of defense against invading microorganisms. Immune competent cells, such as macrophages, dendritic cells, neutrophils, and endothelial cells recognize pathogen-associated molecular patterns on the surface of pathogens, as diverse as Gram-positive and Gram-negative bacteria, viruses, fungi, and *Mycoplasma*. TLRs are a family of pattern-recognition receptors. To date, 12 members of the TLR family have been identified in mammals (5). Upon binding of their cognate ligands, TLRs recruit adaptor molecules to their intracellular signaling domain, leading to the activation of...
several kinases, NF-κB, and direct regulation of immune-responsive genes (6) (Fig. 1).

**Immune mechanisms and vascular pathologies: evidence and controversies**

**Infectious pathogens and atherosclerosis.** Reports dating back over 100 years have suggested that infectious agents could contribute to cardiovascular disease. In the late 1800s, for example, Hektoen (7) described how tuberculosis could infect the arterial wall and lead to degenerative changes, including intimal lesions. Shortly after the turn of the 20th century, Sir William Osler reported the frequent coexistence of tuberculosis and atherosclerosis. Several studies showed that inoculation with Marek disease herpesvirus could produce marked atherosclerosis in animal models (8). However, the idea that infectious agents could contribute significantly to clinical cardiovascular disease was not taken seriously until 1988, when Saikku et al. (9) reported serologic evidence linking an unusual species of *Chlamydia pneumoniae* and coronary atherosclerosis in 70 male patients with myocardial infarction or chronic coronary artery disease. The seroepidemiologic link between *C. pneumoniae* and atherosclerosis-based cardiovascular disease has since been confirmed by at least seven additional case-control and cohort studies. Histopathologic and molecular studies have now confirmed that *C. pneumoniae* is indeed present and viable within atherosclerotic plaque and throughout the susceptible arterial vasculature in both young and old human subjects.

A number of other infectious agents have now been similarly associated with atherosclerotic cardiovascular disorders, including *Helicobacter pylori* (10), CMV (11), EBV (12), HIV (13), HSV1, (14), HSV2 (15), and hepatitis B (16) and C (17). More recent models emphasize the relationship of atherosclerosis to total “infectious burden” rather than specific pathogens (18).

Many have objected that the above evidence is correlative and besets us with a classic “chicken-and-the-egg” dilemma. Koch’s postulate mandates that convincing evidence of a mechanistic link between infectious agents and atherosclerosis requires that atherosclerotic disease should be produced by inoculation with pathogens. In vivo studies in rabbits have now shown that infection with *Chlamydia* can result in the development of atherosclerotic plaque (19).

**FIGURE 1.** TLR signaling pathway and its relevance to atherogenesis. Endogenous and exogenous molecules associated with lesion formation are thought to signal through TLR4 but the potential role of other TLRs has not yet been investigated. Interaction between ligand and receptor leads to the recruitment of adaptor proteins and activation of downstream kinases leading to the activation of NF-κB or IRF3-responsive genes. Endogenous or exogenous ligands can stimulate TLR-bearing cells, such as macrophages, dendritic cells, endothelial cells (EC), and SMC. MM-LDL can stimulate EC and SMC secretion of proinflammatory cytokines and chemokines and also upregulates expression of adhesion molecules. Secreted chemokines attract monocytes to the endothelium, where these EC adhesion molecules facilitate leukocyte retention and subsequent migration into the subendothelial space, where they differentiate into macrophages under the influence of proinflammatory cytokines and growth factors. Uptake of MM-LDL by macrophages via scavenger receptors initially protects EC and SMC from stimulation by modified LDL. However, persistent hypercholesterolemia leads to excessive cellular uptake of MM-LDL, intracellular accumulation of cholesterol esters, formation of foam cell, and eventually apoptosis and release of proinflammatory oxidized lipid derivatives into the plaque, further exacerbating the inflammatory nidus.
of atherosclerosis such as the apolipoprotein (apo) E-deficient genotype, a number of reports have appeared showing that infection with microbes such as CMV, *Chlamydia, H. pylori*, and others can produce or exacerbate atherosclerosis (20–22). A very recent study by Gibson et al. (23) challenged apoE-deficient mice (and wild-type littermate controls) with *Porphyromonas gingivalis*, a frequent causative vector in periodontal disease (24), and found that *P. gingivalis* not only accelerated atherosclerosis, but resulted in up-regulation of TLR2 and TLR4. Furthermore, immunization with *P. gingivalis* before oral challenge prevented exacerbation of atherosclerosis. In contrast, however, Wright et al. (25) reported that atherosclerosis-prone apoE-deficient mice that are free of exposure to germs develop atherosclerosis that appears qualitatively and quantitatively similar to that in apoE-deficient mice raised in an environment of normal exposure to microbes. An important caveat to keep in mind is that experimental animal models of atherosclerosis generally do not accurately reflect all aspects of human atherosclerosis (26).

One potentially important source of inflammatory vascular injury is LPS. Low amounts of LPS can be released into the circulation from Gram-negative bacteria that either colonize or indolently infect the human gastrointestinal, genitourinary, and respiratory tracts. The Bruneck study has provided the first epidemiologic evidence that circulating LPS levels as low as 50 pg/ml constitute a strong risk factor for the development of atherosclerosis, particularly among smokers (27). In this 5-year prospective study of subjects without atherosclerosis at baseline, ~40% of newly developed atherosclerosis was estimated to be attributable to chronic infection (28). Chronic infections conferred an increased risk of atherosclerosis development even in subjects with low coronary risk. Furthermore, in rabbits on hypercholesterolemic diets, weekly injections of LPS accelerated the development of atherosclerotic lesions (29). These observations suggest that systemic proinflammatory mediators such as LPS may be pathogenically linked to the development and progression of atherosclerosis, and that pathogens, and hence TLR-mediated signaling, could play a mechanistic role in atherosclerosis. It is also possible that the effects are indirectly mediated, for example, by producing a generalized proinflammatory condition in which atherogenesis would be facilitated. However, very recent studies have now provided in vivo evidence for a direct mechanistic link between TLR4 signaling and innate immune system activation and atherogenesis (see “Emerging Discoveries: TLRs and Atherosclerosis”).

**Clinical trials with antibiotics.** Indirect epidemiologic evidence suggesting that antimicrobial therapy against *Chlamydia* might provide primary protection against clinical coronary artery disease (30) spurred the undertaking of prospective, randomized interventional studies. Ten clinical trials of antibiotic therapy against *Chlamydia* in a variety of clinical contexts have been published and five other trials are in progress (31). Collectively, studies with cardiovascular end points utilizing treatment regimens with a variety of agents have enrolled >25,000 patients with established coronary heart disease. The largest trial of these is the Azithromycin in Acute Coronary Syndrome study (AZACS), involving 1,450 participants with acute coronary syndromes at our institution (32). Overall, larger randomized trials to date have failed to show significant reductions in cardiovascular events, raising questions about the etiologic role of *Chlamydia* infections and atherothrombosis.

**Is vaccination against atherosclerosis possible?** Atherosclerosis is characterized by chronic inflammation and involves participation of immune cells, but also bears some striking similarities to autoimmune disorders such as lupus, multiple sclerosis, arthritis, and others (33). At least in experimental models, autoimmune diseases can be prevented by pretreatment with vaccines, and evaluation of potential clinical benefit of vaccinations is underway (34). Is it possible that atherosclerosis might also be prevented by vaccination?

Oxidized low-density lipoprotein (ox-LDL) and heat shock protein (HSP) 60 are two autoantigens that have been implicated in atherosclerosis, suggesting potential strategies for development of vaccines. The early stages of atherogenesis involve oxidative modification of lipoproteins, which increases their subendothelial retention (35) and creates an inflammatory nidus, attracting cellular components of the immune system (36). Autoantibodies to epitopes of ox-LDL can be induced during atherosclerosis, and these Abs are genetically and structurally identical to Abs produced by the T15 B cell clone that protects against pathogens such as pneumococci. Indeed, inoculation of atherosclerosis-prone hyperlipidemic mice with *Streptococcus pneumoniae* induces production of ox-LDL-specific Abs, a response that appears to protect against development of atheromatous plaque (37). Studies from our laboratory and others have shown that immunization with ox-LDL inhibits development of atherosclerosis (38, 39) and decreases neointimal proliferation following arterial injury (40) in animal models. Inoculation with another potential autoantigen, HSP65, accelerates atherosclerosis in experimental models (41, 42), for reasons that are not yet clear (43). Induction of immune tolerance to HSP65 by mucosal Ag delivery reduced atherosclerosis in LDL receptor-deficient mice, decreased numbers of CD4+ T cells and extent of IFN-γ immunostaining in plaques, and was accompanied by increased local and systemic production of IL-10 (44), suggesting an adaptive Th2-type immune response induced by oral tolerization.

Recent work toward potential vaccinations against atherosclerosis from our laboratory has focused on apoB-100, the major protein in the LDL particle (45). A case-control study of 227 participants showed that Abs against two apoB-100 epitopes were increased in patients with coronary artery disease. It was also demonstrated that inoculation with two of these apoB-100 peptide sequences reduced atherosclerosis in apoE-null mice by ~60% and increased the collagen content of plaques (46).

These studies suggest that vaccination against modified apoB-100 epitopes might eventually prove beneficial in treating, or perhaps even preventing atherosclerosis and associated vascular diseases (43). However, it will be important to first understand the mechanisms mediating immunity in atherosclerosis; very recently, some progress toward that goal has been achieved (see “Emerging Discoveries: TLRs and atherosclerosis”).
Emerging discoveries: TLRs and atherosclerosis

In vitro evidence that TLR signaling and innate immunity contributes to vascular pathologies. Several reports have documented the expression of TLR4, TLR1, TLR2, and to a lesser extent TLR5 in both human plaques and murine models of atherosclerosis (47, 48), where they appear to be mainly localized to macrophages and endothelial cells. TLR4 expression in macrophages is up-regulated by oxidized but not native LDL (47, 49). But on a mechanistic level, how might TLRs participate in atherogenesis? Are these signaling pathways the long-sought link between immune defense and the chronic fibroproliferative inflammation that is the hallmark of atherosclerotic disease?

Evidence from diverse sources and experimental models has provided a wealth of evidence suggesting that TLRs could affect atherosclerosis in multiple ways. Minimally modified LDL (MM-LDL), a proinflammatory and proatherogenic lipoprotein (50, 51), is recognized by both TLR4 and CD14 on macrophages, and interaction of MM-LDL with TLR4 leads to actin polymerization and macrophage spreading (52). Recognition of MM-LDL by TLR4 on endothelial cells is CD14 and MD-2 independent and results in the secretion of the chemokine IL-8 (49). The potential role of other TLRs in atherosclerotic lesions is still largely unexplored.

Furthermore, evidence for an involvement of an immune response toward HSPs and heat shock transcription factor in the development of atherosclerosis is accumulating (53). HSPs are highly conserved and ubiquitously expressed in almost all mammalian tissues. Immunologic cross-reaction between bacterial (e.g., chlamydial) and human HSP60, which has been detected on the surface of stressed endothelial cells, might be relevant to atherogenesis. Bacterial and human HSP60 signal through TLR4 and/or TLR2 and lead to the activation of NF-κB-dependent proinflammatory gene targets (54, 55). Chlamydial HSP60 promotes human vascular smooth muscle cell proliferation in a TLR4-dependent manner (56). Fragments of fibronectin have been associated with tissue injury and tissue remodeling in response to inflammation, and the extra domain A of fibronectin (EDA) can be recognized by TLR4 (57). Depletion of EDA results in decreased atherosclerosis in apoE-null mice (58), suggesting that the role of TLR4 during atherogenesis is multifaceted and might include both endogenous (MM-LDL, HSP60, EDA) and exogenous (e.g., chlamydial) Ags) molecules among its cognate ligands.

Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoryl-choline (ox-PAPC), the bioactive component of mildly ox-LDLs (59), utilizes TLR4 as a signaling receptor, but also has a direct effect on TLR signaling. ox-PAPC inhibits the activation of IL-8 by TLR4 and TLR2 ligands in endothelial cells or macrophages by disrupting lipid rafts/caveolae (60). The integrity of lipid rafts/caveolae is thought to be essential to LPS-induced cellular activation, since raft-disrupting drugs inhibit LPS-induced cytokine secretion (61). Inhibition of TLRs by ox-PAPC might contribute to down-regulation of the acute phase response to bacterial lipid-containing products and propagate a more chronic inflammation typical of the progression of atherosclerosis. Interestingly, TLRs can directly interfere with cholesterol metabolism in macrophages (62), suggesting an additional mechanism by which TLRs may affect atherogenesis. A more complete understanding of how TLR signaling participates in cholesterol metabolism must await further studies, but will be an important focus because of the potential therapeutic applications. Nevertheless, cross-talk between signaling pathways controlling innate immunity and macrophage cholesterol metabolism may help explain the ability of bacterial and viral pathogens to accelerate atherosclerosis.

Genetic evidence: TLR4 and related polymorphisms and their association with atherosclerosis and vascular disease. Several TLR gene polymorphisms have been described. Most importantly, patients either heterozygous or homozygous for two different single nucleotide polymorphisms (Asp299Gly and Thr399Ile) that map to the extracellular domain of TLR4 are hyporesponsive to a challenge with LPS (63). Since atherosclerosis is characterized by chronic local inflammation (50, 64), a less than aggressive response to LPS might be a disadvantage in pathogen defense, but could diminish cardiovascular risk because of decreased systemic inflammation. Therefore, Asp299Gly and Thr399Ile polymorphisms might be anticipated to be associated with greater cardiovascular protection. Evidence from a population-based epidemiologic study did indeed show that subjects carrying the Asp299Gly TLR4 polymorphism were less susceptible to carotid artery atherosclerosis (65). Other studies have reported that this polymorphism imparts protection from carotid and femoral artery atherosclerosis and acute coronary events (63, 65, 66), as well as greater benefit from statin therapy (67). However, studies in patients with familial hypercholesterolemia failed to show any protection against development of carotid atherosclerosis (68). Expression of inflammatory markers did not correlate with the Asp299Gly TLR4 polymorphism. Analysis of the Southampton Atherosclerosis Study also could not show an association between the TLR4 Asp299Gly polymorphism and either severity of or susceptibility to coronary artery diseases (69). Furthermore, a very recent report from the Stockholm Heart Epidemiology Program found that men with both the Asp299Gly and Thr399Ile polymorphisms had an increased risk of myocardial infarction (70). Since most of these studies involved relatively few subjects, larger clinical studies will be required to reconcile these discordant results.

In vivo evidence that TLR signaling significantly contributes to atherosclerosis. Early publications investigating mouse strain-specific differences in the susceptibility to atherosclerosis revealed that C3H/HeJ mice are atherosclerosis resistant on a high cholesterol diet compared with C57BL/6 mice (71). This resistance is associated with unchanged lipid profiles and apolipoprotein composition of plasma lipoproteins, in particular unchanged high-density lipoprotein levels, upon consumption of a high cholesterol diet (71). Endothelial cells derived from C3H/HeJ mice demonstrated a lack of an inflammatory response toward MM-LDL, supporting the hypothesis that MM-LDL indeed uses TLR4 as a receptor (72). Transfer of bone marrow derived from an atherosclerosis-prone mouse strain into C3H/HeJ mice failed to reverse the phenotype of C3H/HeJ mice, supporting an important role of endothelial cells during initiation of atherosclerosis (73).

Very recently, the first direct in vivo demonstration that MyD88 signaling plays a direct role in pathologic mechanisms leading to atherosclerosis was independently reported by Bjorkbacka et al. (74) and our own group (75). Using a genetic loss of function approach, both studies show an important role for
MyD88 in the development of atherosclerosis in murine models of atherosclerosis. MyD88 deficiency led to a significant decrease in plaque size, lipid content, expression of proinflammatory cytokines and chemokines (IL-12, MCP-1). In addition, we demonstrated that genetic deficiency of TLR4 is also associated with a significant reduction of aortic plaque size, lipid content, and macrophage infiltration in atherosclerosis-prone hypercholesterolemic apoE-null mice (75). These effects did not involve CD14, as the apoE/CD14 double knockout exhibited similar atherosclerosis compared with controls (74). These studies are consistent with in vitro data showing that ox-PAPC (a major bioactive component of MM-LDL) binds to TLR4 to activate endothelial cells without the use of CD14 (49).

Unraveling these complexities will be an important goal for future studies. Since MyD88 also participates in the IL-1R pathway as a downstream adaptor, it is conceivable that TLR signaling might not have been entirely responsible for proatherogenic effects; a role for IL-1 signaling cannot be excluded, particularly since IL-1 signaling accelerates atherosclerosis in apoE-deficient mice (76). However, comparison of data obtained from mice genetically deficient in both apoE and MyD88 with apoE/TLR4 double knockout mice revealed that reduction in the extent of atherosclerosis in the apoE/MyD88 double knockout mice is not entirely due to diminished IL-1 signaling, but is also caused by a lack of TLR4 (75). These findings also raise the possibility that other TLRs that utilize MyD88 might be involved in the development of atherosclerosis.

It is also possible that TLR4 might have complex, perhaps divergent downstream effects. Of potential relevance, NF-κB is a downstream target of TLR4/MyD88 signaling (6), and considerable evidence (albeit largely indirect) is consistent with a proatherogenic function for NF-κB (77). However, effects of NF-κB on atherosclerosis are not straightforward. Kanters et al. (78) found that macrophage-restricted inhibition (via genetic IKK-2 deletion) of the NF-κB pathway accelerates atherosclerosis, yet subsequently reported that bone marrow transplantation using p50-null donor marrow and proatherogenic LDL receptor knockout mice recipients decreased atherosclerosis (79), even though plaques were more inflamed. This raises the interesting possibility that NF-κB has complex functions both to promote and to limit inflammation.

At first glance, these reports appear inconsistent with a previous report suggesting there was no difference in the extent of atherosclerosis between Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>/Apoe<sup>−/−</sup> mice (25). The latter strain was generated by backcrossing Apoe<sup>−/−</sup> with the LPS-hyporesponsive strain C57BL/10ScSn thought to involve a mutation that results in deficient TLR4 signaling. In this study, atherosclerotic burden was not directly measured, but was estimated from cholesteryl ester content in whole aortas. Additionally, Apoe<sup>−/−</sup>/Apoe<sup>−/−</sup> mice reflect only partially a TLR4<sup>−/−</sup> genotype, since those mice also lack two additional genes with unknown function (80). Any influence those two genes might have on the development of atherosclerosis remains unknown. Moreover, a different LPS-hyporesponsive strain (C3H/HeJ) is resistant to development of atherosclerosis (72), consistent with the interpretation that TLR4 signaling is involved in atherogenesis.

Suggestions that TLR signaling might affect plaque vulnerability have begun to emerge. Epidemiologic and clinical data have demonstrated that chronic inflammation and infection increase the risk of developing cardiovascular events such as stroke and myocardial infarction (81), but particularly with regard to infection, how this might occur is unclear. These events are mostly precipitated by rupture or erosion of structurally weakened plaque (4). In more advanced stages of atherosclerosis, extensive compensatory arterial remodeling may occur, which tends to preserve luminal diameter and arterial blood flow. Although outward remodeling of arteries can preserve blood flow and lumen diameter, it is often associated with a more vulnerable plaque phenotype that is thought to be due to structural weakening of the overlying cap by proteases such as matrix metalloproteinases (82). TLRs may be involved in this aspect of the natural history of plaque development as well, since Hollestelle et al. (83) recently demonstrated that TLR4 is involved in outward arterial remodeling in a mouse model. Remodeling occurred either in the presence or absence of exogenous LPS ligand. In the absence of LPS, TLR4-involvement might be dependent on stimulation by endogenous ligands such as EDA or HSP60 since both are up-regulated in surrounding tissue (83).

Conclusions and outlook: emerging evidence, emerging questions, and emerging therapies?

Research into the molecular bases of atherosclerosis and treatments for resultant diseases has turned in an exciting new direction. Until very recently, most of the evidence favoring a link between immunity and vascular pathology has been indirect, but now we have data from experimental animal models that directly implicate signaling pathways normally associated with innate immunity. Before we can begin to develop effective therapies, it will be necessary to expand our understanding of these links. Studies to determine the clinical relevance of these data would be a logical next step, and another important goal will be to develop a much more comprehensive model of the interactions between innate immunity and atherosclerosis. How does acute and chronic infection contribute to vascular pathology? What are the relevant ligands, signaling pathways, and cell types involved? Where can we effectively intervene? Can we prime our defense mechanisms by developing vaccinations to limit, or possibly even prevent, development of the disease? Answering these questions will provide important insights in the years to come. In their long evolutionary journey, ancient defense mechanisms developed to guard against invading pathogens have evidently established a fascinating interplay with the cellular and molecular arsenal against other potential sources of injury long associated with atherogenesis, providing a more profound understanding of the characterization of atherosclerosis by Ross (84) as “a defense mechanism gone awry.”

References


