Emerging role of Toll-like receptors in atherosclerosis

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Abstract **Atherosclerosis is inflammation of the vessel wall of the arterial tree. This inflammation arises at specific areas that experience disturbed blood flow such as bifurcations and the lesser curvature of the aortic arch. Although all endothelial cells are exposed to comparable levels of circulating plasma cholesterol, only endothelial cells overlaying lesions display an inflamed phenotype. This occurs even in the absence of any additional exacerbating disease factors because blood flow controls the expression of Toll-like receptors (TLR), which are initiators of cellular activation and inflammation. TLR2- and 4-expression exert an overall proatherogenic effect in hyperlipidemic mice. TLR activation of the endothelium promotes lipid accumulation and leukocyte accumulation within lesions.—**Curtiss, L. K., and P. S. Tobias. **Emerging role of Toll-like receptors in atherosclerosis. *J. Lipid Res.* 2009.** 50: **S340–S345.**

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INFLAMMATION OF THE VESSEL WALL

Atherosclerosis was once thought to be a simple lipid storage disease that caused pathology by arterial obstruction. The pathology leads to arterial obstruction, but not simply by accumulation of lipid. Atherosclerotic lesions are foci of vessel wall inflammation (1–3). Many hallmarks of inflammation are present and include the expression of soluble inflammatory mediators. Leukocytes, including macrophages, dendritic cells, and lymphocytes, accumulate at lesion sites and the overlying endothelial cells display an inflamed phenotype. Systemically, acute phase proteins are elevated, such as serum amyloid A in mice and C-reactive protein in humans.

Lesions begin early as fatty streaks and progress to pathologic lesions under the influence of both genetic and lifestyle insults (4). Whereas most plaques are the result of many years of gradual asymptomatic disease progression, the final obstructive event is a sudden thrombotic event. During the major part of a lifetime that will elapse between fatty streak formation and overt disease, multiple events will occur to accelerate lesion progression. Genetic diseases like diabetes, hypertension, and notably, hypercholesterolemias lead to severe disease (5). Lifestyle choices including smoking, obesity, and a sedentary lifestyle also will accelerate disease progression. Appreciating that a major component of atherosclerosis is chronic inflammation raises many questions about the relationship between inflammatory processes and disease severity. Inflammation is a normal homeostatic response of the body to wounds or infections. Upon resolution of the problem, inflammation is self limiting and homeostasis is restored. Chronic inflammation occurs when inflammatory responses cannot resolve the problem or when self-limiting mechanisms go awry (6). We know some of the factors that contribute to disease progression, including hyperlipidemia, lipid oxidation, leukocyte accumulation into the arterial wall, and foam cell formation. Inflammatory diseases elsewhere in the body, ranging from periodontitis to autoimmune diseases like lupus, can also have an exacerbating influence on lesion progression (7–9).

Notably, the factors that promote atherosclerosis do not do so uniformly throughout the arterial tree. Site-specific plaque development is the result of disturbed hydrodynamic blood flow (10). Regions of the arterial tree exposed to laminar flow are protected from endothelial activation and atherosclerosis. This antiinflammatory activity is mediated by mitogen-activated protein kinase phosphatase (MKP-1), a negative regulator of p38 and c-Jun N-terminal kinase (11). Lesion susceptible sites are vessel bifurcations and the lesser curvature of the aortic arch. These sites display an inflamed phenotype even in the absence of any additional exacerbating disease factors (12–14). The biochemical mechanisms that enable endothelial cells to detect flow patterns are beginning to be understood (15–17). Arterial endothelial cells possess a single cilium able to detect the mechanical forces of blood flow (18–20) that distinguish disturbed flow from laminar flow. This in turn dictates endothelial cell phenotype. Cells at plaque

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Abbreviations: Hsp, heat shock protein; IL, interleukin; MyD88, myeloid differentiation factor 88; TLR, Toll-like receptors.

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susceptible sites have a proinflammatory phenotype, which is both permissive and causative of plaque development. For example, the proinflammatory endothelial phenotype is permissive in that it allows expression of proinflammatory receptors such as Toll-like receptors (TLRs) and causative in that it permits expression of cell surface adhesion molecules (such as vascular cell adhesion molecule-1), which foster inflammatory leukocyte accumulation into the intima (21–23). No specific disease risk has been identified that would promote a proinflammatory phenotype only at arterial bifurcations and the lesser curvature of the aortic arch. TLRs expressed by endothelial cells may provide a clue to this conundrum.

TLRs AND ATHEROSCLEROSIS

If inflammation is a hallmark of atherosclerosis, the role of TLRs must be understood because they initiate inflammation. They do so every day and the majority of these responses are beneficial by promoting healing and homeostasis. However, occasionally these responses go awry and cause pathology. For example, if a TLR2- or TLR4-mediated innate immune response to infection does not develop in time to curb microbial growth, the host dies of bacteremia (24). Atherosclerosis is one of these pathologic consequences. TLR2 promotes atherosclerosis progression. Hypercholesterolemic LDLr$^{-/-}$ mice with a total deficiency of TLR2 have only minimal lesions (25). Liu et al. (26) recently confirmed the same influence of a TLR2-deficiency in apoE$^{-/-}$ mice and Madan (27) observed a similar level of disease protection in apoE$^{+/-}$ mice.

So far, only TLR4 and TLR2 have been studied in mouse models of disease to determine their role in atherogenesis. However, the innate immune system employs a number of pattern recognition receptor families to respond to DNAs and RNAs, either from invading microbes or within the host. One could easily make the case that other TLRs, especially TLR3, TLR7, and TLR9, should be examined because they also initiate important inflammatory responses (28). In lesions, macrophages, dendritic cells, endothelial cells, and vascular smooth muscle cells can all express TLR2 and TLR4 (29–31). Importantly, although essentially all cells in lesions can express TLRs, this expression is not constitutive. TLR expression is regulated by multiple factors including cell differentiation and the presence of their cognate ligands.

TLR4 signals through the myeloid differentiation factor 88 (MyD88) (as does TLR2) as well as through the Toll/IL-1 receptor domain-related adaptor protein that induces interferon (TRIF). Our laboratory is examining the effect of a TRIF-deficiency on atherosclerosis in LDLr$^{-/-}$ mice, and others demonstrated that MyD88 participates in atherogenesis. MyD88 deficiency leads to reductions in plaque size, lipid content, expression of proinflammatory genes, cytokines, and chemokines such as IL-12 and monocyte chemoattractant protein-1 (32). However, MyD88 is also involved with signaling originating from the IL-1 family of receptors. Because IL-1 and IL-18 are proatherogenic, these effects of a MyD88 deficiency on lesion formation are not sufficient, although necessary, to implicate TLRs in atherosclerosis.

An early study reported that C3H/HeJ mice compared with C57BL/6 mice are resistant to atherosclerosis when they are fed a high cholesterol diet (33). These C3H/HeJ mice carry a point mutation in the intracytoplasmic region of TLR4 that codes for a nonfunctional receptor. Leukocytes from C3H/HeJ mice lack inflammatory responses to minimally modified LDL, supporting the idea that minimally modified LDL initiates atherosclerosis via TLR4 (34). However, transfer of bone marrow-derived cells from an atherosclerosis-prone mouse strain into C3H/HeJ mice does not reverse the phenotype of the C3H/HeJ mice, indicating a key role for endothelial cell TLR4 during early events of atherosclerosis.

TLR4 can directly interfere with cholesterol metabolism in macrophages (35), suggesting a mechanism by which TLR4 may affect disease pathology. A total deficiency of TLR4 is associated with reductions in lesion size, lipid content, and macrophage infiltration in apoE$^{-/-}$ mice fed a high cholesterol diet for six months (32). This deficiency in the double mutant mice results in a 25% reduction in the aortic surface area covered by lesions, as well as a reduction in the lipid content of heart aortic sinus lesions. Importantly, this reduction in disease severity is observed without a significant effect of the TLR4 deficiency on plasma cholesterol levels. Hollestelle et al. (36), demonstrated that TLR4 is a vital receptor in arterial remodeling. A femoral artery cuff exposure to lipopolysaccharides in proatherogenic apoE Leiden transgenic mice increased plaque formation and outward arterial remodeling. Endothelial cells lack the adaptor molecule TRIF-related adapter protein that is required for TLR4 signaling in bone marrow-derived macrophages (37). This could restrict TLR4 signaling in endothelial cells to only the MyD88 pathway. Curiously, CD14, the cofactor for both TLR2 and TLR4, was shown to be irrelevant to atherosclerosis in apo E$^{-/-}$ mice (37).

TLR3, 7, and 9 may also participate in atherosclerosis (38, 39). For example, murine cytomegalovirus exacerbates atherosclerosis and is a ligand for TLR3 and TLR9 (39, 40). Coxsackie B virus is an agonist for TLR7 and promotes cardiac lipid accumulation in mice (41). Herpes simplex virus is a ligand for TLR9 (42) and promotes murine atherosclerosis (43). Finally, antibody complexes of endogenous RNA and DNA are believed to be triggers for autoimmune disease and there is a strong link between autoimmune disease and atherosclerosis (44). Together, this evidence, although indirect, supports the idea that activation of TLRs 3, 7, and 9 also promote atherosclerosis.

ARE TLR AGONISTS EXOGENOUS OR ENDOGENOUS?

TLR agonists can be either endogenous, such as products of sterile tissue damage (45), or exogenous, such as pathogens. The term "endogenous agonist" is used to refer

to disease-associated TLR ligands that arise in mice that are not exposed by direct manipulation to known additional exogenous agonists, such as pathogenic organisms or synthetic mimics of components of pathogenic organisms. One might use the phrase "or unknown endemic exogenous agonist" because the hyperlipidemic mice are not truly sterile, gnotobiotic animals. Study mice are healthy and are kept in pathogen-free rooms, but of course also harbor a variety of commensal organisms. In fact, Erridge (46) has proposed that no true endogenous TLR agonists exist and that only infection- and commensal-derived agonists are recognized by TLRs. However, Wright et al. (47) have reported that true gnotobiotic hyperlipidemic mice do get atherosclerosis and this supports the more commonly held belief that endogenous TLR agonists exist. Importantly, our bone marrow transplantation experiments demonstrate that bone marrow-derived leukocytes do not participate in the early atheroprotective inflammatory responses observed in TLR2$^{-/-}$ mice exposed to only endogenous agonists, but do participate in the proatherogenic responses to an exogenous agonist, such as Pam3, a synthetic TLR2 agonist (25).

If TLR2 deficiency in hypercholesterolemic LDLr$^{-/-}$ mice decreases atheroma formation even in the absence of an administered exogenous agonist, what is the endogenous TLR2 agonist that promotes lesion formation via TLR2-mediated cell activation? Although a proatherogenic role for oxidized lipoproteins and lipids is well established (48), their role as TLR agonists has received minimal attention. Berliner et al. (49) reported that one oxidized LDL phospholipid, specifically 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine, is not a TLR2 agonist and Miller et al. (50) reported that minimally modified LDL is not a TLR2 agonist. However, it was recently reported that 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine and oxidized phosphotidylcholine are ligands for CD36, a TLR2 (and possibly TLR4) coreceptor (51). Because these results are largely negative and do not address other forms of oxidized LDL or its components, a systematic survey is needed of the available forms of modified and oxidized LDL as endogenous TLR agonists.

Several nonlipid candidate endogenous ligands of TLR2 have been identified. These are high-mobility group box 1 protein (HMGB1), hyaluronic acid fragments, and biglycan. HMGB1 was previously known only as a nuclear transcription factor, but is now known also to be a secreted cytokine mediating inflammatory responses to injury and infection (52). Abrogation of its activity in sepsis models improves survival. HMGB1 binds directly to TLR2 and TLR4 and is an activating ligand for both TLR2 and TLR4 (53). HMGB1 has two DNA binding domains, the so-called A-box and B-box, as well as a long acidic tail. The B-box is responsible for inflammatory responses and antibodies to the B-box are therapeutically useful. By contrast, the A-box is antiinflammatory and is therapeutically useful in models of endotoxemia, sepsis, and arthritis (54, 55).

Some of the endogenous ligands that activate TLR4 are known. Sources include necrotic and apoptotic cells (56) that arise from tissue injury, oxidized lipids and proteins (50), saturated fatty acids (57, 58), stress-induced factors such as heat shock proteins (Hsp) (59), fibronectin extra cellular domain A, extra cellular matrix components, and even advanced glycation end products that are formed in diabetics during hyperglycemia (60). Hsps may be potent activators of the innate immune system. Hsp60, Hsp70, and gp96 (the endoplasmic reticulum Hsp90) from a variety of mammalian sources (61) induce the production of proinflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-6, and IL-12 by monocytes, macrophages, and dendritic cells. These Hsp effects, as compared with their molecular chaperone function, are unique in that they require no Hsp-associated peptides, no adenosine 5′-triphosphate hydrolysis, no cofactors, and no protein complex assembly (62). Using C3H/HeJ fibroblasts with point mutation in TLR4 or C57BL/10ScCr fibroblasts with gene deletion of TLR4 transfected with TLR4 complementary DNA, it was found that fibrinogen, surfactant protein-A (63), fibronectin extra domain A (64), heparin sulfate (65), and soluble hyaluronan (66) are endogenous ligands for TLR4. Notably, there are ample examples in the literature that demonstrate how contaminants can lead to misleading conclusions. Recent studies using Hsp preparations essentially free of lipopolysaccharides suggest that the previously reported cytokine function of some Hsps may be a result of endotoxin contaminants (67). Nevertheless, these data collectively suggest that endogenous ligand signaling via TLR2 and TLR4 can impact region-specific disease outcomes.

Each of these observations concerning Hsps, hyaluronic acid fragments, and HMGB1 may be part of a general protective mechanism for detecting and responding to tissue injury (68). Thus, tissue injury leads to generation of matrix component fragments as well as expression of biglycan and HMGB1. These substances in turn may activate favorable repair processes through interaction with TLRs 2 and 4. Tissue injury also occurs in lesions and one would expect hyaluronic acid fragments and HMGB1 to be present. Because TLR2- and 4-expression seem to exert an overall proatherogenic rather than an antiatherogenic effect in hyperlipidemic mice, the TLR-mediated responses to tissue injury in lesions must be considered to be too much of a good thing. Importantly, the presence of TLR endogenous agonists in hyperlipidemic mice and the pathologic effects of these substances on the endothelium need to be detailed.

ENDOTHELIAL CELL TLRs AND EARLY LESION FORMATION

In hypercholesterolemic LDLr$^{-/-}$ mice atherosclerotic lesions develop over extended periods of time (23). Although many techniques have been developed to quantify disease macroscopically, these techniques do not permit visualization of the earliest stages of disease. In humans, an immunohistochemical and mRNA transcript survey of selected vascular beds (including the aorta and subclavian, carotid, mesenteric, iliac, and temporal arteries) has documented the abundance of TLR2 and TLR4. The TLR2 coreceptors, TLR1 and TLR6, have similar distribution pat-

terns, with high expression only in the aorta, carotid, and iliac arteries. In contrast, TLR3 is predominately expressed in the aorta, whereas TLR7 and 9 are minimally expressed only in the iliac (69).

Recently we used laser scanning confocal fluorescence microscopy to examine TLR2 expression during the early onset of disease in LDLr$^{-/-}$ mice (23). We observed that TLR2 expression within the luman of the aorta is confined to the lesser curvature, where it increases progressively with continued weeks of hyperlipidemia. Importantly, the extent of endothelial cell disruption detected by staining with CD31 (PECAM-1) is proportional to the extent of endothelial cell TLR2 expression. CD31 is a homophilic adhesion receptor whose cytoplasmic domain binds catenins and responds to a mechanosensory complex on the endothelium (15). Cytoskeleton actin staining via rhodamine phalloidin demonstrates a similar pattern of altered endothelium (23) and confirms that these morphological changes are not just an artifact of cell activation. As expected, aortic tissue segments from *LDLr*$^{-/-}$*TLR2*$^{-/-}$ mice fed an atherogenic diet are negative for TLR2 staining and the changes in endothelial cell morphology are mitigated in TLR2 knockout mice (23). Thus, endothelial cell TLR2 expression in *LDLr*$^{-/-}$ mice is confined to areas of disturbed blood flow, TLR2 expression is increased with continued arterial exposure to hyperlipidemia, and TLR2 deficiency reduces hyperlipidemic-induced changes in the morphology of the endothelium.

Studies of the earliest events of lesion formation in LDLr$^{-/-}$ mice consuming a high fat diet unexpectedly revealed lipid accumulation within endothelial cells, although lipid accumulation by macrophages and foam cells predominated at later time points. Macrophage lipid accumulation in lesions is the subject of far more study than endothelial cell lipid accumulation, yet endothelial cell lipid uptake is observed before macrophage lipid uptake is apparent (23). It is well appreciated that endothelial cells are quite resistant to lipid accumulation. However, endothelial cells express receptors for modified lipoproteins and have the biochemical pathways for sterol synthesis and receptor-mediated endocytosis of lipoproteins. Cholesterol efflux continues even when cellular cholesterol mass is unchanged. Therefore, cholesterol efflux pathways probably play a key role in endothelial cholesterol homeostasis. Because this lipid accumulation is dependent on TLR2 expression, there must be a mutually reinforcing interaction between endothelial TLR2 expression and accumulation of endothelial lipid.

The expression of scavenger receptors in endothelial cells has been cataloged. However, these receptors in atherosclerosis are not as well understood as macrophage scavenger receptors and there is little study of their regulation by TLR signaling. An excellent review (70) serves to focus on those receptors that are the most highly expressed in endothelial cells and, thus, the most likely to be involved in lipoprotein uptake. These likely candidates are the Class A scavenger receptor with C-type lectin (SRCL also known as CL-P1), the Class B scavenger receptor (CD36), and the Class E scavenger receptor lectin-like oxidized LDL receptor-1 (LOX-1). There are others, such as the Class A scavenger receptor SR-AI that is weakly expressed on endothelial cells, but their function appears to be primarily on macrophages. Export of cellular cholesterol could also regulate endothelial cell lipid content. ABCA1, ABCG1, and SR-B1 are the reverse cholesterol transport receptors that could accomplish this. Genest et al. (71) measured cholesterol efflux from vascular endothelial cells and suggested that none of the ABC transporters or SR-B1 is important for reverse cholesterol transport from human umbilical vein endothelial cells or fibroblasts. However, TLR expression would be expected to regulate this lipid efflux (35, 71) and none of these studies were done in the presence of TLR agonists. Because TLR2 activation modifies lipoprotein accumulation by endothelial cells, reverse cholesterol transporter receptor functions as well as scavenger receptor functions by these cells in early lesions warrants careful examination.

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