Thematic review series: The Immune System and Atherogenesis

Paying the price for pathogen protection: toll receptors in atherogenesis

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Abstract   Atherosclerosis is a chronic inflammatory response characterized by the accumulation of cells of innate and acquired immune systems within the intima of the arterial wall. Macrophages are the predominant participant in innate immune responses in atherosclerosis. Protein receptors expressed by macrophages and endothelial cells recognize components and products of microorganisms and play a vital role in innate immunity. In particular, the members of the toll-like receptor (TLR) family play a critical role in the inflammatory components of atherosclerosis. Both exogenous ligands involved in microbial recognition as well as endogenous ligands involved in sterile inflammation pathways are implicated in the pathology of atherosclerosis.■ In this review, we discuss our current understanding of the role of TLRs and their co-activators in atherosclerosis, with particular emphasis on studies in atherosclerosis-prone hypercholesterolemic mice.— Tobias, P., and L. K. Curtiss. **Paying the price for pathogen protection: toll receptors in atherogenesis.** *J. Lipid Res.* 2005. **46:** **404–411.**

Atherosclerosis is a chronic inflammation of the vascular wall typified by the accumulation of lipid and macrophage-derived foam cells (1, 2). The precursors to atherosclerotic lesions are fatty streaks that develop surprisingly early in life and are commonly found as early as three years of age (3). As the endothelial cells become activated and adhesive, monocytes stick to them, pass between them, and enter the intimal layer of the vessel wall. If the monocytes become activated, they remain in the intimal layer, mature into macrophages, take up lipid to become foam cells, and release a variety of inflammatory mediators, as well as oxidants and proteases. At this point, the inflammation has become chronic and the fatty streak is now well on its way to becoming an atherosclerotic lesion. As lesions mature, they become necrotic and calcified. Ultimately, a lesion may rupture, initiate a thrombus, block an artery, and cause a myocardial infarction or stroke (2).

This concept, that atherosclerosis is a chronic inflammatory disease, leads inevitably to several questions: What receptors might initiate inflammatory responses within arteries? What are the ligands that activate these receptors? And, importantly, what leads to the typical regiospecificity of atherosclerotic lesions? In any current discussion of inflammatory states, the subject of toll-like receptors (TLRs) is bound to arise.

The term "toll-like receptor" refers to the similarity between the *Drosophila* toll receptor, which was discovered as a regulator specifying dorso–ventral polarity in fly embryos, and a group of recently identified mammalian receptors. The similarities among these type I transmembrane proteins encompass both the signaling domains, which contain what is called a toll interleukin-1 (IL-1) receptor (TIR) domain, as well as the ligand receptor domains, which have multiple leucine-rich repeats (**Fig. 1**). Study of TLRs is very recent. Gay and Keith (4) recognized the homology between the intracellular domains of *Drosophila* toll and the IL-1 receptor in 1991. The first sequence of a mammalian TLR homolog was obtained in 1994 (5). However, toll itself was believed to be involved only in *Drosophila* embryonic development, until 1996, when Lemaitre et al. (6) showed that it also regulated antifungal immunity in adult flies. The first demonstration of a functional role for a mammalian TLR came from the work of Medzhitov et al. (7), who showed that a chimera of the intracellular domain of a TLR with the extracellular domain of CD4 would activate NF-κB; the expression of NF-κB-controlled genes for the inflammatory cytokines IL-1, IL-6, and IL-8; and the expression of the costimulatory molecule, B7.1, which is required for the activation of
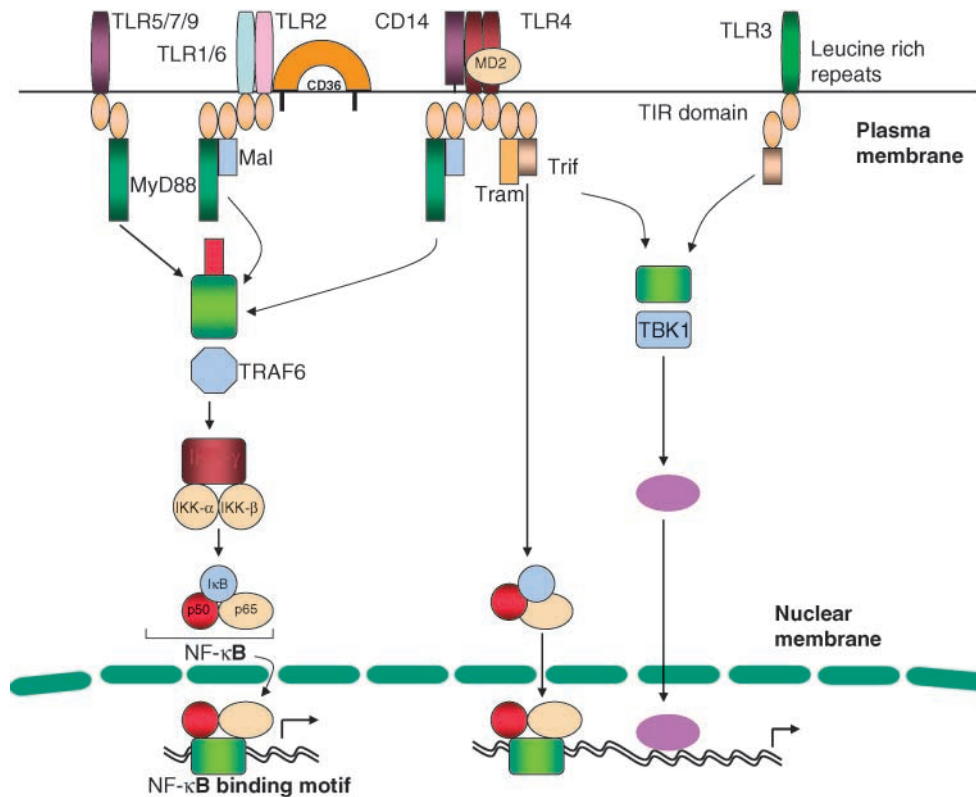
Fig. 1. Signaling pathways carrying TLR-derived signals. Modified from (11).

naive T cells. Whereas no ligand for a TLR was known at the time, this work set the stage for the current exploration of the role(s) of TLRs in innate as well as adaptive immunity. In the next year, 1998, it would be recognized that there was a whole family of mammalian TLRs, that they responded to microbial ligands, and that the long-sought bacterial receptor for lipopolysaccharide (LPS) was TLR4 (8–10). As of this writing, PubMed recalls 910 references during 2004 for "TLR," or an average of more than three per day, and it is difficult to keep up with the torrent in any comprehensive way.

It is now clear that the TLRs are crucial to the proper functioning of our immune systems, both innate and adaptive, and that they are critically involved in responses to infection. In their absence, death from experimental sepsis is significantly enhanced (11). Given that they are such important initiators of immune responses, it would not be surprising if, in some instances, they actually caused disease by inappropriate activation. In this review, we discuss their relevance to atherosclerosis. In this instance, and possibly others (12, 13), we may be paying the price for protection that arises in the form of an alternate disease—slower, but lethal, too.

AN OVERVIEW OF THE TLRS AND THEIR LIGANDS

There are now ten TLRs for which activating ligands are known, an eleventh for which no ligand is known, and rumors that a few more TLRs may exist (14). There is one additional essential component known, MD-2, that associates tightly with the ligand receptor domain of TLR4 and is required for TLR4 function (15, 16). Between them, the TLRs serve to detect most of the known signatures of microbial pathogens, including surface components and nucleic acids. A partial list of ligands is provided in **Table 1**. Defining the ligands for particular TLRs has, in some cases, resulted in mistaken definitions caused by the use of impure reagents (17–19). TLR2 and TLR4, in particular, have broad specificities. For TLR2, some of this breadth derives from its association with either TLR1 or TLR6. These heterodimers have different specificities, most strikingly displayed, in that a TLR2-TLR6 heterodimer detects bis-acylated lipopeptides, whereas a TLR2-TLR1 heterodimer detects tris-acylated lipopeptides (20, 21). Other proteins that assist in presenting ligands to TLRs include LPS binding protein (LBP), CD14, dectin, and CD36 (22–27). LBP and CD14, in conjunction with TLR4, enhance detection of endotoxin and other bacterial lipids, and dectin enhances detection of β-glucans (26). CD36, a known receptor for endogenous ligands, including fatty acids, oxidized LDL, and β-amyloid, has recently been identified as a TLR2 coactivator to sense microbial diacylglycerides, including lipoteichoic acid and MALP-2, a macrophage-activating lipopeptide from *Mycoplasma fermentans* (27).

In their roles as detectors of pathogen attack, the TLRs principally detect ligands derived from microbial pathogens. This allows the host to initiate an innate defensive inflammatory response as well as facilitate the development of an adaptive response by way of dendritic cell activation (28).

TABLE 1. TLR ligands

TLR Component	Pathogen (Exogenous)	Host (Endogenous)	Reference
2 with ?	Fimbriae		(73)
	Yersinia LcrV antigen		(74)
	HCMV		(75)
		Necrotic cells	(76)
2 with 1	Triacylated BLP		(21)
	LPS (Leptospiral)		(77)
	LAM		(78), (79)
2 with 6	PGN-associated lipoteichoic acid		(80)
	Zymosan		(81)
	Diacylated BLP		(20)
	Modulin		(82)
3	dsRNA		(83)
4 and MD-2	LPS		(8)
	Taxol		(84)
	HSP (?)	HSP (?)	(85), (30)
	RSV fusion protein		(86)
		β-Defensin 2	(87)
		Oxidized LDL or PL	(67), (68)
		Hyaluronic acid fragments	(67)
		Heparan sulfate	(29)
5	Flagellin		(88)
7 or 8	Resiquimod		(89)
	ssRNA		(90)
9	CpG DNA		(91)
		Demethylated DNA	(13)
10	None known		
11	Uropathogenic bacteria		(92)

CpG DNA, demethylated CpG containing DNA nucleotides; dsRNA, double stranded RNA; BLP, bacterial lipoprotein; HCMV, human cytomegalovirus; HSP, heat shock protein; LAM, lipoarabinomannan; LDL, low density lipoprotein; LPS, lipopolysaccharide; PGN, peptidoglycan; PL, phospholipid; RSV, respiratory syncytial virus; ssRNA, single stranded RNA; TLR, toll-like receptor.

It has also been proposed that TLRs may detect endogenous "danger signals," initiating sterile inflammatory responses at sites of tissue injury (29). Defining the endogenous ligands has been fraught with difficulty, frequently caused by the use of impure reagents unknowingly contaminated with trace amounts of strongly stimulatory microbial products (30). Of course, "sterile inflammation" can sometimes be caused by as-yet-undiscovered microorganisms; *Helicobacter pylori* is a striking example of this possibility.

Intracellular signaling derived from TLR activation derives principally, if not exclusively, from the association of several intracellular signaling adapters with the TLR signaling domains or TIR (Fig. 1). Three adapters are probably exclusively utilized by the TLRs; these are Trif (TIR domain containing adaptor protein-inducing IFNβ), Tram (Trif-related adaptor protein), and Mal (also known as TIRAP and TICAM-2). A fourth, MyD88 (myeloid differentiation primary response protein 88), is utilized by all the TLRs, except TLR3, and also by the IL-1 and IL-18 receptors. All of these intracellular adaptors associate with their respective TLRs through the TIR domain. However, usage of these adapters varies by TLR. A diagram showing their usage is presented in Fig. 1 (14, 31). In addition, Bruton's tyrosine kinase may be involved in the activity of TLR4, as well as possibly TLRs 6, 8, and 9 (31).

Clearly, TLRs function to signal a ligand binding event across a membrane. It is fairly well accepted that TLR2 (with TLR1 or 6) and TLR5 function at the plasma membrane, because there are no reports of their intracellular function and they serve to report extracellular ligands. There is one report of intracellular TLR2 in ocular mucosal epithelial cells, but its activation was not reported (32). Similarly, TLRs 3, 7, 8, and 9 are all reported to function intracellularly. However, TLR4 clearly functions both at the plasma membrane and intracellularly. Its function at the plasma membrane in myeloid cells is well accepted and it can be readily detected on the cell surface by fluorescence-activated cell sorting (FACS). In epithelial cells and human coronary artery endothelial cells (HCAECs), however, TLR4 appears to function intracellularly (23, 33, 34). TLR4 is not detectable by FACS in HCAECs unless cells are permeabilized. Similarly, TLR4 inhibitory antibodies, which readily inhibit monocyte responses to LPS, do not inhibit coronary artery endothelial cell responses. Interestingly, even with monocytes, the anti-TLR4 antibodies are not fully inhibitory; although there are several possible explanations for this observation, one is that some LPS-initiated monocyte activation could occur intracellularly, where antibodies would not reach (35).

STUDIES IN GENE-DEFICIENT MICE

Although atherosclerosis is certainly a multigenic disease, two gene-targeted knockouts in mice, the LDL receptor and apolipoprotein E, provide excellent models for study (36), and direct in vivo evidence exists that TLR signaling plays a role in atherosclerosis (37, 38). Two groups have demonstrated that MyD88, one of the TLR intracellular signaling molecules, participates in atherosclerosis.

MyD88 deficiency led to a reduction in plaque size, lipid content, expression of proinflammatory genes, and systemic expression of proinflammatory cytokines and chemokines such as IL-12 and MCP-1 (39, 40). Moreover, a total genetic deficiency of TLR4 is associated with reduction in lesion size, lipid content, and macrophage infiltration in hypercholesterolic apoE$^{-/-}$ mice (40). As pointed out earlier, MyD88 also participates in the IL-1 and IL-18 receptor pathways as an intracellular downstream signaling protein, and therefore TLR signaling need not be entirely responsible for the proatherogenic effect of the MyD88 deficiency. Both IL-1 and IL-18 have been shown to be involved in atherosclerotic progression in mouse models (41, 42). Moreover, other TLRs, in addition to TLR4, that utilize MyD88 intracellular signaling pathways could be involved. Interestingly, CD14 was shown not to participate in the proatherogenic response (39). Hollestelle et al. (43) also demonstrated that TLR4 is a key receptor in arterial modeling. Experiments using a femoral artery cuff model in proatherogenic apoE Leiden transgenic mice indicate that exposure to LPS increases plaque formation and outward arterial remodeling, whereas no outward arterial remodeling is observed in TLR4-deficient mice. This arterial remodeling was subsequently shown to occur via upregulation of a potential endogenous TLR4 ligand, the extra domain A of fibronectin, and to NF-κB, because animals deficient in NF-κB demonstrated increased degrees of arterial remodeling during carotid artery ligation (44). Studies by Michelson et al. (40) demonstrated that TLR4 deficiency in double mutant apoE- and TLR4-deficient animals results in a 25% reduction in the aortic surface area covered by lesions, as well as a reduction in lipid content of heart aortic sinus lesions. Importantly, this reduction in disease severity is obtained without a significant effect of the TLR4 deficiency on plasma cholesterol levels. We examined the effect of bone marrow-derived cell expression of TLR4 in atherosclerosis progression in low density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice fed a high-fat, high-cholesterol diet (45). Much to our surprise, no effect on lesion size was found, measured either as an *en face* aortic lesion percentage or as lesion volume within the heart aortic sinus. Our studies suggest that bone marrow-derived cell expression of TLR4 cannot account for the effects of TLR4 on atherosclerosis observed in the above-mentioned studies. If one assumes that TLR4 plays the same role in LDLr$^{-/-}$ mice that it does in apoE$^{-/-}$ mice, expression of TLR4 receptor by other cell types, including endothelial cells, vascular smooth muscle cells, and/or adventitial fibroblasts, is critical to the proatherogenic roles of toll receptor expression.

In contrast to TLR4, the role of TLR2 receptors in atherosclerosis has not been extensively studied. As mentioned earlier, TLR2 mediates responses to lipoproteins derived from multiple pathogens, and, unlike the case for TLR4 ligand specificity, TLR2 functions in heterodynamic interactions with other TLRs or adaptive proteins such as TLR6 and TLR1. Shishido et al. (46) reported that ventricular remodeling after myocardial infarction was more robust in TLR2-deficient animals, suggesting that inflam-

matory responses induced by myocardial infarction may be mediated by TLR2. These observations of decreased cell injury induced by ischemia reperfusion in TLR2 knockout mice have recently been confirmed by Favre and Henry (47). Finally, Schoneveld et al. (48) recently reported that TLR2 stimulation in vitro using peptidoglycan increased cytokine production in adventitial fibroblasts. Use of PAM$_3$ CSK$_4$ (a synthetic TLR2 agonist) in C57BL/6 mice resulted in enhanced cytokine expression and increased neointimal formation following the administration of a periadventitial cuff. Studies of the same cuff model in apoE$^{-/-}$ mice demonstrated that TLR2 stimulation increased arterial plaque size following 3 weeks of periadventitial injury. We now need studies to directly investigate the role of TLR2 in atherosclerosis progression in hyperlipidemic mice models such as LDLr$^{-/-}$ mice. These studies are in progress in our laboratories and indicate that TLR2 can influence atherosclerotic progress in LDLr$^{-/-}$ mice (Mullick, A. E., Tobias, P., Curtiss, L. K., unpublished observations).

TLR LIGANDS POTENTIALLY RELEVANT TO ATHEROSCLEROSIS

Endogenous as well as exogenous factors have been suggested as drivers of atherosclerotic inflammation, and many of these are potential TLR ligands. Of course, during the several decades that typify the development of human atherosclerotic disease, injurious ligands could come from many sources and change over time. Endotoxin, or LPS, is among the possible exogenous agonists driving atherosclerotic inflammation and is the subject of a recent review (49). Several observations suggest that endotoxin could be proatherogenic. Epidemiologically, it has been suggested that atherosclerosis and plasma endotoxin load are positively correlated (50). Furthermore, an inactivating mutation in some patients' TLR4 may be correlated with decreased atherosclerosis (51). Administration of endotoxin to hypercholesterolemic rabbits or to apoE$^{-/-}$ mice results in enhanced disease (52, 53). *Chlamydia pneumoniae* has been found in some atherosclerotic lesions (54). Whether the lesion is there because of chlamydial infection or the infection is there because a lesion and its cells provide a hospitable growth site is unknown. In a mouse model, infection with *Chlamydia* did enhance disease (55, 56). Periodontal disease also has been correlated with atherosclerosis. Infection of apoE$^{-/-}$ mice with *Porphyromonas gingivalis* results in increased atherosclerotic disease, but not by fimbriae-deficient organisms (57). *Chlamydia* sp. and *Porphyromonas* sp. certainly have ligands for at least TLR2 and TLR4.

Peptidoglycans have been reported in atherosclerotic lesions, and serum antipeptidoglycan IgM correlates inversely with intimal-medial thickness in a human study (58, 59). We are unaware of any animal studies involving administration of peptidoglycan to atherosclerosis-susceptible mice. It now seems very likely that peptidoglycan itself is not a TLR ligand; instead, contaminating lipo-

teichoic acids in peptidoglycan preparations are ligands for TLR2 (19, 27).

Suggested endogenous ligands for TLRs have included heat shock proteins, hyaluronic acid fragments, soluble heparan sulfate, fibrinogen extra domain A, oxidized LDL, and β-defensin 2. From a broad perspective, and excepting β-defensin 2, these could be considered as potential proinflammatory agents that might be relevant to atherosclerosis. Heat shock proteins are found in lesions (60); intimal remodeling during lesion development probably leads to hyaluronic acid fragments and soluble heparan sulfate; fibronectin extra domain A is found at lesions (61); and oxidized LDL is undoubtedly proatherogenic. β-Defensin 2 appears to be produced only in epithelial cells, and thus its potential relevance to atherosclerosis seems low. The great difficulty with proposing that an endogenous substance is a TLR agonist (and TLR4 is usually the TLR proposed) is that TLR4 agonists contaminate many recombinant products (30). Ironically, even genuine TLR4 agonists can be contaminated with agonists of other TLRs; the initial mistaken assignment of TLR2 as the endotoxin receptor came about because the endotoxin sample used in the studies was contaminated with Gram-negative bacterial lipoproteins, which are TLR2 agonists (62). Early reports that heat shock proteins 60 or 70 were TLR4 agonists and induced responses strikingly similar to LPS led to the suggestion that these proteins served as danger signals. Ironically, that was true, but not in the sense intended. Subsequent work by at least two labs has shown that the HSPs were LPS contaminated and that the contamination accounted for all the proinflammatory activity (30). It is certainly possible that heat shock proteins could be TLR agonists serving as danger signals, but in our view, that will require their production in eukaryotic rather than bacterial systems. The evidence that hyaluronic acid fragments and soluble heparin sulfate are TLR4 agonists seems somewhat more secure (29, 63). However, there is no evidence that these substances are proatherogenic. The fibronectin extra domain A is present in atherosclerotic lesions, and a recombinant fibronectin extra domain A fragment is a TLR4 agonist (64). However, the activity of this domain was tested only with prokaryotically produced recombinant material, and some skepticism seems advisable because of its possible contamination.

In general, the proatherogenic activity of these endogenous moieties has not been tested directly. How does an acute inflammatory response in a model system relate to the long-term in vivo exposure that occurs over the course of disease development? Only in the case of the fibronectin extra domain A has there been a study in atherosclerosis-prone mice. Mice deficient in expression of the fibronectin alternate splice form containing the extra domain A have been bred with atherosclerosis-prone mice, and these mice develop less disease (61).

The foregoing discussion of potential TLR agonists has so far ignored the potential gorilla in the middle of the room, namely oxidized LDL (OxLDL). Inasmuch as OxLDL is the principal component of a plaque, it would be highly relevant if it were a TLR ligand. A very recent publication shows that the oxidized phospholipid (OxPL) component of OxLDL does promote atherogenic inflammation in murine arteries (65). However, the OxPL does not appear to be activating TLR4, as some have proposed, because it does not elicit the same spectrum of products as LPS (66). In this study (65), the OxPL was topically applied to intact murine carotid arteries in a pluronic gel, and the genes expressed were analyzed by quantitative PCR and immunohistochemistry. The genes expressed were those of atherosclerosisrelated genes, such as MCP-1, KC, TF, and HO-1. However, HO-1, for example, is not one of the genes expressed by LPS stimulation of TLR4 (66), and, thus, OxPL, as a typical TLR4 ligand, cannot explain the results. Others have also observed that OxPL, in the form of minimally modified LDL, does not result in a typical spectrum of LPS/TLR4-induced products or responses (67, Y. Miller, unpublished observations).

Despite the atypical pattern of gene expression observed in vivo, there is evidence that OxPL is, or contains, a TLR4 ligand. Both Miller et al. (67) and Walton et al. (68) have observed that OxPL activates monocytes or endothelial cells in a TLR4-dependent manner. One explanation for the unexpected pattern of responses may be that the activation involves another, as-yet-unknown receptor component. Miller et al. reported that although CD14 was involved, antibodies that inhibit LPS-dependent TLR4 activation did not inhibit minimally modified LDL activation of monocytes (67). And Walton et al. (68) reported that a non-CD14, glycerophosphoryl inositol-tailed component was involved in the activation. It would be interesting to see Furnkranz et al. (65) repeat their study in TLR4-deficient mice. Another explanation could be that despite paying careful attention to possible LPS contamination, current preparations of OxPL or OxLDL are nevertheless multicomponent, perhaps to an extent varying from laboratory to laboratory.

Atherosclerotic lesions are not randomly distributed throughout the vasculature, despite the fact that the cells and lipids that accumulate to form lesions are systemically distributed. Caro, Fitz-Gerald, and Schroter (69) initially proposed the low-shear stress hypothesis of atherosclerosis. Physiologic levels of shear stress $(1–7 \text{ N/m}^2)$ shield against atherosclerosis via effects on the endothelium; these levels of shear inhibit expression of a number of endothelial surface markers (70, 71). Decreased shear stress at branches, bifurcations, and curvatures permits endothelial activation, adhesion molecule expression, and monocyte transmigration. We found that shear stress had differential effects on several receptors involved in inflammation; TLR4 and TNF receptor expression were insensitive to flow, whereas expression of TLR2 was strongly inhibited by high shear stress (72). Inasmuch as there seems to be an effect of TLR2 on atherosclerotic lesion development, and this effect originates in cells other than bone marrow-derived cells, the phenomenon of flow-regulated TLR2 expression in endothelial cells may be relevant to disease. If TLR2 is involved, can TLR2 ligand be far behind?

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