Review

Toll-like receptors in atherosclerosis: a 'Pandora's box' of advances and controversies

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Seminal research over the past 20 years has revealed atherosclerosis to be a chronic inflammatory process that shares features with traditional inflammatory diseases including rheumatoid arthritis. More recently, emphasis has been placed on the role of innate immunity in the development and progression of atherosclerosis. In particular, pattern recognition receptors, including Toll-like receptors (TLRs), have been the focus of much attention as modulators of atherogenesis. This review provides an update on the developments in this area of research in the past 2 years, with a specific focus on the current controversies and how these may affect the design of therapeutics. Specifically, we will address the recent evidence that TLRs elicit both protective and detrimental effects in atherosclerosis and the emerging observation that the outcome of TLR signaling is dependent on the agonist and responding cell type.

Atherosclerosis

Atherosclerosis is the main cause of coronary artery and cerebrovascular disease, which are the leading cause of death worldwide [1]. Atherogenesis is thought to begin with the development of endothelial dysfunction caused by the exposure of the vessel wall to systemic risk factors and local hemodynamics. The ensuing endothelial activation promotes the accumulation of inflammatory cells in the vessel wall. As atheroma (see Glossary) progresses, inflammatory cells produce cytokines and growth factors, which evoke smooth muscle cell migration into the intima. The architecture of the intima changes profoundly leading to the formation of two compartment lesions, the fibrous cap and the necrotic core. Inflammatory cells may also produce matrix degrading enzymes that disrupt the integrity of the fibrous cap or procoagulant molecules such as tissue factor, ultimately leading to plaque rupture and thrombosis [2].

Despite the reduction in mortality that has been achieved over the past decades, 70% of cardiovascular events cannot be prevented with the treatment of known risk factors [3]. Aggressive glucose metabolism control in type 2 diabetes did not automatically result in prevention of cardiovascular disease (CVD) [4] and lipid-lowering agents beyond statins have not yet delivered the expected reduction in cardiovascular events [5], highlighting a complex relationship between inflammation and hyperlipidemia. Inflammation is the important component of the pathogenesis of CVD that is not yet therapeutically targeted.

The role of the adaptive branch of immunity, which is acquired during an individual's lifetime and generates antigen-specific responses, was one of the first components of the pathogenesis of atherosclerosis to emerge, and its study led to the recent discovery of antigenic peptides within native lipoproteins [6,7]. Although the involvement of innate immune cells (e.g., monocytes, macrophages, etc.) has been known for a long time in atherogenesis [8,9], the determinant role of the innate arm of host defense is more recent and follows the discovery of pattern recognition receptors (PRRs) as the most potent inducers of inflammation ever known [10,11].

Features of innate immunity

Innate immunity is the first line of host defense and as such requires rapid deployment. PRRs facilitate a rapid response as they are a set of 'ready-made' receptors that recognize common pathogen constituents known as pathogen-associated molecular patterns (PAMPs). The mammalian host defense system makes use of at least 50 PRRs, which belong to one of three distinct groups: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Glossary

Apolipoprotein E deficient (ApoE$^{-/-}$) mice: mice deficient in apolipoprotein E, which are hypercholesterolemic and spontaneously develop atherosclerotic lesions.
Atheroma: accumulation of lipid and inflammatory cells inside arterial vessel walls.
Intima: innermost layer of an artery.
Myeloid cells: non-lymphocyte blood cells.
Statin: a class of lipid-lowering drugs.

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Together, PRRs provide surveillance of both intracellular and extracellular compartments. RLRs and NLRs are cytosolic PRRs that survey the intracellular space, whereas TLRs are transmembrane PRRs. TLRs, of which there are at least 13 in mammals, are the most extensively studied of the PRR families (reviewed in [12]) and will be the focus of this review. For all TLRs (except TLR3), signaling relies on the signaling adapter myeloid differentiation primary response gene 88 (MyD88) and results in the nuclear translocation of nuclear factor-κB (NF-κB) and interferon response factor (IRF) family members (Figure 1).

Each TLR has specificity for certain ligands, the best characterized of which are exogenous. Yet, a growing number of endogenous agonists, which can be released by activated or dying cells or that are generated as a result of tissue damage, injury, and oxidation have been established [13]. Potential endogenous TLR ligands (reviewed in [14]) include high-mobility group box (HMGB)-1, cholesterol crystals, modified lipid fragments, and the recently identified carboxy(alkylpyrrole) protein and globotetraosylceramide [15,16]. Broadly, TLRs can be divided according to the compartment where they exert sensing of the relevant molecular patterns. Whereas TLR1, 2, 4, 5, and 6

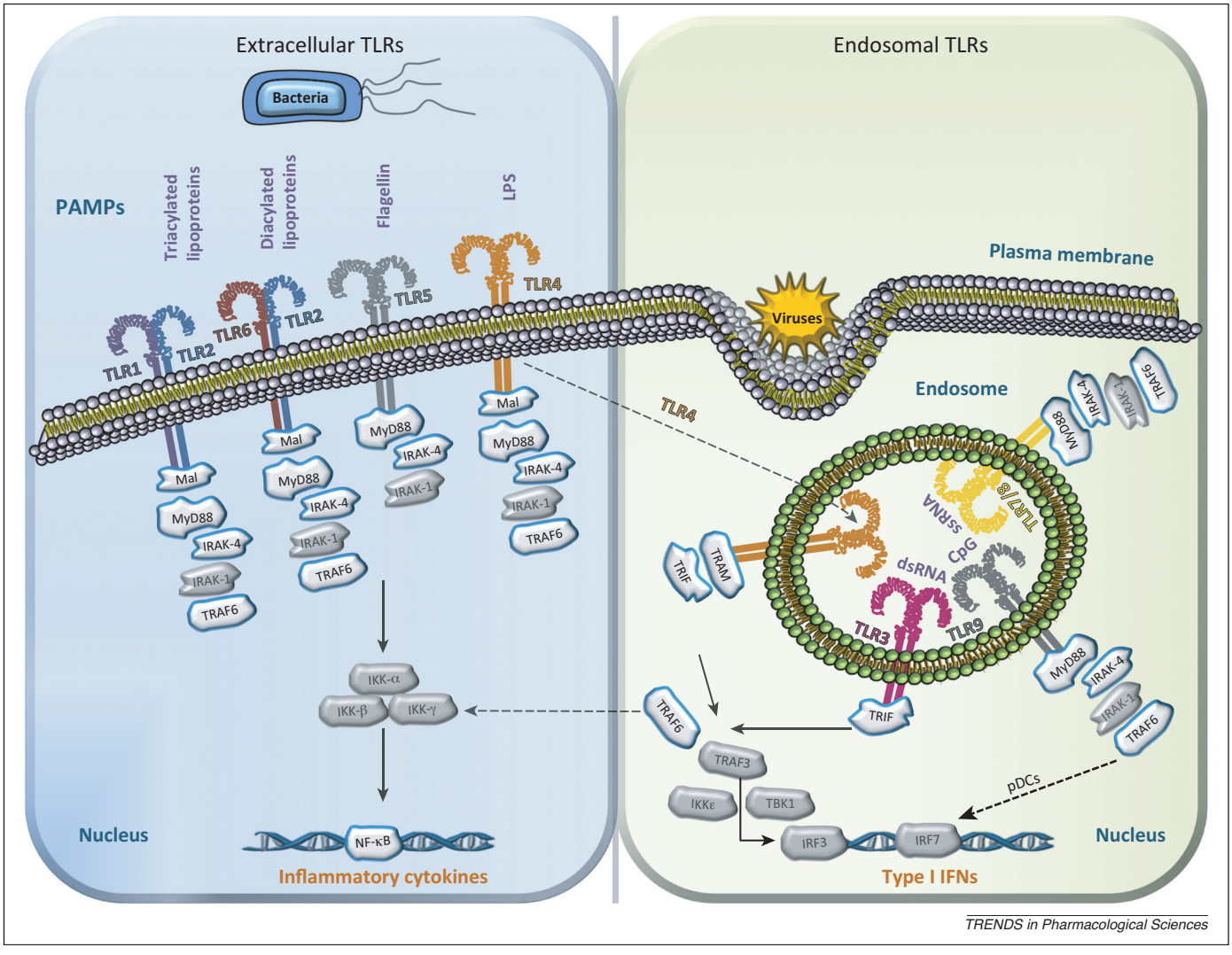

Figure 1. Toll-like receptor (TLR) signaling in atherosclerosis. Exogenous (including components of bacteria and viruses) and endogenous TLR ligands engage extracellular and intracellular/endosomal TLRs to trigger two distinct signaling cascades. All TLRs possess a Toll/interleukin-1 (IL-1) receptor (TIR) domain, which is required for signaling via downstream adapters of which there are five: myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter inducing interferon (IFN)-β (TRIF), MyD88-adapter-like (Mal)/TIR domain-containing adapter protein (TIRAP), TRIF-related adapter molecule (TRAM), and sterile-α and armadillo motif-containing protein (SARM). Molecules grayed out in the figure are those which have not yet been examined in atherosclerosis. **MyD88-dependent signaling pathway**: with the exception of TLR3, all TLRs signal via the adaptor protein, MyD88. TLR2 and TLR4 also require Mal/TIRAP to bridge their TIR domains with MyD88. Structurally, MyD88 consists of a TIR domain and a death domain (DD). Following ligand activation of TLRs, the MyD88 DD interacts with the DD of IL-1 receptor-associated kinase (IRAK)-4. Subsequently, other members of the IRAK family, including IRAK-1 and IRAK-2, become activated and associate with tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), an E3 ubiquitin ligase. The resulting polyubiquitin chains enable the recruitment of transforming growth factor (TGF)-β-activated kinase-1 (TAK-1) binding proteins, TAB-2 and TAB-3, which in turn activate TAK-1. TAK-1 then activates the IKK complex, which includes IKKα, IKKβ, and NEMO/IKK-γ, enabling nuclear factor-κB (NF-κB) translocation into the nucleus and, eventually, the transcription of inflammatory genes. Furthermore, TAK-1 mediates activation of mitogen-activated protein kinases such as Erk1, Erk2, p38, and Jnk, leading to the transcription of activating protein-1 (AP-1). **TRIF-dependent signaling**: the TRIF signaling pathway is used by TLR3 and translocated TLR4 (via TRAM). TRIF binds TRAF3 and TRAF6. TRAF3 associates with IKK-related kinases, TBK1 and IKKε, allowing interferon response factor (IRF)-3 nuclear translocation and, eventually, the production of type 1 interferons (IFNs). TNFR associated death domain protein (TRADD) is required for TRIF-dependent signaling. TRIF also binds TRAF6 and activates TAK-1, leading to the phosphorylation of IκBα by IKKα and IKKβ and driving NF-κB activation.

are located on the cellular membrane (for surveillance of the extracellular space), the remaining TLRs are placed on the endosome and lysosome membranes (for surveillance of the lumen of these intracellular vesicles) (reviewed in [17]). The only exception is TLR4, which can translocate from the surface to the endosomes and induce signaling with all four of the signaling adapters [18].

In this review, we aim to provide an update on the requirement of TLRs in the initiation and development of atherosclerosis and explore the current controversies in the field. The knowledge that TLRs may be detrimental in atherosclerosis emerged in the mid-2000s [19]. However, the field is rapidly expanding, including reports over the past 2 years that TLRs may also exert atheroprotective functions. Thus, a review of the role of TLRs in atherosclerosis is timely and required. Because the sensing compartment of the TLR is relevant to the overall effect on the modulation of atherosclerosis, we will discuss intracellular and extracellular TLRs separately.

TLRs as extracellular sensors in atherosclerosis

One of the first pieces of evidence suggesting a detrimental role for TLR signaling in atherogenesis came from the landmark study of Bjorkbacka *et al.* [19], demonstrating that the whole body deficiency of MyD88 reduced both atherosclerotic lesion development and macrophage accumulation in Apolipoprotein E deficient ($ApoE^{-/-}$) mice. This study was confirmed [20] and was followed shortly by the demonstration that whole body genetic deletion of TLR2 and TLR4 in murine models of atherosclerosis results in an approximate 50% reduction in lesion formation via inhibition of lipid deposition and inflammatory cell recruitment and activation [20–23] (Table 1). Similarly, in arterial injury models, MyD88, TLR2, and TLR4 deficiency lead to protection from injury [24–26].

Expression of TLR2 and TLR4 in human atherosclerotic plaques is colocalized with the nuclear translocation of the p65 NF-κB family member in lesional endothelial cells and macrophages [27]. Although conflicting results have

Table 1. Functional effects of targeting TLRs in atherosclerosis

TLR	Main signaling pathway(s)	Effect on atherosclerosis	Mechanism of effect	Refs
TLR1	MyD88/ TIRAP (Mal)	• No difference in lesion development in $LDLR^{-/-}TLR1^{-/-}$ versus $LDLR^{-/-}$ mice • ↓ Atherosclerosis in $LDLR^{-/-}TLR1^{-/-}$ versus $LDLR^{-/-}$ mice following Pam3 stimulation	Not determined	[31]
TLR2	MyD88/ TIRAP (Mal)	• 55% ↓ in atherosclerosis in $LDLR^{-/-}TLR2^{-/-}$ versus $LDLR^{-/-}$ mice • No effect of myeloid-specific TLR2 deletion in $LDLR^{-/-}$ in the absence of exogenous ligand • ↓ Atherosclerosis in $LDLR^{-/-}$ with myeloid-specific TLR2 deletion following Pam3 stimulation • ↓ Atherosclerosis in $ApoE^{+/-}TLR2^{-/-}$ versus $ApoE^{+/-}TLR2^{-/-}$ mice following high-fat feeding and bacterial infection • ↓ Plaque vulnerability features in TLR2 blocked human atheroma cells	• ↑ Macrophage infiltration, lipid accumulation, apoptosis • ↓ Smooth muscle cells • NF-κB activation, production of proinflammatory cytokines and MMPs	[22,30,37]
TLR3	TRIF	• 40% ↑ in atherosclerosis in $ApoE^{-/-}TLR3^{-/-}$ versus $ApoE^{-/-}$ mice • ↑ Atherosclerosis in $LDLR^{-/-}$ $TLR3^{-/-}$ versus $LDLR^{-/-}$ mice • ↑ Atherosclerosis in $ApoE^{-/-}$ mice following exogenous poly(I:C) stimulation • ↓ Atherosclerosis in $LDLR^{-/-}$ with myeloid-specific TLR3 deficiency	• Protection from intimal and medial vascular injury • Exogenous TLR3 activation induces endothelial dysfunction and production of proinflammatory cytokines	[33,34,47,50]
TLR4	MyD88/TIRAP (Mal) or TRIF/TRAM	• ↓ Atherosclerosis in $ApoE^{-/-}TLR4^{-/-}$ versus $ApoE^{-/-}$ mice	• ↓ Lesional lipid content and ↑ lesional macrophage infiltration • ↓ Proinflammatory cytokine production in $ApoE^{-/-}TLR4^{-/-}$	[20]
TLR5	MyD88	Not determined	Not determined	
TLR6	MyD88/ TIRAP (Mal)	• No difference in lesion development in $LDLR^{-/-}TLR6^{-/-}$ versus $LDLR^{-/-}$ mice • ↓ Atherosclerosis in $LDLR^{-/-}TLR6^{-/-}$ versus $LDLR^{-/-}$ mice following MALP2[a] stimulation	Not determined	[31]
TLR7	MyD88	• ↑ Atherosclerosis in $ApoE^{-/-}TLR7^{-/-}$ versus $ApoE^{-/-}$ mice	• ↑ Necrotic core formation, lipid deposition, macrophage infiltration, and proinflammatory cytokine production, ↓ smooth muscle cells and collagen	[57]
TLR8	MyD88	Not determined	Not determined	
TLR9	MyD88	Not determined	Not determined	

[a]MALP2, macrophage-activating lipopeptide 2.

emerged from studies examining TLR polymorphisms in humans (reviewed in [28]), data from our laboratory has provided functional evidence that signaling through TLRs is strongly involved in the activation of human atherosclerotic lesions. We pioneered a method for the isolation of live cells from surgical carotid endarterectomies. The isolated cells comprise a mixed population representing the major cell types resident in human atherosclerotic plaques (e.g., macrophages, smooth muscle cells, and T lymphocytes), which spontaneously produce a wide range of proinflammatory cytokines and chemokines without extrinsic stimulation [29]. We utilized this system to perform functional studies on the requirement of TLR signaling in NF-κB activation and cytokine production in live human cells from the disease site. In this study, blockers of TLR2 and MyD88 signaling almost abolished NF-κB activation, the production of the inflammatory molecules chemokine (C–C motif) ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1), interleukin-6 (IL-6), CXCL8/IL-8, and the generation of the matrix degrading enzymes matrix metalloproteinase (MMP)-1, -2, -3, and -9. Conversely, TLR4 and its signaling adaptor, TRIF-related adaptor molecule (TRAM), were not required for cytokine production but had a selective role in MMP-1 and -3 production [30].

Recent studies revealed significant intricacies of TLR ligand sensing in atherosclerosis. The first advance is the identification of differences in the signaling requirements of endogenous or exogenous agonists for TLR2 in the context of atherosclerosis. TLR2 forms heterodimers with either TLR1 or TLR6, and the specificity of each heterodimer (TLR1/2 and TLR2/6) is different for specific molecular structures. The intraperitoneal administration (a common systemic delivery route in murine models) of either TLR1/2 or TLR2/6 synthetic agonists mimicking bacterial PAMPs enhanced local lesion formation (e.g., in the abdominal aorta) in low density lipoprotein (LDL) receptor deficient ($LDLR^{-/-}$) mice fed a high fat diet [31]. This augmentation was lost in $LDLR^{-/-}TLR1^{-/-}$ mice and $LDLR^{-/-}TLR6^{-/-}$ mice, respectively [31], as well as in $TLR2^{-/-}$ mice [23], indicating that TLR1/2 and TLR2/6 heterodimers are necessary for enhancing atherogenesis in the presence of bacterial PAMPs. Yet remarkably, when no exogenous agent was administered, TLR1 and TLR6 were redundant for lesion development in hyperlipidemic animals, suggesting that hypercholesterolemia acts through different pathways to exogenous ligands [31]. Very recent findings start to shed light on the identity of hyperlipidemia-derived endogenous ligands in human pathology. High density lipoproteins (HDLs) from patients with chronic kidney dysfunction markedly reduced nitric oxide bioavailability in human aortic endothelial cells and resulted in an increase of systemic arterial blood pressure in wild type mice via TLR2 in a TLR1- or TLR6-independent pathway, suggesting that HDL from these patients can induce TLR signaling [32].

The second recent development is the enhanced understanding of the downstream signaling pathways that mediate the effect of TLRs on atherogenesis. TLRs induce intracellular signaling via adapter molecules that relay the signaling cascades from the surface receptor to the downstream intracellular signaling molecules. In addition to MyD88, other signaling adapters used by TLRs include MyD88 adaptor-like (MAL), TRAM, and TIR domain-containing adaptor-inducing interferon-β (TRIF) (Figure 1). A TRIFLps2 lack-of-function mutation was atheroprotective in hyperlipidemic $LDLR^{-/-}$ mice via reduction of local and systemic inflammation [33]. Because $LDLR^{-/-}$ mice deficient in TLR3 showed some enhancement of disease (discussed further below), the authors concluded that hyperlipidemia gives rise to endogenous activation of the TRIF signaling pathway via TLR4 with proatherogenic consequences [33]. Moreover, hematopoietic deficiency of TRAM and TRIF, but not MAL, reduces atherosclerosis without affecting cholesterol metabolism via attenuation of systemic and vessel inflammation [34].

Finally, it is becoming clear that the expression of TLRs in specific cell types has a bearing on the effect that TLR signaling has on lesion formation. The cellular expression patterns of TLRs in atherosclerosis are already reviewed in detail in [14]. Briefly, TLR expression varies across different vascular beds with expression being higher in arteries compared with veins [35], and it is not isolated to myeloid inflammatory cells. In atherosclerotic lesions in the aortic root of $ApoE^{-/-}$ mice, TLR4 is selectively expressed by macrophages [36]. However, TLR2 expression increases in endothelial cells in atherosclerosis-susceptible regions upon inception of hypercholesterolemia [37]. In human atheroma compared with healthy vessels, TLR1, TLR2, and TLR4 expression is increased [27], particularly in endothelial cells and macrophages [27,36]. Several studies have described increased TLR2 and/or TLR4 expression and responsiveness on circulating monocytes in patients with a spectrum of coronary syndromes [38–40]. More recently, Kashiwagi *et al.* observed that TLR4 was more frequently expressed on the non-classical $CD14^{+}CD16^{+}$ monocytes in patients with acute myocardial infarction, in particular in vessels draining from the culprit coronary artery compared with systemic blood [41]. Further studies are needed to identify the systemic or local factors that are specifically involved in increased expression of TLRs in peripheral blood mononuclear cells and whether it is linked to pathogenesis or prognosis of disease.

The effect of TLR signaling on atherosclerosis is strongly dependent on the cell type that is expressing it. Surprisingly, bone marrow (BM) transfer studies have revealed that BM cell derived TLR2 expression is not involved in responses to endogenous agonists produced in the presence of hypercholesterolemia but is required for responses to mimics of bacterial PAMPs [23]. Similar results have been obtained with TLR4-deficient mice [42], undermining the natural assumption that the proatherogenic role of TLRs is solely linked to their expression in hematopoietic cells. Even more strikingly, a recent study has shown that when MyD88 expression is selectively lost in cells expressing CD11c (commonly dendritic cells), atherosclerotic lesion formation increases – rather than decreasing as in whole body deficiency of MyD88 [19] due to the loss of formation of T regulatory cells and loss of their protective effect on lesion formation via transforming growth factor (TGF)-β-mediated MCP-1 reduction [43]. Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) is an E3 ubiquitin ligase involved in downstream signaling of

IL-1/TLR family members and certain TNFR members. Endothelial cell specific TRAF6 deficiency in female ApoE$^{-/-}$ mice attenuated atherosclerosis as a result of reduced monocyte recruitment via reduced adhesion molecule and proinflammatory gene expression [44]. Conversely, myeloid TRAF6 was atheroprotective via promoting IL-10 expression, reduced endoplasmic reticulum stress, increased capacity to clear apoptosis, and reduced sensitivity to oxidized LDL-induced apoptosis [44]. These studies indicate that TLR signaling is a complex balance of heterogeneous cellular responses that need to be taken into account when designing therapeutics to address this area of pharmacology.

Intracellular TLRs in atherosclerosis

In the past 2 years, the spotlight has been placed on the role of endosomal TLR signaling as a modulator of atherosclerosis. One of the key features of endosomal TLRs is the activation of the interferon pathway as well as cytokine pathways (Figure 1). Earlier studies had already shown that TLR9 stimulation with a synthetic oligonucleotide carrying unmethylated CpG-containing sequences (usually contained in bacterial DNA) induced interferon (IFN)-α production from segments of human carotid plaques in culture, presumably due to activation of plasmacytoid dendritic cells, that are strong producers of IFNα upon viral infection [45]. IFNs can also, in turn, enhance the expression of intracellular PRRs on vascular tissues. IFN-γ induces expression of the endosomal RNA sensor TLR3, and the intracytoplasmatic members of the RLR family MDA5 and RIG-I in non-atherosclerotic human coronary artery rings [46]. Indeed, we observed that TLR3 expression is significantly increased in smooth muscle cells from diseased tissue (AthSMC) compared with control cells. This increased TLR3 expression in AthSMC led to a 40-fold enhancement of TLR3 signaling and generation of both proinflammatory and anti-inflammatory cytokines [47].

In 2011, we were the first group to describe TLR-mediated atheroprotection [47]. We observed that in a murine model of arterial injury, intraperitoneal administration of the synthetic TLR3 ligand Poly(I:C) – which mimics viral long double-stranded RNA – attenuates neointima formation and also reduces injury-induced medial damage in C57BL/6 mice. TLR3-mediated protection of the media from damage was also observed in the absence of exogenous stimuli, suggesting that endogenous protective TLR3 ligands are produced during the response to injury to avoid medial damage. In addition, in chow-fed ApoE$^{-/-}$ mice deficient in TLR3$^{-/-}$, lesion development was accelerated, with disease enhancement at early (15 weeks of age) but not late (30 weeks of age) time points, confirming that TLR3 has protective functions in the vessel wall [47]. This enhancement of atherogenesis in ApoE$^{-/-}$TLR3$^{-/-}$ mice was observed in the absence of exogenous TLR3 stimulation, suggesting that a protective endogenous agonist for TLR3 may be generated during the initiation of atherogenesis. Our observations were recently confirmed in another strain of hyperlipidemic mice. Richards *et al.* crossed TLR3$^{-/-}$ mice with LDLR$^{-/-}$ mice, resulting in increased lesion formation in the resultant LDLR$^{-/-}$TLR3$^{-/-}$ double knockout mice [33]. As yet, the endogenous TLR3 ligands involved in atherosclerosis are unknown, but mRNA from necrotic cells and stathmin, a microtubule regulatory protein have both been identified as potential endogenous TLR3 ligands [48,49].

The outcome of TLR3 signaling in the context of vascular responses is, however, not univocal. Zimmer *et al.* published work examining endothelial cell responses to TLR3 activation [50]. In this study, the authors showed that the intravenous administration of Poly(I:C) augmented endothelial dysfunction and reactive oxygen species (ROS) production in a TLR3-dependent manner. Following electrical denudation of carotid arteries, the administration of Poly(I:C) impaired re-endothelialization, despite significantly increasing the number of circulating endothelial progenitor cells. No effect on the development of neointima formation was reported in this study. Finally, increased lesion development in high-fat fed ApoE$^{-/-}$ mice was observed after Poly(I:C) stimulation [50]. Echoing these results, a recent link has been demonstrated between activation of endosomal TLR signaling with arterial and gestational hypertension [51–53]. In particular, TLR3 activation induces the production of the vasoconstrictor peptide endothelin-1 in pulmonary arterial hypertension [54].

The divergence of effect of TLR3 on atherosclerotic lesion formation [33,47,50] is difficult to explain fully, yet it is potentially dependent on the different doses and administration routes, as well as the presence or absence of a high-fat diet in the experimental setting, indicating that the effect of TLR stimulation is context-dependent. In another recent study, Lundberg *et al.* have shed some light on the possible root of the current discrepancies. The authors examined atherosclerotic lesion formation in high-fat fed LDLR$^{-/-}$TLR3$^{-/-}$ BM chimeras in which hematopoietic cells are selectively TLR3-deficient [34]. This study observed a protection from lesion development in the chimeric mice, suggesting that TLR3 activation on hematopoietic cells is detrimental to lesion development. This observation is in keeping with earlier studies showing that elective deficiency of IFN-β in BM decreases atherosclerotic lesion formation [55]. Data from a very recent study suggest that the detrimental role of myeloid TLR3 may be mediated through MMP2 [56]. TLR3 deficiency in BALB–ApoE$^{-/-}$Npc1$^{-/-}$ mice was associated with reduced MMP2 activity and increased lesional collagen and smooth muscle cell content [56], suggesting a role for TLR3 in degrading the extracellular matrix in lesions. Collectively, this body of information indicates that the outcome of TLR3 stimulation is dependent on the overall contribution of the different cell types bearing this receptor.

A protective role for TLR7 in atherosclerosis has also been recently described. ApoE$^{-/-}$TLR7$^{-/-}$ mice display enhanced lesion development compared with ApoE$^{-/-}$ mice [57]. Furthermore, lesions in ApoE$^{-/-}$TLR7$^{-/-}$ mice displayed increased macrophages and lipids and reduced smooth muscle cell content within the lesions. Reduced production of proinflammatory cytokines and chemokines was associated with TLR7 in human plaques [57]. Similar to TLR3, TLR7 is surrounded by some controversy. In a femoral artery cuff model, blockade with a TLR7/9 antagonist reduced neointimal thickening, macrophage infiltration, and cytoplasmic HMGB-1 expression, which indicates

reduced cell stress [58]. Given the protective role of TLR7 observed in ApoE$^{-/-}$ mice, it is possible to speculate that the antagonist mediates its beneficial effects by acting through TLR9. Indeed, in a transverse aortic constriction model of heart failure, it was recently shown that TLR9$^{-/-}$ mice had a better outcome including improved cardiac function and less macrophage infiltration than control mice [59]. However, in a rabbit model of collar-induced injury, administration of the synthetic exogenous TLR7 agonist imiquimod augmented lesion formation, cytokine release, and plaque infiltration [60]. Thus, whereas TLR7 appears to confer atheroprotective functions in the setting of unperturbed hypercholesterolemia, administration of TLR7 agonists may have the reverse effect. It is possible that similar to TLR2 and TLR3, the effects of TLR7 activation are different depending on exogenous versus endogenous ligand activation.

Emerging therapeutics and future challenges

The therapeutic potential of targeting TLRs in atherosclerosis and associated conditions is currently almost unexplored. In terms of preclinical studies, TLR2 is leading the way as the therapeutic target of choice in the context of vascular disease. Blocking TLR2 signaling reduces proinflammatory pathways in human atherosclerosis [30]. In addition, the TLR2-specific monoclonal antibody OPN301 blocks TLR2-induced proinflammatory cytokine signaling and has been shown to maintain heart function and reduce infarct size in murine ischemia/reperfusion (I/R) injury [61]. In a recent study, the clinical grade humanized version of this antibody, OPN305, was shown to also reduce infarct size and increase cardiac function in a porcine model of myocardial I/R injury [62]. Given the failure of translation of therapeutic successes from small animal models to humans, success in a porcine model, which has anatomy and physiology more similar to humans than rodent models, suggests better hope for translation. Although whole body expression of TLR4 has a role in murine atherosclerosis, its role in human atherosclerosis appears to be more limited [30]. An anti-TLR4 antibody has recently been shown to lower blood pressure in rats [63]. Few studies have examined TLR4 blockade in murine models. Administration of a TLR4 antagonist, *Rhodobacter sphaeroides* lipopolysaccharide (Rs-LPS), had no effect on early atherosclerosis in ApoE$^{-/-}$ mice in a very recent study [64]. However, Rs-LPS did attenuate lesion formation in diabetic ApoE$^{-/-}$ mice revealing a potential context-dependent beneficial effect of TLR4 blockade in atherosclerosis [64].

Several challenges remain before the promise of therapeutic modulation of TLR signaling becomes a clinical reality in atherosclerosis. Firstly, there are unanswered questions. The majority of the available studies only report the effect of TLR modulation in terms of lipid-rich lesion area. This solely reflects the lipid accumulation process, which is only one of the many features of atherogenesis. What is the role of TLRs on other crucial aspects of human CVD pathogenesis such as plaque vulnerability, lesion remodeling, and cell death? In addition, further studies are warranted to ascertain how TLRs affect the various pathways that lead to CVD in women and men.

Moreover, the potential modulation of TLR signaling for clinical purposes will have to withstand an assessment of the potential safety risks. TLRs play a central role in host defense against pathogens, and the blockade of their signaling may cause undesirable increased infection risk in patients. For instance, MyD88-deficient mice are highly vulnerable to at least 35 pathogens in the experimental setting [65]. In humans, tuberculosis is a particular concern in carriers of genetic variants of TLRs (recently reviewed in [66]). However, some reassurance for the feasibility of TLR blockade in humans comes from patients with inherited deficiencies of MyD88 [65] and its neighboring signaling protein interleukin-1 receptor-associated kinase-4 (IRAK-4) [67]. Both MyD88-deficient children and MyD88-deficient mice exhibit the same lack of *in vitro* MyD88-dependent functional responses. Yet the clinical phenotype of these children was surprising because no severe viral, parasitic, and fungal diseases were found. Importantly, the range of bacterial infections was narrow and related to invasive pneumococcal disease and noninvasive bacterial infections of the upper respiratory tract and the skin, mostly caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively. Spontaneous improvement occurred in adolescence [68], suggesting that in humans at least MyD88-dependent responses may play a more restricted role in host defense than previously anticipated. Further therapeutic developments for CVD in this area will need to undergo a proper risk/benefit ratio assessment taking into account the observed differences between mice and humans.

An alternative approach to blocking TLRs and their signaling mediators would be to focus on the search for and selective targeting of athero-relevant molecular patterns that can be recognized by TLRs, eliminating the risk to host defense. Additionally, it may be possible in the future to harness/promote the antiatherogenic functions of intracellular TLRs with selective agonists. Critical to this will be the full elucidation of the consequences of exogenous versus endogenous TLR ligand activation pathways.

Concluding remarks

Reflecting the increasing perception of innate immunity as the key initiator of immune responses, the study of innate immune receptors in human disease is a recent focus of research [10,11]. The vascular field is not immune from this transition. Increasing evidence supports roles for TLRs as key modulators of the initiation and development of atheroma. Their effects are not limited to the activation of inflammatory cells but also alter the behavior of resident vascular cells [23,46,47,50]. The therapeutic payoff of this new knowledge on the interaction between innate immunity and vascular disease is likely to be very high. Yet, the development of therapeutic tools will necessitate the further dissection of cell specific as well as agonist-specific effects.

The emerging paradigm from whole body genetic deficiencies is that extracellular TLRs mediate proatherogenic signaling in the majority of cases, whereas endosomal TLRs mediate atheroprotection. This difference is unlikely to solely reflect differences in downstream signaling due to the convergence of most TLRs (except TLR3) on MyD88.

Thus, the main contributor to the outcome of TLR stimulation is the compartment where TLRs are sensing in specific conditions. The effect of several TLRs on the development of atherosclerosis in the absence of exogenous agonists indicates that the disease itself is associated with the emergence of endogenous agonists, generated upon the establishment of hypercholesterolemia or cell damage. Intriguingly, the outcome of TLR stimulation in atherogenesis is different when exogenous agonists are introduced compared to when the disease model is left unperturbed. This is not surprising in light of the fact that TLRs are well known to signal differently upon encountering endogenous or exogenous agonists [69].

An ulterior level of complexity is the fact that TLRs give rise to context-specific responses that are dependent on the cell type that is expressing them. Indeed, myeloid-specific deficiency studies have in some instances given divergent results from whole body genetic deficiencies, raising questions on the cellular subsets that are responding to signaling in each disease context. Targeting strategies will also need to consider that the cellular composition of atherosclerotic lesions changes during plaque progression as the consequences of blocking/stimulating a given TLR may differ at different points in disease development.

TLR signaling has the ability to modulate atherosclerosis in ways that were previously unsuspected. Yet the deeper we explore them, the more it becomes a 'Pandora's box'. According to Greek mythology, Pandora was the first woman on Earth, and – following a long tradition of female ancestors – was betrayed by her curiosity and opened against all advice a forbidden jar liberating all the evils into the world. In the case of TLRs in atherosclerosis, it is not necessarily 'evils' but 'complexities' that recent research has set free. Developing therapeutics against TLR signaling will entail understanding these complexities further and targeting (i) the right agonist, (ii) the right cell, (iii) at the right time to avoid proatherogenic consequences. The last resource that remains, as for Pandora, is hope (that we get the biology right).

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