EXPERT REVIEWS

Treating atherosclerosis: the potential of Toll-like receptors as therapeutic targets

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Atherosclerosis is an inflammatory disease with a strong involvement of innate immunity. Toll-like receptors (TLRs) are the best-characterized pattern recognition receptors of the innate immune system. Almost all cell types in lesions, inflammatory leukocytes and resident vascular cells alike express TLRs. TLRs are able to sense modified lipids, enhance foam cell formation, induce leukocyte recruitment, and increase cytokine and matrix metalloproteinase production within atherosclerotic lesions. As such, TLRs represent an important link between atheroma and inflammation, making them attractive targets for the treatment of cardiovascular disease. Novel TLR-specific biologics are being developed and tested in other inflammatory diseases. This article will describe the exciting potential of TLRs as therapeutic targets for the treatment of atherosclerosis and will also highlight the potential challenges in the clinical application of TLR-based therapeutics in cardiovascular disease.

Keywords: atherosclerosis • inflammation • therapeutics • Toll-like receptor

Cardiovascular diseases account for a fifth of deaths worldwide [1] and thus constitute a large socioeconomic burden. Atherosclerosis, a complex disease involving both resident vascular and blood-borne cells, is the major underlying cause of cardiovascular disease. Atherosclerosis is, in the majority of patients, a slow progressive disorder, silently developing over years to decades of an individual's life. However, its manifestations are often acute and dramatic, and include myocardial infarction, stroke and sudden death.

Lipid accumulation at sites of nonlaminar flow is currently the most characterized initiating mechanism of atherosclerosis [2]. In these areas the recruitment sequence – involving increased expression of adhesion molecules and chemokines by endothelial cells (ECs) – is activated and leukocytes, predominately monocytes, infiltrate the subendothelial space [3–5]. Two main populations of monocytes have been described in both mouse and man, characterized by differences in chemokine receptor expression, size and granularity [6–8]. In addition, a third subset of human monocytes expressing markers placing them as an intermediate subset between the two main populations has also been described [9]. 'Inflammatory' monocytes are preferentially recruited into murine atherosclerotic plaques through the chemokine receptors CCR2, CCR5 and CX3CR1 and their ligands [10]. 'Resident' monocytes, which display anti-inflammatory and proangiogenic roles in a murine model of myocardial ischemic injury [11], are also recruited into atheroma, albeit with less frequency than 'inflammatory' monocytes, via CCR5 [10]. Recruited plaque monocytes become activated following exposure to a milieu of growth factors, upregulate scavenger receptor expression and take up modified lipids. Lipid uptake by monocytes/macrophages promotes their differentiation into large, lipid-laden foam cells leading to their entrapment in the vessel wall. The accumulation of inflammatory cells within the intima leads to the production of reactive oxygen species and cytokines [12]. Monocytes and ECs are not the only cells that participate in lesion formation. Other inflammatory cells, including T lymphocytes, mast cells and different subsets of dendritic cells (DCs), also contribute to lesion formation through antigen recognition and cytokine production [13]. Smooth muscle cells of medial or progenitor origin make their entrance at a later stage and surround the increasing inflammatory infiltrate, which is becoming fatty and necrotic in an attempt to support tissue integrity by forming a fibrous cap. A mature atherosclerotic plaque is now formed. As such, the plaque may be asymptomatic as long as the

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expansive remodeling accommodates the protruding lesion and the fibrous cap remains intact. Plaque rupture is the most common precursor of acute plaque thrombosis; however, other mechanisms of acute plaque thrombosis have been described [14,15]. Both T lymphocytes and macrophages may compromise the strength of the fibrous cap by hampering collagen synthesis via IFN-γ [16] or producing matrix metalloproteinases, respectively.

The hallmarks of chronic inflammatory diseases – leukocyte recruitment, antigen presentation, upregulated cytokine production and tissue destruction – are all present in atherosclerosis [17]. To a significant extent, atherosclerosis may be considered to be an inflammatory disorder [18]. Increasing evidence strongly supports roles for both the innate and adaptive immune systems in lesion formation [16]. One of the recent advances in the last decade has been the appreciation that innate immunity is far from being simply activated in a blind fashion. It is rather activated by specific molecular patterns expressed by pathogens and probably modified self-molecules via pattern-recognition receptors (PRRs). Several classes of PRR have been described including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, RNA helicases and C-type lectin receptors. TLRs have been the most extensively examined and characterized. At least 13 TLRs have been described in mammals, each one with a certain degree of specificity for a range of both exogenous and endogenous ligands [19]. TLR2, TLR4 and TLR5 are located on the cell surface whereas TLR3, TLR7, TLR8, TLR9 and murine TLR13 are expressed on the endoplasmic reticulum, endosomes and lysosomes. TLR ligation leads to activation of downstream signaling cascades, and the subsequent production of cytokines and activation of receptors involved in the immune synapse. By doing so TLRs dictate the type of adaptive immune responses required for host defence and disease alike. The realization of such unforeseen specificity in the innate immune response has shifted the focus from the adaptive to innate immunity in terms of development of therapeutics [20].

In this article, we aim to present the recent evidence supporting the role of TLRs as the missing link between atheroma and inflammation, through their contribution to leukocyte recruitment and activation, foam cell formation and tissue destruction. TLRs are attractive therapeutic targets for atherosclerosis and many other inflammatory diseases. We will detail existing data supporting roles for TLRs in the development of atherosclerosis and we will discuss potential strategies for treating atherosclerosis through therapeutic targeting of TLRs. Finally, we will highlight the potential challenges that may be faced in the development of such therapeutics.

TLR signaling pathways

On the basis of structural homology in the cytoplasmic region, TLRs are members of a larger superfamily that includes IL-1 receptors (IL-1Rs) [21]. All TLRs share the Toll/IL-1 receptor (TIR) domain, which is essential for downstream signaling through homophilic interactions with numerous adaptor molecules [19]. The extracellular region of the TLRs contains leucine-rich repeats that form a horseshoe-shaped solenoid structure [22].

Recognition of pathogen-associated molecular patterns (PAMPs) by TLRs leads to transcriptional upregulation of inflammatory genes depending on the TLRs involved. TLRs mainly sense as homodimers, although TLR2 can heterodimerise with TLR1 or TLR6 [23] and TLR4 can heterodimerise with TLR6 [24]. In addition, the initiation of signaling relies on the participation of a variety of coreceptors to the signaling complex. Scavenger receptors feature among these coreceptors [25]. The differences in TLR response can be explained by the distinct and divergent signaling pathways that are activated following TLR ligation. A family of five adaptor proteins are recruited to the TIR domain of the TLR, including myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TRIF-related adaptor molecule (TRAM), and sterile-α and armadillo motif-containing protein (SARM) [23]. TLR signaling is composed of two distinct pathways depending on whether the adaptor molecule MyD88 is used following activation (Figure 1).

MyD88-dependent signaling pathway

MyD88 is essential for the signaling of TLRs with the exception of TLR3. MyD88 starts interactions with members of the IRAK family, resulting in activation of TNF receptor-associated factor 6 (TRAF6). A complex of TAK1, TAK-1 binding protein (TAB)1, TAB2 and TAB3 is subsequently activated. The complex translocates to the cytosol where TAK1 becomes activated, phosphorylating IKK-β and MAPK pathways [21]. Phosphorylated IκB undergoes degradation by the ubiquitin-proteasome system, thereby freeing NF-κB to translocate to the nucleus and initiate transcriptional activation of proinflammatory gene expression. Activation of the MAPK cascade is responsible for the formation of another transcription factor complex, activated protein (AP)-1, which targets proinflammatory cytokine genes [26].

Myeloid differentiation primary response gene 88-deficient ($MyD88^{-/-}$) mice do not show responses including macrophage production of inflammatory mediators, B-cell proliferation or endotoxin shock in response to the TLR4 ligand lipopolysaccharide (LPS) [27]. Similarly, attenuated cellular responses to TLR2 ligands (peptidogylcan and lipoproteins), bacterial flagellin (which stimulates TLR5), imidazoquinoline (which acts as a ligand for TLR7) and the TLR9 ligand CpG DNA motifs were observed in $MyD88^{-/-}$ mice [28–31]. These findings clearly demonstrate that the adaptor protein MyD88 is essential for the inflammatory responses mediated by several members of the TLR family.

MyD88-independent signaling pathway

Toll-like receptor 3 signals independently from the other TLRs by using the adaptor molecule TRIF instead of MyD88 [21]. TLR3 recognizes dsRNA, an intermediate of viral replication, and is responsible for the production of type 1 IFNs, which initiate the host antiviral immune response [32–34]. On ligation, TLR3 homodimerizes and initiates signaling through the adaptor protein TRIF, which activates the major transcription factors NF-κB and IFN regulatory factor (IRF)-3 [35]. In humans, SARM functions

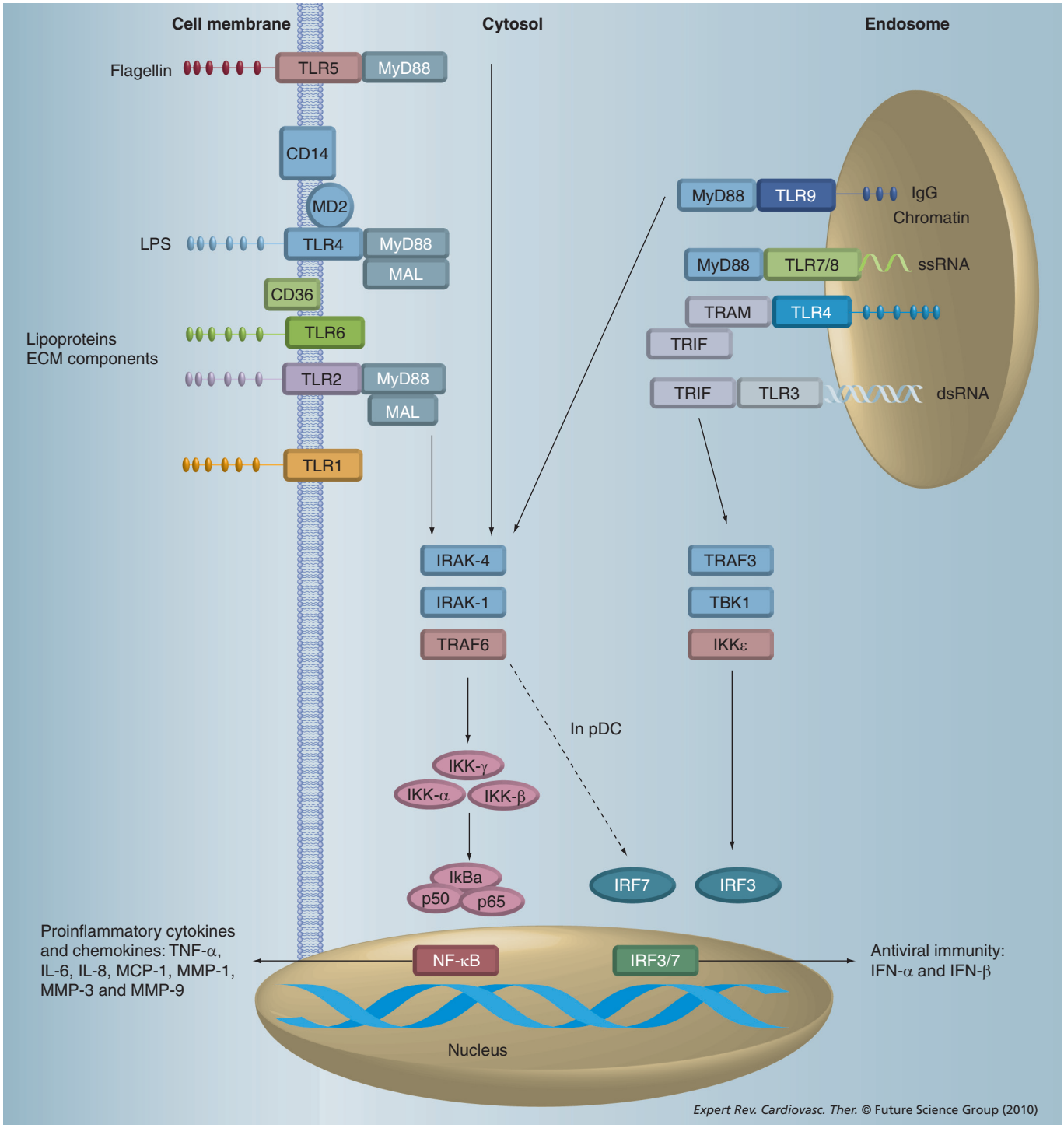

Figure 1. Toll-like receptor signaling pathways. The specific signaling pathways downstream of TLR ligation depend on the signaling adaptor recruited to the TIR domain. Ligands of intracellular TLRs are endocytosed and delivered to the endosome to initiate signaling. Recognition of ligands by intracellular TLRs is dependent on endosomal acidification and, thus, can be blocked by drugs that block acidification, such as chloroquine. With the exception of TLR3, all TLRs recruit MyD88, which then recruits IRAK-4 and TRAF6, leading to NF-κB activation. The MyD88-independent signaling pathway instead uses the adaptor protein TRIF, which then associates with TRAF3 and TBK-1, resulting in interferon (IRF) activation. TLR4 also uses the adaptor molecule TRAM to activate TRIF.
ECM: Extracellular matrix; IRF: Interferon regulatory factor; LPS: Lipopolysaccharide; MMP: Matrix metalloproteinase; MyD88: Myeloid differentiation protein 88; pDC: Plasmacytoid dendritic cell; TIR: Toll/IL-1 receptor; TLR: Toll-like receptor; TRAF6: Tumor necrosis factor receptor-associated factor 6; TRAM: TRIF-related adaptor molecule; TRIF: Toll–IL-1 receptor domain-containing adaptor inducing IFN-β.

as an inhibitor of TRIF-mediated signaling [36]. TRIF associates with TRAF3 and TRAF6. TRAF3 is important for activating two noncanonical IKK-related kinases, Traf family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKK-ε, which both activate IRF-3 [37]. IRF3 induces proinflammatory cytokines, type 1 IFNs and increases expression of IFN-inducible genes. NF-κB is also activated through the phosphorylation of the inhibitory molecule IκBα by IKK-α and IKK-β [38].

TLR4 is unique in its signaling pathways as it can signal using both the MyD88-dependent and -independent pathways using four adaptor molecules, which are activated sequentially. TLR4 recruits the adaptor protein TRAM to activate TRIF in addition to using MyD88 and Mal directly after ligand binding to signal [39]. IFN-inducible genes, such as glucocorticoid-attentuated response gene-16 (*GARG16*), immunoresponsive gene 1 and the gene encoding CXC chemokine ligand 10, have been detected in the MyD88-independent pathway through TLR4 but not TLR2 stimulation [40]. Hence, these genes are upregulated in a TLR4-dependent, MyD88-independent manner. LPS stimulation of TLR4 in MyD88-knockout mice results in the activation of the transcription factor IRF3, consequently activating IFN-β production [41,42].

TLR ligands in atherosclerosis

As a group of receptors, TLRs recognize and respond to a diverse array of ligands. Each receptor is specialized in the recognition of a particular set of ligands expressed in a certain cellular or extracellular compartment. TLR ligands can be broadly classified as either exogenous or endogenous. Both exogenous and endogenous TLR ligands have been detected in atherosclerotic lesions and thus may contribute to TLR responses in the plaque.

Exogenous TLR ligands

Components of bacteria, yeast and viruses are all exogenous ligands for TLR2. Lipoteichoic acid, a major component of the cell wall of Gram-positive bacteria, is a ligand of TLR2/6 heterodimers [43]. TLR2 is essential for the detection of Gram-positive bacteria [44,45]. TLR2/6 heterodimers also recognize mycoplasma diacylated lipoproteins peptide (using CD36 as a coreceptor) [25,46,47], zymosan (with cooperation from dectin-1 and CD14) [45,48,49] and peptidoglycan [49]. However, peptydoglican-derived molecules are also sensed by nucleotide oligomerization-binding domain (NOD)-like receptor family members [50]. In addition, TLR2/6 heterodimers sense triacylated lipopeptides [51,52], and atypical LPS from *Porphyromonas gingivalis* is sensed by TLR2 [53,54]. TLR3 is implicated in recognition of viral dsRNA. TLR3 interacts with CD14 and c-Src for ligand uptake and signal transduction [55,56]. TLR4, in complex with CD14 and the coreceptor MD2, senses LPS (endotoxin), a component of the outer membrane of Gram-negative bacteria [57,58]. TLR5 detects bacterial flagellin and TLR9 is required for responses to unmethylated CpG DNA, typically of bacterial origin [23].

Both bacterial and viral infectious agents, including *Chlamydia pneumonia*, *P. gingivalis* and cytomegalovirus, have been detected in atherosclerotic plaques [59–61]. These infectious agents can be recognized by TLR2 and TLR4 [62–64]. Infectious disease has been linked to increased risk of developing atherosclerosis [65–67]. Endotoxemia is also a risk factor for the development of atherosclerosis [68]. Human atherosclerotic plaques also contain nucleic acids [69,70], peptidoglycan [71] and exogenous heat shock proteins [72].

Endogenous TLR agonists

Activation of TLRs can occur in the absence of exogenous stimuli by endogenous ligands generated as a result of tissue damage and inflammation. This possibility is often described as 'sterile inflammation' [73]. Many of these endogenous TLR ligands, in particular lipoproteins, material from necrotic cells and extracellular matrix proteins, are present within atherosclerotic plaques.

Saturated fatty acids induce inflammatory gene expression through activation of TLR4 [74], although recent data has cast doubt over whether saturated fatty acids can directly initiate TLR signaling [75]. ApoCIII, a component of very-LDL (VLDL), is a TLR2 ligand and can induce proinflammatory signals in monocytes [76]. Modified lipids are also able to elicit TLR signaling. Minimally modified LDLs induce TLR4 activation in macrophages [77,78]. Interestingly, the signal pathway engaged following TLR4 ligation determines the cellular response to ligand engagement, with signaling via the MyD88-dependent pathway inducing cytokine production [78] and signaling via the MyD88-independent pathway generating reactive oxygen species [77]. Oxidized LDL induces inflammatory responses including increased chemokine gene expression in macrophages through a newly described TLR4/6 heterodimer [24].

Tissue damage and remodeling results in the breakdown of components of the extracellular matrix – some of these molecules have been identified as TLR ligands. The alternative splice of fibronectin, extra domain A (EDA), fibrinogen and tenascin C are all ligands for TLR4 [79–81]. MyD88-dependent signaling pathways via both TLR2 and TLR4 are activated by hyaluronan (a large glycosaminoglycan component of the extracellular matrix) [82–84] and biglycan [85]. In addition, the large extracellular matrix proteoglycan versican can activate tumor-infiltrating myeloid cells via TLR2, TLR6 and CD14 [86]. mRNA from necrotic cells is an endogenous ligand for TLR3 and can induce DCs to produce IFN-α [87].

Many studies have identified heat shock proteins (HSPs) as being present in murine atherosclerotic plaques and as being ligands for TLR2 and TLR4 [88–96]. The nuclear protein HMGB-1, which is expressed in human atherosclerotic cells [97], is also a ligand for TLR2 and TLR4 [98].

The list of endogenous TLR agonists is being progressively extended. Recently, the microtubule regulator stathmin has been found to be an endogenous activator of TLR3 [99]. There are probably many other endogenous TLR ligands relevant to atherogenesis that remain to be identified.

TLR expression in atherosclerosis

Atheroma is composed of a complex mix of both inflammatory leukocyte populations and resident vascular cells, which all differentially contribute to further plaque progression and outcome.

Therapeutic targeting of TLRs in atherosclerosis therefore requires a robust knowledge of the expression patterns of these receptors in both health and disease. In keeping with the observation that arteries are more susceptible than veins to atherosclerotic plaque formation, human primary arterial cells are responsive to a broader range of TLR ligands than venous cells [100]. Furthermore, the expression profiles of various TLRs vary across different vascular beds. For example, TLR3 mRNA is expressed in the aorta, whereas the temporal and iliac arteries do not express TLR3 but instead express TLR8 mRNA. The carotid artery, however, expresses mRNA for both TLR3 and TLR8 [101]. In contrast to healthy vessels, TLR expression (in particular expression of TLR1, TLR2 and TLR4) is increased in human atherosclerotic vessels [102].

All populations of leukocytes, including monocytes/macrophages, DCs and T and B lymphocytes, appear to express TLRs (recently reviewed in [103]). However, in the context of atherosclerosis, TLR expression on monocytes/macrophages and DCs is the best characterized. TLR2 and TLR4 are the most highly expressed TLRs on human blood monocytes and their activation on these cells leads to secretion of the proinflammatory cytokines TNF-α and IL-6 [104,105]. Peripheral monocytes from patients with coronary disease have an increased expression of TLR4 and TLR2 compared with controls [106–109]. Enhanced TLR signaling is also present in patients with acute coronary syndromes [110–112]. Recently, TLR2 expression has also been identified as being increased on blood monocytes from patients with arteriosclerotic diseases such as coronary artery disease, aortic aneurysm or peripheral arterial disease in comparison to age- and sex-matched healthy controls [109]. Furthermore, TLR2 expression on these cells was found to be an independent risk factor for disease development (in parallel with conventional risk factors) [109]. ApoE$^{-/-}$ mice with advanced atherosclerosis also display increased TLR2 and TLR4 expression on circulating cells [113].

To our knowledge, the expression of TLRs on various monocyte subsets has not been examined in detail, although differential LPS responsiveness has been described in two populations of CD14^{+} human blood monocytes [114]. Given that the two main populations of blood monocytes are proposed to differentiate into different cell populations within tissues (with 'inflammatory' monocytes preferentially becoming macrophages and 'resident' monocytes preferentially becoming DCs), it can be postulated that different monocyte populations will express different TLRs and utilize different TLR signaling pathways [115]. Indeed, Barbalat *et al.* have described type I IFN production via TLR2 activation specifically in murine inflammatory monocytes derived from the bone marrow or spleen [116]. Elucidating differences in TLR expression and signaling in various monocyte/macrophage subsets will be key to the design of effective therapeutics.

Exposure to oxidized lipids and foam cell formation induces increased TLR2 and TLR4 expression *in vitro* on monocyte-derived macrophages and a monocytic cell line [117,118]. Correspondingly, increased expression of these TLRs has been observed on CD68-positive macrophages in human and murine atherosclerotic lesions [102,118]. Increased TLR expression within atherosclerotic lesions may lead to increased activation of proatherogenic downstream signaling pathways. Several therapeutics, including angiotensin II blockade, statins and insulin, have been shown to reduce protein expression of TLR2 and TLR4 in murine and human cells [119–123]. These studies suggest that therapeutic reduction of TLR expression should be achievable. Further studies are needed to determine whether altering TLR expression is possible in patients with cardiovascular disease and to assess if such reductions can inhibit TLR responses.

Dendritic cells are emerging as important immune cells in pathogen sensing although their role in atherogenesis remains to be determined. Networks of DCs have been observed in the intima of healthy vessels in both humans and mice [124–127], in particular at regions predisposed to atherosclerotic lesion formation [125,128]. Furthermore, the presence of both of the best-described subsets of DCs, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), has been observed in human atherosclerotic lesions [123,129]. In contrast to mDCs, which express and respond to TLR2, TLR3 and TLR4, pDCs only weakly express mRNA for these receptors but instead strongly express TLR9 [129–132]. Both mDCs and pDCs express TLR7 and respond to stimulation with the synthetic TLR7 ligand R848, albeit by secreting different cytokines; pDCs express IFN-α, while mDCs express IL-12 [133]. Thus, both of these DC subsets may differentially contribute to the development of atherosclerosis.

Mast cells release preformed mediators including proteases, histamine and cytokines following activation. They are recognized as playing important roles in host defence to infections and in allergy. They have also been implicated in atherosclerosis. Increased numbers of mast cells are observed in human atherosclerotic lesions at sites of plaque erosion, rupture and hemorrhage [134]. Mast cells express TLR1–9, although expression of TLR5 remains controversial [135–139]. Recently, Bot and colleagues observed that substance P mediates adventitial mast cell activation and intraplaque hemorrhage in a collar-induced model of atherosclerosis [140]. Interestingly, substance P has also recently been shown to upregulate TLR2 expression on cultured human mast cells and to enhance responses to lipoteichoic acid and the synthetic TLR2 agonist Pam3CSK4 [141]. Thus, TLR2 on mast cells may contribute to a vulnerable plaque phenotype.

Although it is generally considered that innate immune cells, such as monocytes, macrophages and DCs, are the main cellular expressers of TLRs, it is worth noting that the earliest expression of TLRs during atherogenesis appears to be by resident vascular cells. TLR2 expression is increased in ECs at atherosclerosis-prone sites such as the inner curvature of the aortic arch in LDL receptor (LDLR)$^{-/-}$ mice [142], supporting *in vitro* data showing that exposure to laminar flow leads to reduced TLR2 expression on human coronary artery ECs [143]. Furthermore, this endothelial TLR2 expression at predisposed sites was associated with early atherosclerotic processes including monocyte recruitment, although whether endothelial TLR2 expression is the cause or consequence of monocyte recruitment is unknown [142]. TLR4

is also expressed by ECs. A recent study has found that TLR4 expression on human umbilical vein ECs is regulated by JNK [144], which suggests that TLR4 may be capable of positively regulating its own expression during atherogenesis.

Smooth muscle cell (SMC) expression of TLRs has also been described. At the mRNA level, SMCs constitutively express TLR1, TLR3, TLR4 and TLR6 [145]. *C. pneumoniae*, TLR3 and TLR4 ligand stimulation can induce mRNA expression of TLR2 in human coronary artery smooth muscle cells, whereas TLR2 is constitutively expressed in murine aortic smooth muscle cells [146]. Secretion of proinflammatory cytokines including monocyte chemoattractant protein (MCP)-1 and IL-6 by cultured smooth muscle cells in response to LPS or Poly(I:C) stimulation has confirmed functional expression of TLR4 and TLR3 on these cells [145–148]. In addition, exposure of SMCs to *C. pneumoniae* leads to TLR2-dependent MCP-1 release [146].

Toll-like receptor expression is not confined to the intima. In the adventitia, which is increasingly being acknowledged as an important site of inflammation and tertiary lymphoid organ formation in the atherosclerotic vessel wall [149–151], TLR4-positive cells have been identified [152]. Interestingly, not all TLR4-positive cells were macrophages and *in vitro* data showed that primary human adventitial fibroblasts express TLR4. Primary human adventitial fibroblasts also express TLR2 [153]. This suggests that resident vascular cells in the adventitia may also express TLRs. When Vink *et al.* applied LPS to the adventitia in a periadventitial cuff arterial injury model, neointima formation was increased. No effect of LPS on neointima formation was observed in TLR4$^{-/-}$ mice [152]. Similar results have been observed with the synthetic TLR2 ligand Pam3Cys-SK4, with perivascular Pam3Cys-SK4 stimulation inducing neointima formation in wild-type but not TLR2$^{-/-}$ mice [153]. TLR activation of both adventitial macrophages and fibroblasts might mediate these effects. Despite this and the limited amount of data showing the effects of TLR ligation on ECs and smooth muscle cells *in vivo*, it should still be recognized that TLR activation on resident vascular cells in all layers of the vessel wall may contribute to atherogenesis.

Differences in cellular TLR expression patterns exist between mouse and man, and they may hinder the extrapolation of murine findings into man and delay the development of effective and safe therapeutics. For example, whereas differential expression of TLR2, TLR4 and TLR9 have been described on mDCs and pDCs in humans, these TLRs are equally expressed by both murine DC populations [154], at least at the mRNA level. Expression of TLR2 in cultured murine ECs is higher than human ECs. In murine ECs, TLR2 is located on the cell surface while in human ECs TLR2 is unavailable to agonists as it is sequestered intracellularly unless cells are stimulated with IFN-γ or IL-1β [155]. TLR4 expression is confined to macrophages [118], while TLR2 is expressed on ECs in murine lesions [142]. In human lesions TLR2 and TLR4 expression are less segregated [102]. Whether these differences reflect true evolutionary differences between species or reflect differences between early and advanced lesions is currently unknown.

Roles of TLRs in atherosclerotic lesion formation

Toll-like receptors have many functions that may impact on atherosclerotic lesion formation (Figure 2). TLR engagement on monocytes can contribute to foam cell formation via supporting lipid uptake and disruption of cholesterol efflux mechanisms. Activation of TLR2, TLR4 and TLR9 on murine macrophages induces lipid uptake and foam cell formation [156–159]. Exogenous TLR ligands such as *C. pneumoniae* can induce foam cell formation using TLR2 and TLR4 via both MyD88-dependent and -independent pathways [64,160,161]. TLR4 can mediate macropinocytosis (fluid-phase uptake) of lipids [162] whereas other TLRs may induce lipid uptake via other mechanisms. Activation of TLR3, -4 and -9 can induce scavenger receptor expression and, thus, may indirectly promote foam cell formation [158,163]. Activation of these receptors can also lead to increased expression of fatty acid binding proteins such as aP2 and Mal1, which may promote lipid uptake [164,165]. Engagement of TLR3 and TLR4 can inhibit LXR transcriptional activity through IRF3 and can thus reduce cholesterol efflux by attenuating expression of genes including ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) [166].

Consistent with these proatherogenic roles of TLRs in atherosclerotic lesion formation, data from murine models of atherosclerosis in which specific TLRs have been deleted provide strong support towards developing antagonists of TLR signaling for the treatment of atherosclerosis. C3H/HeJ mice possess a missense mutation in the third exon of the *TLR4* gene, which results in histidine replacing proline at position 712 of the polypeptide chain [167,168]. Of interest, these mice are resistant to diet-induced atherosclerosis [169,170]. However, when bone marrow transplantation (BMT) was performed from C3H/HeJ mice into ApoE$^{-/-}$ mice, no effect on lesion development was observed [171], suggesting that resident vascular cell expression of TLRs may be of more significance in atherogenesis than TLR expression on hematopoietic cells. A similar phenomenon is observed with TLR2. When Mullick *et al.* performed BMT from TLR2$^{-/-}$ to LDLR$^{-/-}$ mice, no effect on lesion development was observed [172]. However, performing the same bone marrow transfer prior to Pam3CSK4 (synthetic TLR2 agonist) stimulation led to a reduction in lesion development [172]. Together, these data suggest that TLR2 expression on hematopoietic cells may be important for recognition of exogenous but not endogenous TLR2 ligands.

Atherosclerotic lesion size is reduced by approximately 60% in MyD88$^{-/-}$ApoE$^{-/-}$ double knockout mice with a concomitant 75% reduction in macrophage infiltration [173]. Crossing of mice with an IRAK4 kinase-inactive knock-in with ApoE$^{-/-}$ mice led to an 89% reduction in lesion size and reduced lesional macrophage infiltration and lipid accumulation following carotid ligation [174]. However, both MyD88 and IRAK4 are also downstream of IL-1 and IL-18 receptors. Deletion of IL-1β attenuates aortic root lesion development by 30% in ApoE$^{-/-}$ mice and reduces aortic VCAM-1 and MCP-1 expression [175]. A reduction in lesion size is also observed in ApoE$^{-/-}$ mice overexpressing the endogenous IL-1 inhibitor IL-1R antagonist (IL-1RA) [176]. Conversely, deficiency of IL1-RA in ApoE$^{-/-}$ leads to increased

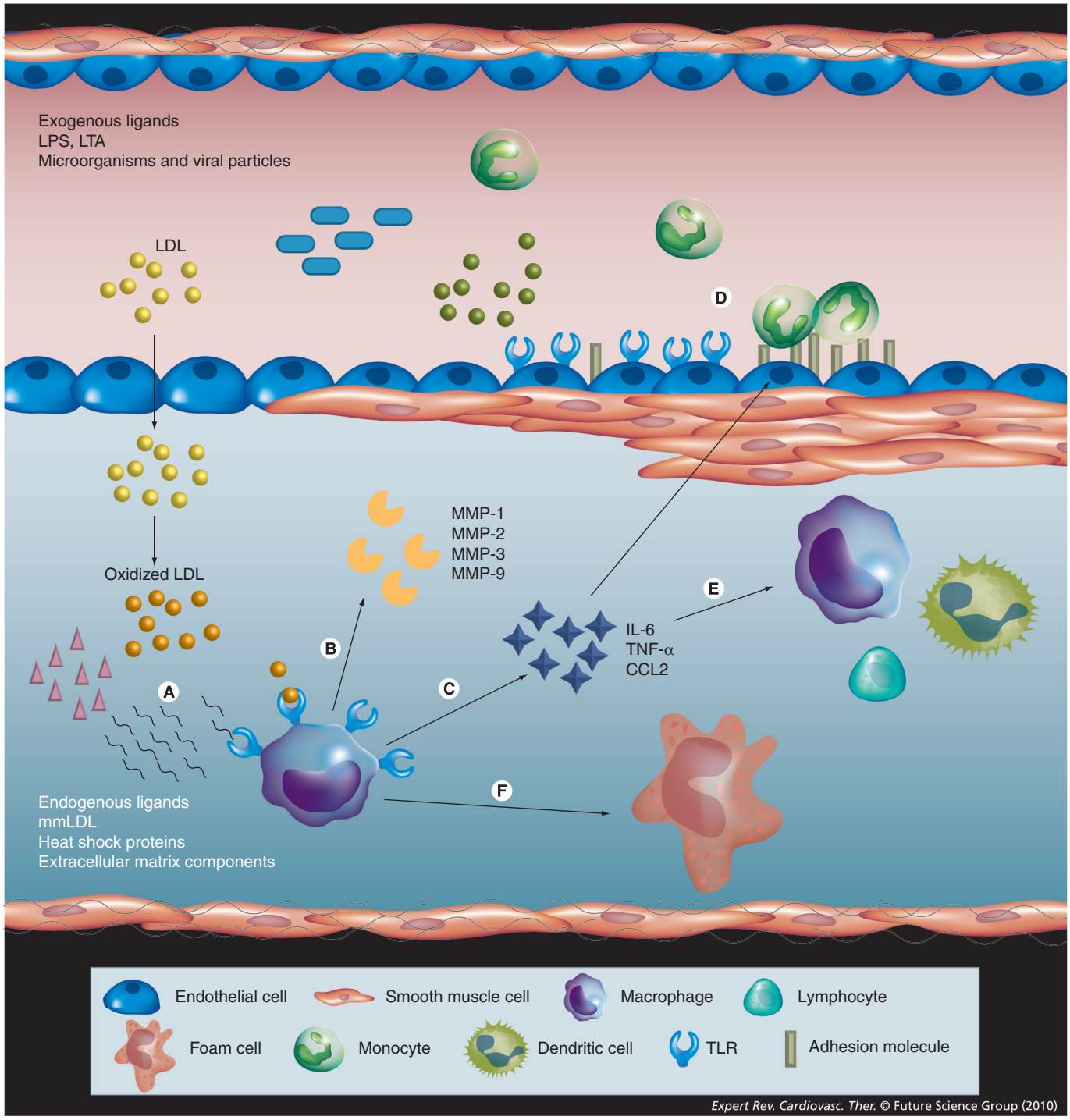

Figure 2. The roles of Toll-like receptors in atherosclerotic lesion formation. TLRs have many functions that may contribute to atherosclerotic lesion development and progression, including: **(A)** recognition of exogenous and endogenous ligands including modified lipoproteins, **(B)** activation of plaque cells to release matrix-degrading enzymes, **(C)** activation of plaque cells to release proinflammatory cytokines and chemokines, **(D)** induction of adhesion molecule expression on endothelial cells, which promotes leukocyte recruitment into the subendothelial space, **(E)** activation of other leukocyte populations in the plaque and **(F)** facilitation of foam cell formation. The precise effects of TLR engagement in the atherosclerotic plaque will depend on the cell types and TLR ligands present.
LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; MMP: Matrix metalloproteinase; TLR: Toll-like receptor.

lesion formation at early time points [177]. Smaller lesions with a more stable phenotype are also observed in ApoE$^{-/-}$IL-18$^{-/-}$ double knockout mice [178].

Whole-body deficiency of TLR2 in LDLR$^{-/-}$ mice results in a 55% reduction in atherosclerotic lesion development after 10 weeks of high-fat feeding [172]. In addition, TLR2 agonist

administration significantly increases atherosclerotic lesion development in LDLR$^{-/-}$ mice [172]. Lack of TLR2 in the ApoE$^{-/-}$ murine model of atherosclerosis confirms TLR2 deletion to be atheroprotective [179]. Similar to TLR2 deficiency, global deficiency of TLR4$^{-/-}$ in ApoE$^{-/-}$ mice leads to a 55% reduction in lesion size and a 65% reduction in macrophage infiltration [173]. Bone marrow transfer of TLR4$^{-/-}$ macrophages into agouti LDLR$^{-/-}$ mice fed a low-fat diet also leads to reduced lesion formation compared with mice transplanted with TLR4$^{+/+}$ macrophages. However, no difference was observed when recipient mice were fed a high-fat diet or a high-fat diet with added saturated fatty acids, suggesting take-over of alternative mechanisms in severe hypercholesterolemia [180]. Reduced atherosclerotic lesion development in TLR4$^{-/-}$ApoE$^{-/-}$ and TLR2$^{-/-}$LDLR$^{-/-}$ mice was associated with a reduction in serum CCL2 levels, a key chemokine involved in monocyte recruitment into atherosclerotic plaques [10,172,173,181]. Deletion of TLR/ILR signaling components has differential effects on serum cholesterol levels. Whole-body deletion of TLR4 and IL-1β does not affect serum cholesterol levels [173,175], whereas TLR2$^{-/-}$LDLR$^{-/-}$ mice exhibit reduced plasma cholesterol levels [175]. By contrast, IL-1RA$^{-/-}$ApoE$^{-/-}$ mice and IL-18$^{-/-}$ApoE$^{-/-}$ mice display increased serum cholesterol levels [177,178]. Serum cholesterol data in MyD88$^{-/-}$ApoE$^{-/-}$ knockout mice are less clear. Whereas Michelsen *et al.* observed no effect of MyD88 deficiency on serum cholesterol levels, Bjorkbacka *et al.* observed an increase in serum cholesterol in MyD88$^{-/-}$ApoE$^{-/-}$ mice [173,182]. Differences in the time point and sex of mice used in these studies may explain these differences.

Studying the effects of TLR polymorphisms on cardiovascular disease has enabled attempts to be made to establish a definitive link between TLRs and human cardiovascular disease. The effect of two single-nucleotide TLR4 polymorphisms (Asp299Gly and Thr399Ile) on the development of cardiovascular disease has been examined. These polymorphisms map to the extracellular domain of TLR4 and are associated with a diminished response to inhaled LPS [183]. These polymorphisms were consequently anticipated to be atheroprotective. Individuals carrying these polymorphisms have lower circulating levels of proinflammatory cytokines and soluble adhesion molecules, which have been associated with atherogenesis [184]. However, despite the large number of studies now performed (reviewed in [185]), there is no clear indication of the effect of being a carrier of these alleles on development of cardiovascular disease, with many studies yielding conflicting results. Reduced atherosclerosis and intima–media thickness was observed in carriers of the Asp299Gly polymorphism in one study [186]. However, in a subsequent and larger study, no association between intima–media thickness and a beneficial clinical end point was observed with this polymorphism [187]. Furthermore, a large study using 5000 participants found no conclusive association between the TLR4 Asp299Gly polymorphism and myocardial infarction [188]. In addition, in the Coronary Artery Progression Study these TLR4 polymorphisms were not linked to intima–media thickness or any change in clinical outcome [187]. A relatively small study has identified the TLR2 polymorphism Arg753Gln to be associated with restenosis and an increased risk of developing mycoplasmic disease [189]. It is worth noting that much of the discrepancy between the outcomes of the polymorphism studies may be explained by the complex activation and regulation of TLRs *in vivo*. In addition, the relevance of a given TLR will depend on the ligands present during disease progression. Much larger and more recent clinical trials are required to provide a more conclusive outcome regarding the effect of carrying TLR polymorphisms on atherosclerosis.

Therapeutic potential of targeting of TLRs in atherosclerosis

Given that TLRs and their ligands are present and exert numerous functions that may contribute to atherosclerotic lesion formation, TLRs thus provide an attractive therapeutic target for treating disease. Following the successful and effective use of anti-TNF therapies in the treatment of rheumatoid arthritis [190], developing drugs that specifically target TLRs to treat chronic inflammatory diseases, such as atherosclerosis, may have significant clinical benefit.

Tol-like receptor 2 and TLR4 are the most extensively studied TLRs in atherosclerosis. Since genetic deletion of TLR2 and TLR4 is athero-protective in murine models of atherosclerosis [172,173], antagonism of these receptors is an attractive prospect for treating atherosclerosis. TLR4 has been identified as the key driver for the pathological production of the proinflammatory cytokine TNF-α during sepsis. Attempts have been made to prevent TLR4 activation by developing antagonists to its ligand LPS. Eritoran is one of the most advanced TLR4 antagonists [191,192]. It works by interfering with the interactions between TLR4 and its coreceptor MD-2. In a Phase II clinical trial, eritoran reduced the mortality rate due to sepsis by 6.4% compared with the placebo group [193]. Attempts have also been made to modulate TLR signaling by modifying the structure of lipid A, the biologically active part of the LPS molecule, through rational drug design [194]. Targeting TLR2 and TLR4 with specific antagonists has been attempted in some preliminary studies. Blockade of TLR2 using neutralizing antibodies against the receptor and MyD88 inhibition in human atherosclerosis has revealed a predominant role for TLR2 and MyD88 in NF-κB activation and proinflammatory cytokine production. In contrast with murine studies, inhibition of TLR4 and the TLR4 signaling adaptor protein TRAM was not rate limiting for cytokine and chemokine production [195]. TLR2 and MyD88 blockade also inhibited production of matrix metalloproteinases (MMPs)-1–9, whereas blockade of TLR4 and TRAM selectively attenuated production of the matrix-degrading enzymes MMP-1 and -3 [195]. These findings may point towards preferential TLR2-dependent signaling through a MyD88-dependent pathway in human atherosclerosis both in terms of inflammation and matrix degradation. Surprisingly, it appears that TLR4 has a more restricted role in human atherosclerosis, with a focus on matrix degradation.

Monoclonal antibodies against TLR2 have been developed and are in clinical trials. One such example is OPN-305, which is a TLR2-specific monoclonal antibody that inhibits proinflammatory cytokine production by blocking TLR2 in a range of

inflammatory diseases [20]. Recently, antibody blockade of TLR2 has been shown to reduce infarct size and maintain heart function through reduction of proinflammatory mechanisms in a murine model of myocardial ischemia/reperfusion injury [196].

All TLRs share the TIR cytoplasmic domain, which is essential for signaling through homotypic interactions with downstream adaptor proteins. Hence blockade of the TIR domain provides an intracellular site for anti-inflammatory drug action by preventing TLR signaling globally. Compound 4a (hydrocinnamoyl–valyl pyrrolidine) is a low-molecular-weight MyD88 mimic that interferes with interactions at the TIR domain. It inhibits p38 MAPK activation and attenuates the release of proinflammatory cytokines [197].

Toll-like receptor downstream signaling pathways offer additional possibilities for targeting blockade. NF-κB nuclear translocation – a sign of activation – has been identified in atherosclerotic plaques [198] and its activation is more prominent in acute complications of atherosclerosis (reviewed in [199]). Blockade of IκB kinase β/2 – a key enzyme involved in the canonical pathway of activation of NF-κB – abolishes cytokine and MMP production in human atherosclerosis [200]. Conversely, the alternative pathway of NF-κB activation was not involved. The use of statins has been demonstrated to block NF-κB activation by upregulating the expression of the inhibitory IκBα complex in clinical trials [201,202]. Specific inhibitors targeting the IκB kinases have been extensively developed, and although they are very effective in abating inflammation there is toxicity mainly linked to the role of NF-κB in cell survival [190]. However, NF-κB blockade has a different outcome. Gareus *et al.* inhibited NF-κB activation either by ablation of NF-κB essential modulator (NEMO) or by expression of a dominant-negative IκBα specifically in ECs in $ApoE^{-/-}$ mice. Endothelial NF-κB inhibition resulted in a 33–60% inhibition of aortic root lesion size (reduction dependent on sex of mice and method of NF-κB inhibition), reduced macrophage recruitment and reduced expression of adhesion molecules, cytokines and chemokines [203]. However, IκB kinase 2 deletion in macrophages in $LDLR^{-/-}$ mice led to larger atherosclerotic lesions with increased necrosis [204]. Conversely, deletion of the p50 subunit of NF-κB in hematopoietic cells in $LDLR^{-/-}$ mice led to attenuated lesion formation with reduced uptake of oxidized LDL, although increased inflammation in lesions was also seen [205]. Like NF-κB, the p38 and JNK pathways are also activated following TLR activation and culminate in proinflammatory gene expression and initiation of acute inflammation. Developing drugs that target these pathways may have therapeutic potential. p38 inhibitors have been developed and are currently in Phase II clinical trials for rheumatoid arthritis and psoriasis [206].

Novel therapeutics that target TLRs are being identified. One such example that has significant potential is the use of miRNAs. miRNAs are short (~22 nucleotides) endogenous noncoding RNA molecules that can regulate gene expression at many levels. A key role of miRNAs in the regulation of genes involved in immune defence is being uncovered [207]. miRNAs act as positive or negative regulators of TLR signaling by controlling gene expression at the post-transcriptional level. miR-146 is an immunoregulatory miRNA that targets two critical proteins involved in TLR signaling: TRAF6 and IRAK1. miR-146 is upregulated in response to LPS, the ligand for TLR4, negatively regulating the mRNA levels of both TRAF6 and IRAK1 [20]. miR-146, along with other inhibitory miRNAs such as miR-155 [208,209], may be consequently used in atherosclerosis to inhibit TLR signaling and the induction of proinflammatory gene expression. Interestingly, circulating levels of miR-155 have recently been shown to be reduced in patients with coronary artery disease [210], suggesting that these patients may have dysregulated inhibition of TLR signaling pathways. Albeit very promising, more investigation is needed to overcome the potential caveat with the use of miRNAs of off-target side effects owing to the fact that a single miRNA is able to regulate the expression of multiple genes. Elucidation of the full roles of various miRNAs will be particularly important given the recent data that maintenance of cholesterol homeostasis can be regulated by miRNA [211,212].

The study of molecules used by viruses for evading immune responses in the host led to some success in TLR antagonism. Vaccinia virus is a pox virus containing several genes that act as decoy receptors, including *A46R* and *A52R*, which block TLR downstream signaling [213,214]. *A46R* interferes with MyD88-dependent signaling while *A52R* disrupts the protein interactions between IRAK2 and TRAF6 [214].

With future research providing more information regarding TLR signaling pathways and the specific function of TLRs in atherosclerosis, the ability to develop drugs through rational design that can specifically target TLRs in atherosclerosis will be a possibility. Given the role of TLRs in the pathogenesis of atherosclerosis, therapeutic targeting of TLRs by drugs could have tremendous clinical potential. TLR2 and TLR4 are the best characterized in terms of ligand identification and genetic deficiency in murine models of atherosclerosis. Therefore, they probably represent the best targets so far.

With new opportunities come new challenges. The growing realization of the inability of murine models to predict outcome in clinical use leads to caution in the extrapolation of murine data to human disease. This caution also exists in the development of TLR antagonists. Whereas IL-1, TLR2 and TLR4 deletion is equally effective in murine models of atherosclerosis, only TLR2 has a predominant role in human disease [195]. In addition, differences in cellular TLR expression patterns between mouse and man (discussed previously) may hinder the generation of safe and effective blockers of TLR signaling. Consideration should also be given to the cellular and vessel-specific expression patterns of the planned target in both health and disease when developing strategies aimed at targeting TLR signaling pathways in atherosclerosis. This issue can affect the type of strategy and also the timing of intervention. Throughout the natural history of atherosclerosis, the plaque will have different representation of cell types. For example, a macrophage foam cell-rich fatty streak versus a mature lesion with a smooth muscle cell-rich cap. Thus, there may be differential effects of targeting a specific TLR depending on the disease stage at which intervention occurs. The outcome of TLR inhibition may very well vary with lesion composition and agonist

involved. For example, bone marrow chimera studies in TLR2$^{-/-}$ did not prevent the formation of diet-induced atherosclerotic lesions as whole-body deficiency did [172]. However, when an exogenous TLR2 agonist was administered, the subsequent increase in atherosclerotic lesion formation was preventable by the transfer of TLR2-deficient bone marrows [172]. These findings suggest that endogenous agonists induce proatherogenic TLR2 signaling on nonmyeloid cells, while exogenous agonists are detected by myeloid cells enhancing atherosclerosis development. These differences might have consequences in the therapeutic setting.

Conclusion

In the past 10 years the molecular determinants of atherosclerotic plaque activation have finally been unravelled. One of the major contributions to this exciting discovery was the identification of TLRs as key inducers of innate immunity. TLRs sense modified lipids in the intima and signal cell recruitment within the lesion, as well as cytokine and MMP production. These discoveries paved the way for the development of biologics that may offer hope for the treatment of cardiovascular disease beyond risk factor abatement.

Expert commentary

There is increasing evidence that innate immunity responses engaged by TLRs have a profound impact on the development and outcome of atherosclerotic lesions. Human evidence is also pointing in the same direction. TLRs act by activating the downstream signaling pathways NF-κB and IRF. The choice between these two pathways is made depending on the cell type, the TLR agonist and the compartment where it is sensed. While TLRs are viewed by some as sensors of nonself pathogen-associated molecular patterns, reports of endogenous ligands are flooding in, enriching the list of danger-associated molecular patterns. Endogenous molecules recognized by TLRs include modified lipoproteins, supporting the possibility that TLRs are the main inducers of plaque activation. Thus far, TLR2 and -4 are the most attractive therapeutic targets, and promising tools to block TLR2 and -4 have been developed. In particular, blocking TLR2 is effective in dampening activation of human atherosclerotic lesions and in reducing the area of myocardial infarction in murine models of acute ischemia. However, some caution should be exerted for two reasons. First, the results of TLR2 and -4 blockade differ between human and mouse. Second, the outcome of genetic deletion of these TLRs differs in the myeloid and nonmyeloid cell compartments. Hence, the effect of targeting might be different according to the cellular composition of the lesion, the stage of disease and timing of intervention. The exciting possibility of targeted biologics for cardiovascular disease is on the horizon, once these last reasons for concern are addressed.

Five-year view

Toll-like receptors are a relatively new field of biology. Since the first description of a TLR in humans in 1997 there has been an exponential increase in the number of studies examining these receptors. Research over the past few years has clearly shown that TLRs and their ligands play key roles in various aspects of atherosclerotic lesion formation. However, the full picture remains to be uncovered. Over the next 5 years, we anticipate that our understanding of the cellular expression patterns, agonists (especially endogenous ligands) and signaling pathways of TLRs in health and cardiovascular disease will increase exponentially. Therapeutics targeting TLRs are already in preclinical development for other inflammatory diseases including rheumatoid arthritis (reviewed in [20]). The outcome of these trials will guide the development of TLR-based therapeutics in cardiovascular disease in the near future.

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Key issues

- Innate immunity is the first line of host defence against pathogens carried out by phagocytic cells such as macrophages, neutrophils and dendritic cells, as well as natural killer cells, mast cells, eosinophils and basophils.
- Atherosclerosis is an inflammatory disease with a strong involvement of innate immunity.
- Toll-like receptors (TLRs) are pattern-recognition receptors that sense conserved motifs leading to innate immunity.
- Both exogenous and endogenous ligands can activate TLRs and many ligands of both types can be found in atherosclerotic lesions.
- TLRs are expressed by all cells in atherosclerotic lesions including leukocytes and resident vascular cells, although various TLRs are differentially expressed by different cell populations.
- TLRs contribute to lesion formation by promoting leukocyte recruitment and activation, foam cell formation and by inducing cytokine and matrix metalloproteinase production via downstream signaling molecules.
- TLRs are attractive targets for the development of therapeutics to treat atherosclerosis; biologics have been developed and are being tested in other inflammatory diseases.
- Further work is required to fully elucidate TLR expression patterns and functions in different cell types in health and disease to allow the most effective therapies to be developed.

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