

The Role of Toll-like Receptors in Atherothrombotic Cardiovascular Disease

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Cite This: *ACS Pharmacol. Transl. Sci.* 2020, 3, 457–471

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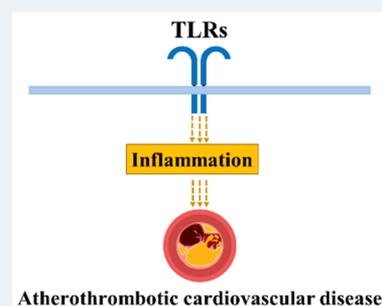
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ABSTRACT: Toll-like receptors (TLRs) are dominant components of the innate immune system. Activated by both pathogen-associated molecular patterns and damage-associated molecular patterns, TLRs underpin the pathology of numerous inflammation related diseases that include not only immune diseases, but also cardiovascular disease (CVD), diabetes, obesity, and cancers. Growing evidence has demonstrated that TLRs are involved in multiple cardiovascular pathophysiologies, such as atherosclerosis and hypertension. Specifically, a trial called the Canakinumab Anti-inflammatory Thrombosis Outcomes Study showed the use of an antibody that neutralizes interleukin-1 β , reduces the recurrence of cardiovascular events, demonstrating inflammation as a therapeutic target and also the research value of targeting the TLR system in CVD. In this review, we provide an update of the interplay between TLR signaling, inflammatory mediators, and atherothrombosis, with an aim to identify new therapeutic targets for atherothrombotic CVD.

KEYWORDS: TLR, cytokines, inflammation, atherosclerosis, thrombosis



Cardiovascular disease (CVD) with atherosclerosis as its major pathology, remains as the most serious life threatening disease in modern society, contributing to an estimated 17.7 million deaths worldwide.^{1,2} The risk factors that contribute to CVD consist of both modifiable risk factors and nonmodifiable risk factors. The modifiable risk factors include unhealthy diet, physical inactivity, sedentary or screen time, smoking, diabetes, obesity, hyperlipidaemia, hypertension, mental illness, infection, and chronic inflammation. The nonmodifiable risk factors comprise age, gender, race, and genetics. Of all the modifiable risk factors, chronic inflammation has a central role in CVD and can be enhanced by other factors.³ Inflammation is a naturally protective response to injury, infection, and tissue stress or malfunction, such as tissue lipid accumulation, but can become a cause of chronic inflammation.⁴ Recently, virus- and bacterial infection-evoked inflammation has emerged as a critical contributor to the progression of CVD.⁵

Accumulating evidence underscores the contention that infection is a risk factor in the pathogenesis of CVD.^{5,6} The association between pathogen infection and CVD was first observed in 1988, when elevated antibodies for *Chlamydia pneumoniae* in serum were observed in patients with acute myocardial infarction and coronary artery disease.⁷ Additionally, in a group of patients with a high rate of coronary artery disease, 75% of these subjects had at least three of five tested pathogens in their blood.⁸ Moreover, periodontal disease, a disease caused by chronic Gram-negative bacterial infection of

the gums, has been suggested to exacerbate CVD.⁹ Numerous studies have shown that atherosclerotic plaques contain multiple different pathogens, including *Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis*, and *Escherichia coli*.^{10–12} These observations indicate that pathogens not only stimulate inflammation at the local infection sites, but also travel to distant sites to propagate their effects. The underlying mechanistic relationship between the pathogen infection and atherothrombosis is proposed to increase systemic inflammation,¹³ noting that knowledge of precise mechanisms linking inflammation with CVD is limited.

To initiate inflammatory responses, the invading pathogens must first be recognized, which occurs via a family of pattern recognition receptors (PRRs).¹⁴ As suggested by the name, PRRs recognize substances relying on unique evolutionarily conserved structures on pathogens. Toll-like receptors (TLRs) are essential members of PRRs which can recognize and respond to a large repertoire of conserved molecular patterns presenting on both exogenous invading substances (pathogen-associated molecular patterns, PAMPs) and endogenous molecules from injured tissue or cells (damage-associated molecular patterns, DAMPs).¹⁵ In innate immunity, invading

Received: November 7, 2019

Published: February 6, 2020

Table 1. Human Toll-like Receptor Expression and Their Ligands

TLRs	expressed on human	exogenous ligands	endogenous ligand ^a
TLR1	endothelial cells (ECs); Vascular smooth muscle cells (VSMCs); monocyte; macrophages; B cells; neutrophils; NK cells; platelets	cooperate with TLR2 to recognize triacyl lipopeptides	NA
TLR2	ECs; VSMCs; fibroblasts; monocyte; dendritic cells (DCs); NK cells; platelets	peptidoglycans; lipoproteins; LPS; beta-glucan	human β -defensins; acute serum amyloid A; histones; cooperate with TLR2 to recognize HMGB1; HSPs; ECM
TLR3	ECs; VSMCs; macrophages; T lymphocytes; DCs	dsRNA; ssRNA	NA
TLR4	ECs; VSMCs; macrophages; mast cells; B and T lymphocytes; platelets	LPS; glycoproteins; fibronectin	HSP60; HSP70; HMGB1, fibronectin; hyaluronic acid; biglycan; oxLDL; mLDL; fetuin-A; Ang II; serum amyloid A; histones; S100 proteins
TLR5	ECs; VSMCs; monocytes; macrophages; DCs; neutrophils; T cells	flagellin	NA
TLR6	ECs; VSMCs; macrophages; B cells; platelets	cooperate with TLR2 to recognize diacyl lipopeptides	cooperate with TLR2 to recognize HMGB1; HSPs; ECM
TLR7	ECs; VSMCs; DCs; monocytes; macrophages; T cells; B cells; platelets	ssRNA	guanosine and its modified derivatives; siRNA
TLR8	Monocytes; Macrophages; DCs; Neutrophils	ssRNA	siRNA
TLR9	ECs; VSMCs; macrophages; plasmacytoid DCs; T cells; B cells; platelets	CpG DNA	histones; mitochondrial DNA
TLR10	monocytes; neutrophils; plasmacytoid DCs; B cells	unknown	NA

^aNA: not available.

pathogenic microbes are sensed by either components on their outer membranes, intracellular substances, or structural components.¹⁶ For example, *Porphyromonas gingivalis* is recognized by TLR4 or TLR2 via its outer membrane constituent lipopolysaccharide (LPS).^{17–19} Apart from the PAMPs, TLRs also recognize DAMPs leading to sterile inflammation.²⁰ Atherosclerosis is considered to commence with the deposition of lipid in the vascular wall.^{21,22} The remarkable discovery of TLRs as the receptors of endogenous lipid derivatives underpins their critical pathogenic role in CVD.^{23,24}

Several examples have emerged to support the activation and contribution of TLRs to the progression of vascular atherothrombotic diseases. The vasculature system expresses all of the known human TLRs,²⁵ and under vascular pathophysiology, the levels of TLRs are found to be upregulated. For instance, human atherosclerotic lesions have an increased expression of TLR1, TLR2, and TLR4.²⁶ The presence of TLR ligands accelerates the progression of atherosclerosis^{27,28} and the disruption of TLR signaling can affect the progression of diseases. For example, the deficiency of TLR2²⁷ or TLR4²⁹ attenuates the development of atherosclerosis in mouse models. In this review, we will discuss the association between TLRs and CVD from the aspect of TLRs, their ligands, signaling pathways, and therapeutic implications.

1. OVERVIEW OF TLR-MEDIATED SIGNALING SYSTEM

1.1. Overview of TLRs. TLRs are named for their structural similarity to Toll, a receptor first discovered in the fruit fly *Drosophila melanogaster*.^{30,31} TLRs are transmembrane proteins, or more specifically, type I integral membrane glycoproteins with three structural domains: a leucine-rich repeat (LRR) motif, a single helical transmembrane domain, and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain. The LRR domain serves as the terminal binding dock for invading molecules, while the TIR domain recruits the signaling adaptors to initiate downstream signaling.^{32,33}

The first human homologue of a TLR was cloned and characterized in 1997 and is now known as TLR4.³¹ Since

then, there have been 13 mammalian TLRs discovered, with TLR1–TLR10 expressed in human and TLR1–TLR9 along with TLR11–TLR13 present in mice.^{15,34} The location of the receptors in the cell varies, with TLR1, 5, 6, and 10 being located on the cell surface, TLR3, 7, 8, and 9 being located in intracellular endosome, and TLR2 and TLR4 being expressed both on the cell surface as well as in intracellular compartments.^{35,36} TLR1–TLR9 are widely expressed in human blood vessels³⁷ as well as various vasculature cells.³⁸

1.2. Ligands Recognized by TLRs. TLRs as a family of PRRs, are capable of recognizing both PAMPs and DAMPs.¹⁵ When an exogenous invader attacks the host, TLRs recognize it first, and evoke the defense system via activation of innate immunity and also adaptive immunity.³⁹ The PAMPs from the invading pathogenic microbes include LPS, lipoproteins, glycoproteins, teichoic acid, viral and bacterial DNA, single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) (Table 1). TLRs also recognize endogenous molecules released from injured or damaged tissue sites. The first evidence of an endogenous activator of TLRs dates back to 2000, when heat shock protein 60 (HSP60) was reported to trigger cytokine production via TLR4.⁴⁰ Since then, the endogenous ligand repertoire has expanded to include several HSPs, fibrinogen, high-mobility group box-1 (HMGB1), self-DNA, mRNA, ssRNA, oxidized LDL (oxLDL), angiotensin II (Ang II), free fatty acids, amyloids, and extracellular matrix (ECM) components, such as fibronectin, heparin sulfate, and hyaluronic acid.^{41–43} Each type of TLR can sense a specific group of ligands (Table 1). TLR2 tends to sense the patterns from Gram-positive bacteria while TLR4 is responsible for the recognition of Gram-negative bacterial components.⁴⁴ TLR5 recognizes flagellin.⁴⁵ TLR3, TLR7, and TLR8 are receptors for viral and synthetic RNAs,^{46,47} while TLR9 is a receptor for DNA.⁴⁸

1.3. TLR Signaling Pathways and Related Adaptors or Accessory Proteins. Upon the engagement of TLR ligands, the specific receptor will undergo a conformational change and then directly or indirectly recruit intracellular adaptors via its TIR domain.⁴⁹ These adaptors include myeloid differentiation factor-88 (MyD88), MyD88 adapter-like protein (also known as TIR domain-containing adaptor protein, MAL or TIRAP),

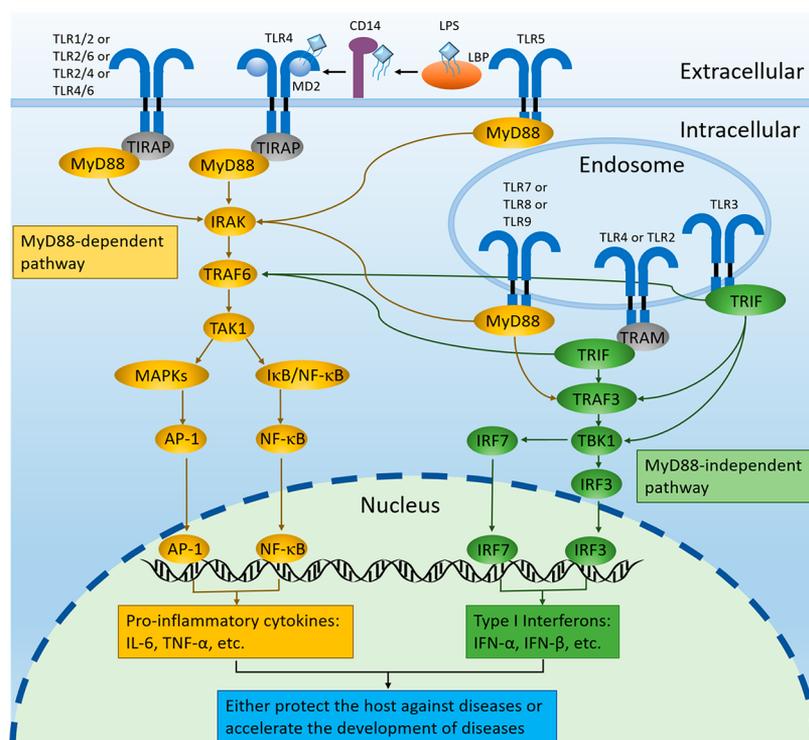


Figure 1. The canonical signaling pathway of TLRs. The canonical pathway of TLR signaling includes the MyD88-dependent pathway and MyD88-independent pathway. In the MyD88-dependent pathway, the binding of TLR ligands causes the attachment of MyD88 to TIR, resulting in the phosphorylation of IRAKs and downstream TRAF6 and TAK1 which leads to the nuclear translocation of inflammatory transcription factors such as NF- κ B and AP-1 as well as the activation of mitogen-associated protein kinases (MAPKs), leading to transcription of pro-inflammatory cytokine genes. However, the TIR domain of TLR7, TLR8, and TLR9 directly recruits MyD88 to either activate TRAF6 or TRAF3 which leads to IRF7 activation and interferons secretion. The MyD88-independent pathway is a major model adopted by TLR3 and TLR4. The recruited TRIF activates TRAF3 and its downstream TBK1 and IRF3 to regulate the gene transcription of type I interferons. TRIF also activates TRAF6 which is responsible for late stage pro-inflammatory cytokines. The final recognition of LPSs to TLR4 is a concerted result of different accessory proteins: LBPs, CD14, MD-2. Abbreviations: LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response protein 88; TIRAP, MyD88 adaptor-like protein; IRAK, interleukin-1 (IL-1) receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; TAK-1, transforming growth factor- β -activated kinase-1; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; NF- κ B, nuclear factor- κ B; TRIF, toll/IL-1 receptor domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; TBK, TANK-binding kinase; IRF, interferon response factor; LBP, LPS binding protein; CD14, cluster of differentiation 14 protein; MD-2, lymphocyte antigen 96.

TIR domain-containing adaptor protein inducing interferon- β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α and armadillo-motif containing protein (SARM).⁵⁰ TIRAP and TRAM are the adaptors which promote the recruitment of MyD88 and TRIF to the targeting intracellular TIR domains, respectively. However, the TIR domain can also recruit MyD88 or TRIF independent of other adaptors, for example, TLR5, TLR7, and TLR9 can directly recruit MyD88 without the assistance of TIRAP,⁵¹ while TLR3 recruits TRIF independent of TRAM.⁵² Depending on the involvement of MyD88 or TRIF, TLR signaling pathways have been subdivided into MyD88-dependent signaling pathways and TRIF dependent pathways that are also known as MyD88-independent signaling pathways (Figure 1). Except for TLR3, all other TLRs can signal through the MyD88-dependent signaling pathway and TLR4 can signal through both MyD88-dependent signaling pathways and MyD88-independent signaling pathways.¹⁷ In the MyD88-dependent pathway, the binding of TLR ligands causes the attachment of MyD88 to TIR, resulting in the phosphorylation of interleukin-1 receptor-associated kinase 4 (IRAK4), which eventually phosphorylates IRAK1/2.¹⁵ IRAK1/2 complex interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6) leading to transforming growth factor- β (TGF- β) activated kinase-1

(TAK1) activation. These processes consequently result in the nuclear translocation of inflammatory transcription factors such as NF- κ B and AP-1 as well as the activation of mitogen-associated protein kinases (MAPKs), leading to transcription of pro-inflammatory cytokine genes.⁵³ However, TLR7, TLR8, and TLR9 via MyD88 can also activate TRAF3 leading to interferon (IFN)-regulated factor 7 (IRF7) activation. The MyD88-independent pathway acts through TRIF to activate interferons to modulate the immune system. The MyD88-independent pathway is mainly adopted by TLR3 and TLR4. The recruited TRIF activates TRAF3 and its downstream TBK1 and IRF3 to regulate the gene transcription of type I interferons. TRIF also activates TRAF6 which is responsible for late stage pro-inflammatory cytokines.¹⁵ In contrast to the above well studied adaptors, SARM is the newest identified member of the TLR adaptor family and has a unique role which mainly inhibits the downstream signaling of TLRs by interacting with TRIF and MyD88.⁵⁴

The final recognition of certain patterns by TLRs requires accessory proteins. The response of TLR4 to LPS requires the cooperation of LPS binding proteins (LBPs), CD14, a typical glycosylphosphatidylinositol-anchored protein, and lymphocyte antigen 96 (MD-2)¹⁷ (Figure 1). The LBP is the first protein interacting with LPS in the plasma.⁵⁵ CD14 is a cell

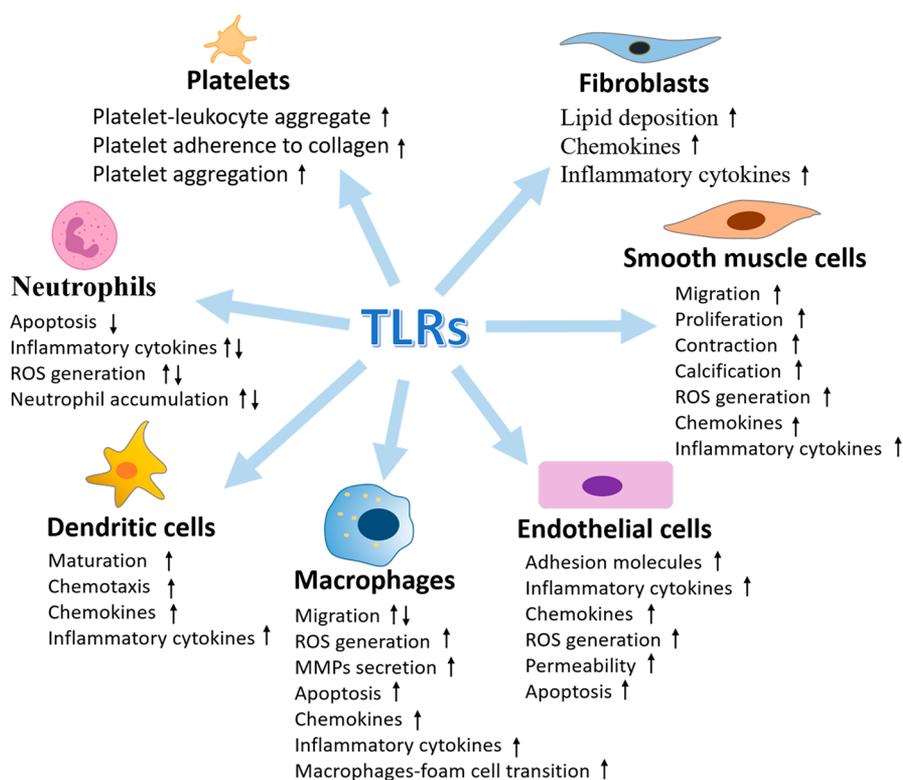


Figure 2. Complex effects of TLRs in vasculature cells. Via their signaling pathways TLRs have diverse effects on both the vascular cells and blood cells. These effects are both protective and destructive to the host.

surface protein which facilitates the transfer of LPS from LBPs to the TLR4/MD-2 complex.^{56,57} MD-2 is the accessory protein that senses the attachment of CD14-delivered LPS,⁵⁸ leading to the homodimerization of TLR4.⁵⁹ TLRs often form homodimers to transduce signals from the extracellular to intracellular environment. TLRs can also form heterodimers, such as a TLR1/2 dimer and TLR2/6 dimer, to expand the TLR ligand spectrum (Figure 1).^{60,61} These conformational changes and the pairing of TIRs in the cytoplasm lead to the recruitment of downstream adaptors which then elicit exquisite signaling networks.⁵⁰

1.4. The Prominent Product of TLR Signaling. TLR signaling stimulates pro-inflammatory cytokines and type I IFNs via the MyD88-dependent pathway or MyD88-independent pathway¹⁵ (Figure 1). Pro-inflammatory cytokines include interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, lymphokines, monokines, and chemokines, while type I IFNs mainly consist of IFN- α and IFN- β .^{62,63} The secretion of these cytokines is concomitant with their downstream inflammation biomarker, such as IL-6, C-reactive protein (CRP), and fibrinogen.^{64,65} The upstream and downstream inflammatory biomarkers are closely associated with an increased incidence of cardiovascular events.^{66–68} Briefly, levels of inflammatory cells and diverse cytokines are elevated in atherosclerotic lesions. Elevated pro-inflammatory cytokines, particularly IL-1, IL-6, IL-18, and TNF- α stimulate attractants to activate inflammatory cells as well as increase the expression of adhesion molecules of endothelial cells (ECs), such as selectins, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) to promote the recruitment or migration of inflammatory cells (monocytes, neutrophils, lymphocytes, and macrophages) into the vascular wall and consequently

accelerate the development of atherosclerosis.⁶⁸ However, there are also cytokines, particularly TGF- β , IL-10, and IFN- α that down-regulate inflammatory reactions and protect the host against atherogenesis, which is the inherent function of the innate immune defense system.^{62,66} The activation of TLRs can also evoke other biological mediators in a cell-dependent manner, including platelet activation factor, reactive oxygen species (ROS), and nitrogen species to orchestrate the progression of diseases.^{69,70} The overall responses of vasculature cells to TLR activation is displayed in Figure 2.

2. THE ROLE OF TLR1/2/6 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

TLR1 and TLR2 are highly expressed in human atherosclerotic plaques.²⁶ In LDL receptor knockout (LDLr^{-/-}) mice, aortic ECs exposed to disturbed blood flow show increased TLR2 expression and are the source of the TLR2 expression in early atherosclerotic lesions.⁷¹ High fat diet (HFD)-induced hyperlipidaemia increases TLR2 expression, and deficiency of TLR2 decreases EC dysfunction, intimal leukocyte accumulation, and lipid accumulation.⁷¹ In another LDLr^{-/-} mice model, the deficiency of TLR2 results in a 50% reduction in aortic atherosclerosis, implying the detrimental role of TLR2 in this model.²⁷ In the same study, the depletion of TLR2 also prevents the development of atherosclerosis occurring in response to a synthetic TLR1/2 agonist, a trisacylated lipopeptide Pam3CSK4.²⁷ As TLR2 is closely associated with TLR1 and TLR6 to transmit signals, to identify which TLR2 coreceptor is involved in TLR2 mediated atherosclerosis, the TLR1 or TLR6 deficient LDLr^{-/-} mice model was developed. However, the depletion of TLR1 or TLR6 had no effect on lesion progression in the absence of exogenous agonists.⁷² In contrast, the deficiency of TLR1 or TLR6 did attenuate the

progression of atherosclerosis elicited by exogenous TLR1 and TLR6 ligands, respectively, suggesting the redundant role of TLR1 and TLR6 for the unknown endogenous TLR2 ligands.⁷² In apolipoprotein E knockout (ApoE^{-/-}) mice, the deficiency of TLR2 also shows a protective role in the development of aortic atherosclerotic plaque^{73,74} which may be due to decreased lipid accumulation and macrophage recruitment in the aortic sinus as well as reduced monocyte chemoattractant protein-1 (MCP-1) levels.^{75,76} Treatment with OPN-305, a humanized anti-TLR2 antibody, reduces myocardial ischemia/reperfusion injury in pigs.⁷⁷ These *in vivo* studies show the pro-atherogenic role of TLR2.

In human umbilical vein endothelial cells (HUVECs), the oscillating flow via the TLR2-TAK1-IKK2 signaling pathway promotes ICAM-1 and VCAM-1 secretion,⁷⁸ and LPS from *Helicobacter pylori* or *Porphyromonas gingivalis* stimulates TLR2 to induce the secretion of pro-inflammatory factors, specifically TNF- α and IL-8.¹⁹ In the presence of neutrophils, agonists of TLR2, including endogenous hyaluronan, trigger substantial stress and apoptosis in cultured ECs. In human atherosclerotic plaques, the number of luminal apoptotic ECs is correlated with neutrophil accumulation, and TLR2 staining in smooth muscle cell (SMC)-rich plaques indicates the involvement of TLR2 in the superficial erosion of human atherosclerotic plaques.⁷⁹ TLR2 deficiency reduces intimal neutrophil adherence in regions of local flow disturbance and mitigates the injury of ECs and formation of local thrombosis.⁸⁰ These results suggest the central pathogenic role of EC TLR2 under the assistance of neutrophils.

TLR2 induces VSMC migration via an IL-6-Rac1 dependent pathway.⁸¹ TLR2 also interacts with Nox1 to induce ROS generation, inflammatory cytokine production, matrix metalloproteinase-2 (MMP-2) secretion, and VSMC migration.⁸² Furthermore, the activation of TLR2 increases VSMC calcification and chondrogenic differentiation.⁸³ TLR2 deficiency attenuates TLR2 agonist initiated VSMC chondrogenic differentiation and consequent calcification. In addition, the deficiency of TLR2 inhibits HFD induced advanced atherosclerotic calcification which is triggered by IL-6-mediated RANKL (receptor activator of nuclear factor κ B ligand) induction and osteoprotegerin suppression.⁸³ Vitamin K2, one of the major clinical agents for calcification treatment, inhibits HFD-induced aortic intimal calcification in ApoE^{-/-} mice and *in vitro* calcification of SMCs. This phenomenon is interestingly concomitant with the reduced expression of TLR2 and TLR4.⁸⁴ These observations suggest the potential effects of TLR2 on vascular calcification via the modulation of VSMCs.

In human monocytes, dietary palmitic acid can induce pro-IL-1 β expression and inflammasome-mediated IL-1 β secretion by inducing the dimerization of TLR1 with TLR2.⁸⁵ TLR2 also interacts with Nox2 to mediate ROS production in monocytes⁸⁶ and macrophages.⁸⁷ Atherogenic lipids also signal via CD36 and TLR2 to trigger apoptosis in macrophages that are undergoing endoplasmic reticulum stress.⁸⁸ In an *ex vivo* culture system of human atherosclerotic plaques that contain a high percentage of macrophages, the blocking of TLR2 reduces MCP-1, IL-8, and IL-6 secretion and MMP-1, MMP-2, MMP-3, and MMP-9 generation, suggesting the critical role of TLR2 in pro-inflammatory cytokine secretion and matrix degradation.⁸⁹

TLR2⁹⁰ and its counterparts⁹¹ are abundantly expressed in human platelets. In addition, TLR2 mRNA expression is

elevated in platelets of patients with acute coronary syndrome.⁹² Upon the stimulation of ligands, TLR2 elicits downstream signaling cascades and platelet activation either by inducing the assembly of the TLR2/TLR1 complex^{93–95} or the TLR2/TLR6 complex.⁹⁶ The activation of TLR2 in platelets causes P-selectin and cytokine secretion, integrin activation, and platelet aggregation.^{93,95} Especially, in the hyperlipidemic ApoE^{-/-} mouse model, the deficiency of TLR2 showed a strong thromboprotective effect.^{93,96} These data demonstrate the potent therapeutic role of targeting platelet TLR2 for CVD treatment.

3. THE ROLE OF TLR3 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

TLR3 is an intracellular receptor for dsRNA from both viral and dead host cells.⁹⁷ After binding dsRNA, TLR3 activates the transcription factors IRF3 and NF- κ B, which are important for initiating synthesis of many inflammatory proteins. In a rabbit model of hypercholesterolemia, mRNA expression of TLR3 is strongly upregulated in the aorta.⁹⁸ Systemic administration of TLR3 agonists impairs endothelial function in wild type mice but not in TLR3 null mice.⁹⁹ In the LDLr^{-/-} mice, the deficiency of TLR3 in hematopoietic cells protects mice against atherosclerosis without the alteration of circulating lipids.¹⁰⁰ However, in the ApoE^{-/-} mouse model, TLR3 shows a protective role in arterial injury and early atherogenesis as the deletion of TLR3 promotes the development of atherosclerosis.¹⁰¹ In addition, TLR3 deficient mice display increased collagen and SMC content in the atherosclerotic lesions, suggesting the important role of TLR3 in causing plaque instability, by partially regulating the activity of MMP-2 and MMP-9 in macrophages.¹⁰² There is also a study showing that human platelet TLR3 potentiates platelet aggregation, ATP release, and integrin activation.¹⁰³ This evidence suggests the potential role of TLR3 in modulating the development of CVD.

4. THE ROLE OF TLR2/4/6 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

TLR4 is highly expressed in the vasculature, especially in patient atherosclerotic plaques.^{26,104} The stimulation of TLR4 using LPSs enhances the inflammatory response in whole blood from patients with established atherosclerosis and these responses are potentiated by obesity.¹⁰⁵ The concentration of gut-derived LPSs from *Escherichia coli* correlates with the level of TLR4 and pro-inflammatory molecules in human carotid plaques.¹² In the same study, the activation of TLR4 by LPS upregulates human monocyte Nox2, which could contribute to oxidative stress.¹² In murine femoral artery cuff models, adventitial stimulation by LPS augmented neointima formation, which is reduced by TLR4-deficiency.¹⁰⁶ A deficiency of TLR4 in ApoE^{-/-} mice also reduces aortic atherosclerosis,²⁹ along with reduced intimal lipid accumulation by 75% and reduced gene expression of VCAM-1, MCP-1, and TLR2 in the lesion.⁷⁶ However, this occurs without the alteration of total serum cholesterol and triglyceride levels.⁷⁶ In a LDLr^{-/-} mouse model, the deficiency of TLR4 reduces atherosclerosis without affecting inflammation which occurs via modulation of plasma cholesterol levels.¹⁰⁷ The TLR4 antagonist LPS-RS also inhibits monocyte and macrophage recruitment and collagen accumulation in the intima and consequently inhibits atherosclerosis in diabetic LDLr^{-/-} mice without affecting

the serum inflammatory modulators.¹⁰⁸ *In vivo*, TLR4 mediates Ang II-induced vascular remodelling via the regulation of NADPH oxidase activity.^{109,110} HMGB1 as an endogenous ligand of TLR4 via both MyD88-dependent and MyD88-independent pathways stimulates intimal hyperplasia and vascular remodelling in a mouse carotid wire injury model.¹¹¹ Genetic deletion or neutralization of HMGB1 or TLR4 silencing all prevent intimal hyperplasia *in vivo*.¹¹¹

The primary ligand for TLR4 is LPS.¹⁷ LPS consists of an O-antigen, a hydrophilic polysaccharide and a hydrophobic lipid A end consisting of mainly fatty acids.¹¹² The lipid A component of LPS is the structure that TLR4 specifically recognizes.¹¹³ TLRs are tightly associated with the function of the endothelium.¹¹⁴ In human ECs, LPS acts via a TLR4-Nox4-NF- κ B dependent pathway to elicit intracellular inflammatory factors,¹¹⁵ including TNF- α , IL-8, as well as ROS, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) generation.¹¹⁶ In human microvascular ECs, LPS stimulates the expression of inflammatory mediators via the TLR4, PPAR- γ , and PI3K/Akt/mTOR signaling pathway.¹¹⁷ HMGB1 is a DNA binding cytokine and is elevated in HUVECs under oxidative stress.¹¹⁸ Via the TLR4-caveolin-1 pathway, HMGB1 regulates EC hyperpermeability.¹¹⁹ OxLDL up-regulates the oxidized LDL receptor-1 (LOX-1), MCP-1, and VCAM-1 expression¹²⁰ leading to human aortic EC (HAEC) apoptosis.¹²¹ LPS is also able to induce LOX-1 expression via the TLR4-MyD88-Nox4-ROS-p38-NF- κ B pathway in HUVECs.¹²²

VSMCs of human atherosclerotic coronary arteries show an intense expression of TLR4. LPS elicits diverse effects on VSMCs, including ROS generation,¹²³ inflammation,¹²⁴ and proliferation/migration.¹²⁵ Hyperelongated glycosaminoglycan chains on proteoglycans initiates lipid retention in the neointima as a very early pathogenic stage during the development of atherosclerosis.^{21,22,126,127} Pharmacologically blocking the effects of growth factors on VSMC proteoglycan synthesis inhibits lipid binding and deposition *in vitro* and *in vivo* and is a valid target for preventing atherogenesis.^{127–129} Traditional cardiovascular agonist TGF- β ,¹³⁰ thrombin,^{131,132} signals via the Smad2 linker region which leads to the modification of proteoglycans. We have recently found that LPS via its canonical TLR4-TAK1 pathway stimulates the Smad2 linker region phosphorylation to regulate glycosaminoglycan chain initiation and hyperelongation in human aortic SMCs (HASMCs) (unpublished data). HSP60, an endogenous cell-stress marker which is highly expressed in atherosclerotic lesions,¹³³ mediates the migration of human VSMCs via TLR4 and ERK signaling.¹³⁴ In addition, HSP70 signals via the TLR4-MyD88-dependent pathway to play an important role in the pathophysiology of diabetic vasculopathies.¹³⁵ In HASMCs, HSP70 stimulates the TLR4/ERK/JNK/AP-1 pathway to induce the expression of TGF- β 1 and consequently leads to increased ECM production, such as fibronectin or type I collagen.¹³⁶ HMGB1 is highly expressed in human atherosclerotic lesions by activated VSMCs¹³⁷ as well as by macrophages.¹³⁸ In turn, HMGB1 via TLR4 induces HASMC migration without affecting cell viability.¹¹¹ In human coronary artery VSMCs, oxLDL induces inflammatory mediator secretion and contractile protein expression depending on the collaboration of urokinase receptor, CD36 and TLR4.¹³⁹ Furthermore, similar to that for TLR2, treatment with oxLDL increases the expression of TLR4 in cultured human VSMCs, and this response coincided with calcification

of VSMCs. The calcification is attenuated when silencing TLR4 or inhibiting NF- κ B but is reversed by C2-ceramide treatment, suggesting the role of TLR4-NF- κ B-ceramide in mediating vascular calcification.¹⁴⁰ TLRs also interplay with other critical receptors in vasculature cells. In cultured VSMCs, the level of TLR4 is increased in the presence of Ang II.¹⁴¹ Similarly, in rat VSMCs, TLR4 modulates Ang II mediated production of TNF- α and MMP-9.¹⁴²

TLR4 is highly expressed in human adventitial fibroblasts, and the addition of LPS can cause intimal hyperplasia via the activation of the NF- κ B pathway and downstream cytokine secretion, including IL-6, MCP-1 and TNF- α .¹⁰⁶ LPS also via the TLR4-NF- κ B pathway promotes lipid accumulation by the up-regulated adipose differentiation-related protein in human adventitial fibroblasts. Silencing of adipose differentiation-related protein and using a TLR4 antibody or NF- κ B inhibitor all reduced the lipid deposition in these fibroblasts.¹⁴³ Oncostatin M, a gp130 cytokine, synergizes with LPS to augment the secretion of MCP-1, IL-6, and vascular endothelial growth factor (VEGF) along with the activation of STATs (signal transducer and activator of transcription proteins), MAPKs, and NF- κ B signaling in human aortic adventitial fibroblasts and HASMCs, suggesting the involvement of fibroblast TLR4 in the pathogenesis of atherosclerosis.¹⁴⁴

Lysophosphatidic acid is an endogenous pathogenic factor in the development of CVD.¹⁴⁵ In a human monocytic cell line (THP-1), lysophosphatidic acid signals via TLR4 to stimulate MMP9 expression.¹⁴⁶ Palmitic acid, a saturated fatty acid, has been associated with the incidence of CVD events.¹⁴⁷ In human dendritic cells (DCs), palmitic acid via TLR4 stimulates the secretion of IL-1 β .¹⁴⁸ The recognition of palmitic acid has been suggested to involve the direct binding to MD-2.¹⁴⁹ In macrophages and DCs, HMGB1 signals directly via TLR4 to induce the secretion of proinflammatory cytokines.¹⁵⁰ There are also reports which demonstrate that the signal transduction of HMGB1 needs the collaboration of MD-2 and TLR4.¹⁵¹ In addition, HMGB1 binds to LPS to strengthen the response of TLR4 to LPS in human monocytes.¹⁵²

In mouse RAW264.7 macrophages, LPS induces nitric oxide and IL-6 via ERK1/2 and p38 MAPK but not c-Jun signaling.¹⁵³ In mice macrophages, minimally oxidized LDL (mmLDL) activates TLR4 signaling to induce lipid uptake and foam cell formation.¹⁵⁴ Notably, *in vivo*, intravenous injected mmLDL rapidly accumulates in circulating monocytes, and this phenomenon is attenuated in TLR4-deficient mice.¹⁵⁴ In cultured human macrophages, treatment with oxLDL stimulates macrophage-foam cell transition, cytokine secretion, human leukocyte antigen-DR isotype (HLA-DR) and CD86 expression.¹⁵⁵ The inhibition of TLR2, TLR4, and CD36 decreases the secretion of IL-1 β , IL-6, and IL-8, the expression of HLA-DR and CD86, implicating the cooperation of these three receptors in regulating immune responses; whereas only TLR4 and CD36 participate in the formation of foam cells.¹⁵⁵ Similarly, in an earlier report, the recognition of oxLDL by macrophages required formation of a heterodimeric complex of TLR4, TLR6, and scavenger receptor CD36, indicating TLRs are able to collaborate and sense endogenous pro-atherogenic stimuli and are therefore tightly associated with atherogenesis.²³

TLR4 is functionally expressed both in mice and human platelets.^{90,156,157} LPS were able to activate platelets and

promote platelet aggregation at blood detectable concentrations.¹⁵⁸ In a thrombocytopenic mouse model, LPS-stimulated TNF- α seems to be dependent on platelet TLR4.¹⁵⁹ Via platelet TLR4, LPS also induced adhesion of platelets to neutrophils,¹⁶⁰ an important process in the development of atherosclerosis.¹⁶¹ Histone mediated thrombin generation and the procoagulant phenotype in human platelets is blocked by TLR2 and TLR4 antibodies, suggesting the involvement of these two receptors in blood coagulation.¹⁶² The role of TLR4 in platelet function, thrombosis, and hemostasis has recently been reviewed by others.¹⁶³ It is worth noting that platelets, as anucleated blood cells, do not have all the signaling proteins and nuclear DNA that play vital roles in the TLR signaling system of nucleated cells. Thus, further research is required to delineate the signaling pathways of TLRs in platelets.

5. THE ROLE OF TLR5 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

Compared to other TLRs, TLR5 has emerged as the topic of much research on its role in atherothrombotic disease. According to an immune-histochemical analysis of TLR proteins, the expression of TLR5 is elevated in all examined atherosclerotic lesions.²⁶ In a mouse model of atherosclerosis, the deficiency of TLR5 attenuated high fat-induced atherosclerosis with a decrease of TLR4 expression.¹⁶⁴ Similarly, hematopoietic TLR5 deficient mice show attenuated atherosclerotic lesion formation by around 25% with reduced macrophage accumulation and defective T-cell responsiveness in LDLr^{-/-} mice.¹⁶⁵ In another model, TLR5 does not affect macrophage maturation but rather the expression of MCP-1 and IL-6,¹⁶⁵ indicating the regulatory role of TLR5. Using mice aortic ECs and HAECs as *in vitro* models, TLR5 via the MyD88-independent pathway interacts with Nox4 to mediate bacteria flagellin-induced peroxide generation.¹⁶⁶ The silencing of Nox4 or TLR5 in the HAECs shows reduced nuclear localization of NF- κ B, suggesting the downstream role of NF- κ B. This TLR5-Nox4-NF- κ B axis also enhances IL-8 secretion and ICAM-1 expression, which are critical for monocyte adhesion to ECs and trans-endothelial migration.¹⁶⁶ More recently, this flagellin-TLR5-Nox4-NF- κ B axis has been reported to promote IL-6 secretion as well as the migration of VSMCs via a JNK-RhoA and Rac1 dependent pathway.¹⁶⁷ In ApoE^{-/-} mice, Nox4 knockdown protects the mice against atherosclerosis in the presence of the TLR5 challenge.^{166,167} These results demonstrate that the TLR5-Nox4-NF- κ B axis plays an essential role in atherogenesis.

6. THE ROLE OF TLR7/8 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

TLR7 and TLR8 are intracellular receptors that sense endogenous or exogenous ssRNA.⁴⁷ Both TLR7 and TLR8 can regulate inflammatory responses,^{168,169} such as the secretion of TNF- α , IL-1 β , and CCL3 in human monocytes.¹⁶⁸ Additionally, in acute ischemic stroke patients, the level of TLR7 and TLR8 in peripheral blood is associated with poorer outcomes and greater inflammatory responses,¹⁷⁰ implicating the involvement of TLR7 and TLR8 in inflammatory related diseases. Moreover, in a rabbit model fed with HFD, the mRNA expression of TLR8 is upregulated and is correlated with the progression of atherosclerosis in the aorta,⁹⁸ suggesting the potential role of TLR8 in atherothrombotic

CVD modulation. In human atherosclerotic lesions, TLR7 is abundantly expressed and a higher expression is associated with better outcomes in patients with severe atherosclerosis as observed in a follow-up study over a maximum of 8 years,¹⁷¹ indicating the protective role of this receptor. This phenomenon is possibly due to increased IL-10 secretion.¹⁷¹ Consistently, in ApoE^{-/-} mice, TLR7 deficiency accelerates the development of atherosclerotic plaques by constraining inflammatory cell activation and pro-inflammatory cytokine secretion.¹⁷² Functional TLR7 also is expressed in human platelets, and its activation can lead to increased interaction of platelets with granulocytes and increased platelet adherence to collagen, without thrombosis formation.¹⁷³ However, the deficiency of TLR7 acts against the development of atherosclerosis in ApoE^{-/-} mice¹⁷⁴ and the administration of a TLR7 and TLR9 antagonist reduces neointimal remodelling and foam cell accumulation.¹⁷⁵ These results suggest that the role of TLR7 in atherosclerosis needs further investigation.

7. THE ROLE OF TLR9/10 IN ATHEROTHROMBOTIC CARDIOVASCULAR DISEASE

Mitochondrial DNA (mtDNA), a DAMP recognized by TLR9, has various effects on vascular dysfunction and CVD.^{176,177} The plasma mtDNA levels in trauma patients are many folds higher than that in healthy volunteers and this elevated mtDNA can stimulate the TLR9-NF- κ B pathway to activate neutrophils thus contributing to injury.¹⁷⁷ Infusion of Ang II increases the circulating levels of cell-free DNA, an endogenous TLR9 ligand that positively correlates with inflammatory features of coronary atherosclerosis.¹⁷⁸ Genetic deletion or pharmacologic blockade of TLR9 attenuates atherogenesis in ApoE^{-/-} mice with reduced lipid deposition and macrophage infiltration in atherosclerotic lesions.^{178,179} Restoration of TLR9 in bone marrow promotes atherogenesis in TLR9-deficient mice.¹⁷⁸

In mice Raw264.7 cells, TLR9 activation enhances LOX-1 and Nox1 expression via the TLR9-p38 MAPK signaling pathway, leading to foam cell formation.¹⁸⁰ More recently, cell-free DNA activated TLR9 promotes activation of macrophages via p38 MAPK to mediate vascular inflammation and atherosclerosis.¹⁷⁸ *In vitro*, the activation of TLR9 in macrophages with a CpG-containing oligodeoxynucleotide results in increased lipid accumulation and augmented foam cell formation in a NF- κ B- and IRF7-dependent manner.¹⁸¹ In addition, the activation of TLR9 on plaque-residing plasmacytoid DCs leads to enhanced IFN- α secretion, a cytokine correlated with plaque instability, and increased cytotoxicity of CD4⁺ T cells toward SMCs.¹⁸² These data suggest a pathogenic role of the TLR9-MyD88-IRF-7-IFN- α pathway. TLR9 is expressed in human platelets.⁹¹ TLR9 promotes platelet activation and aggregation *in vitro* and accelerates thrombosis *in vivo* in a MyD88-dependent manner.¹⁸³ However, in early stasis venous thrombogenesis, it is the TLR9 signaling of polymorphonuclear neutrophils, not platelets, that plays a role.¹⁸³

In contrast, there are reports which support the protective activity of TLR9 in atherothrombotic vascular disease. In a group of HFD fed ApoE^{-/-} mice, genetic deletion of TLR9 exacerbated atherosclerosis accompanied by increased lipid deposition and recruitment of macrophages, DCs, and CD4⁺ T cells. Moreover, in the same disease model, the administration of a TLR9 agonist (type B CpG oligodeoxynucleotide 1668) resulted in a reduction of lesion severity, indicating an

antiatherogenic role of TLR9.¹⁸⁴ IRF5 is a critical downstream mediator of TLR7/8/9 via the TRAF3 pathway; however, it has been shown that the deficiency of IRF5 in a mouse model promotes atherosclerosis, indicating the complex biological role of this protein.¹⁸⁵ This protective role may be due to the enhanced secretion of IL-10 in response to TLR9 activation.^{186,187} These findings indicate that specific TLRs can exert distinct pro- and antiatherogenic effects, and further investigations are required.

Human TLR10 is the newest member of the human TLR family to date. However, the ligand of TLR10 has not yet been identified as well as its functional role has not been characterized. As TLR10 is genomically close to TLR1 and TLR6, it may function closely in collaboration with TLR2.¹⁸⁸ In both TLR10 overexpression cell models and TLR10 transgenic mice model, TLR10 has been demonstrated to suppress TLR signaling.¹⁸⁹

8. THE THERAPEUTIC POTENTIAL OF TARGETING THE TLR SIGNALING SYSTEM

Gold standard statins and more recently protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are mainstream cardiovascular drugs which act by reducing plasma cholesterol levels. However, despite clinically successful control of hyperlipidemia, their ability to reduce the mortality of CVD is still limited, suggesting other sources of cardiovascular risk remain.^{190–192} Therefore, seeking therapeutic strategies via nonlipid lowering mechanisms are promising pathways to reduce CVD. Numerous experimental and clinical studies have suggested the pivotal involvement of inflammation in the pathogenesis of atherosclerosis.¹⁹³ Several anti-inflammatory candidates have gone through different phases of clinical trials, such as the p38 MAPK (mitogen-activated protein kinase) inhibitor, losmapimod (NCT02145468), low-dose methotrexate (NCT01594333), colchicine and IL-1 β neutralizing antibody, canakinumab.¹⁹⁴ Among these intensively tested anti-inflammatory entities, colchicine¹⁹⁵ and canakinumab¹⁹⁶ are the most promising candidates for treating CVD. As a broad anti-inflammatory agent, colchicine exerts its function via the inhibition of tubulin polymerization. Through the disruption of the cytoskeleton, colchicine therefore orchestrates the inflammatory signaling networks known as the inflammasome and pro-inflammatory cytokines in addition to *in vitro* platelet aggregation.¹⁹⁷ Currently, several clinical trials investigating the use of colchicine in CVD management are ongoing, including COACS (Colchicine for Acute Coronary Syndromes, NCT01906749), LoDoCo2 (Low Dose Colchicine for secondary prevention of cardiovascular disease, U1111-1139-8608), COLCOT (Colchicine Cardiovascular Outcomes Trial, NCT02551094) and Colchicine-PCI (Colchicine in Percutaneous Coronary Intervention, NCT02594111). In a small cohort study, colchicine reduced inflammatory cytokines in patients with acute coronary syndrome.¹⁹⁸ An investigation of the anti-inflammatory effects of colchicine in PCI is still ongoing (NCT01709981). Canakinumab, a human monoclonal antibody that neutralizes IL-1 β , has been approved for the treatment of rare hereditary IL-1 β -driven disorders. In a phase II clinical trial, canakinumab reduced CRP, IL-6, and fibrinogen without major effects on lipid levels in a group of individuals with high cardiovascular risk.¹⁹⁹ The ability to reduce inflammatory markers without any effect on lipid levels or platelet function makes canakinumab an ideal agent to test the independent benefit

of inflammatory therapy in patients with atherosclerosis. In the trial CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study), a randomized double-blind placebo-controlled trial of over 10 000 patients with a history of acute coronary syndromes, canakinumab significantly reduced recurrent cardiovascular events independent of lipid lowering in a median follow-up of 3.7 years.^{194,196} The success of the CANTOS trial points to the therapeutic potential of targeting inflammation to reduce the incidence of CVD. However, the use of canakinumab is associated with high risk of infection.¹⁹⁶ In addition, as an antibody, canakinumab is very costly and only available via injection, indicating it is not an ideal therapeutic agent.

TLR signaling triggers the transcriptional activation of different pro-inflammatory cytokines, including pro-IL-1 β , which were then processed into their active forms (IL-1 β) by activating inflammasome. Therefore, targeting the TLR signaling pathway is a plausible and complementary strategy to manage abnormal inflammation and vascular conditions. Direct inhibition of TLR signaling has been developed for diseases such as rheumatoid arthritis, cancer, autoimmunity, allergies, and microbial inflammation. So far, five drugs have been approved in different countries. Picibanil (OK-432), a lyophilized mixture of *Streptococcus pyogenes* acting at least partially via TLR4, was approved in Japan as an anticancer agent in 1975.²⁰⁰ Bacillus Calmette-Guérin, a TLR2, TLR4, TLR9 agonist, is widely used as a vaccine and diagnostic test for tuberculosis, as well as immunotherapy in the treatment of bladder cancer.²⁰¹ Monophosphoryl lipid A, a derivative of *Salmonella Minnesota* LPS, acting via TLR4 has been approved by FDA as an immunological adjuvant in human vaccines to enhance antibody responses.^{202,203} Mifamurtide, a ligand of NOD2 (nucleotide-binding oligomerization domain-containing protein-2) and also TLR4, has been approved in Europe for postoperative combination chemotherapy for osteosarcoma.²⁰⁴ Imiquimod as a TLR7 and TLR8 agonist, is approved by FDA to treat anogenital warts, actinic keratosis, and superficial basal cell carcinomas.²⁰⁵ However, to date, no compound has been approved to treat CVD. To the best of our knowledge, there is also no compound in clinical trial that directly targets TLRs to treat vascular disorders. Nonetheless, novel TLR signaling modulators and devices are still being investigated,^{206,207} and it is anticipated in the future there will be a TLRs targeting clinical drug for the treatment of CVD.

9. CONCLUSIONS

TLRs as pivotal regulators in the immune system control of the secretion of inflammatory mediators which are associated with atherothrombotic diseases. Developing treatments targeting the TLR system will potentially reduce the inflammatory cascades and therefore slow the progression of atherosclerosis and reduce the burden of CVD. However, TLRs also provide the host with crucial advantages as the first line of defense against pathogenesis. One of the major challenges of targeting this system is to protect the host defense system while reducing the negative long-term cardiovascular effects of TLRs. In addition, most of the current research is still in cell or animal models and these results may face difficulties when translated to humans. Further investigations of the specific role of each TLR isoform, the specific molecular mechanism, as well as the extensive study of the effect of its downstream pro-inflammatory cytokines are needed to advance this area. Targeting a distinct receptor, a specific intermediate, or

cytokine product might lead to the discovery of an efficacious agent for the prevention of atherothrombotic CVD.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Funding supporting the studies in our laboratory and referenced in this review has been received from the National Health and Medical Research Council (no. 1022800) of Australia, National Heart Foundation of Australia (no. G09M4385), Diabetes Australia Research Trust (P.J.L.), and academic support packages from the University of Queensland (P.J.L.). Y.Z. is supported by the Research Training Scholarship of The University of Queensland. D.K. is supported by NHMRC-Peter Doherty (no. 1160925) and National Heart Foundation (no. 102129) fellowships. H.T.T. is funded by National Heart Foundation (no. 102761). S.X. is funded by American Heart Association (no. 18CDA34110359).

ABBREVIATIONS

Ang II, angiotensin II; AP-1, activator protein-1; ApoE^{-/-}, apolipoprotein E knockout; CD14, cluster of differentiation 14 protein; CRP, C-reactive protein; CVD, cardiovascular disease; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; dsRNA, double-stranded RNA; ECM, extracellular matrix; ECs, endothelial cells; HAEC, human aortic EC; HASMCs, human aortic SMCs; HFD, high fat diet; HMGB1, high-mobility group box-1; HSPs, heat shock protein; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; ILs, interleukins; IRAK, interleukin-1 (IL-1) receptor-associated kinase; IRF, interferon response factor; LBP, LPS binding proteins; LDLr^{-/-}, LDL receptor knockout; LOX-1, LDL receptor-1; LPS, lipopolysaccharides; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MD-2, lymphocyte antigen 96; MMP, matrix metalloproteinase; mtDNA, mitochondrial DNA; MyD88, myeloid differentiation primary response protein 88; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; Nox, NADPH oxidase; oxLDL, oxidized LDL; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; ROS, reactive oxygen species; SARM, armadillo-motif containing protein; SMC, smooth muscle cell; ssRNA, single-stranded RNA; TAK-1, transforming growth factor-β-activated kinase-1; TBK, TANK-binding kinase; TGF, transforming growth factor; TIR, toll-interleukin-1 receptor; TIRAP, MyD88 adapter-like protein; TLRs, toll-like receptors; TNFs, tumor necrosis factors; TRAF, tumor necrosis factor receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, toll/IL-1 receptor domain-containing adaptor protein; VCAM-1, vascular cell adhesion molecule-1; VSMCs, vascular smooth muscle cells

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