A Review: The Significance of Toll-Like Receptors 2 and 4, and NF-κB Signaling in Endothelial Cells during Atherosclerosis

Baoxin Yan1,2,†, Xiaoxian Yu1,†, Xianzhen Cai1,2, Xiaojun Huang1, Bin Xie1, Danchun Lian1, Jinhao Chen1,2, Weiwen Li1,2, Ying Lin1,2, Junjun Ye1,2, Jilin Li1,*,††

1Department of Cardiology, Second Affiliated Hospital of Shantou University Medical College, 515000 Shantou, Guangdong, China
2Shantou University Medical College, 515000 Shantou, Guangdong, China
*Correspondence: lijilin@126.com (Jilin Li)
†These authors contributed equally.

Abstract

Atherosclerosis (AS) is a chronic inflammatory vascular disease that begins with endothelial activation followed by a series of inflammatory responses, plaque formation, and finally rupture. An early event in endothelial dysfunction is activation of the nuclear factor-κB (NF-κB) signaling axis. Toll-like receptors (TLRs) in endothelial cells (ECs) play an essential role in recognizing pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and lifestyle-associated molecular patterns (LAMPs). Activation of the canonical NF-κB pathway stimulates the expression of cytokines, chemokines, and an array of additional genes which activate and amplify AS-associated inflammatory responses. In this review, we discuss the involvement of TLR2/4 and NF-κB signaling in ECs during AS initiation, as well as regulation of the inflammatory response during AS by noncoding RNAs, especially microRNA (miRNA) and circular RNA (circRNA).

Keywords: TLR2; TLR4; NF-κB; atherosclerosis

1. Introduction

Based on ample experimental and clinical evidence, atherosclerosis (AS) is now recognized as a chronic inflammatory vascular disease [1,2]. The first step in AS pathology is endothelial activation, followed by inflammatory responses, plaque formation, and eventually rupture [3]. These disease stages lead to vascular stenosis, or the blockage of blood flow [4]. It is now clear that endothelial cell (EC) dysfunction is pivotal in the initiation and progression of AS lesions [3,5].

The pathological process of AS broadly involves inflammation and immunity. Toll-like receptors (TLRs) and nuclear factor-κB (NF-κB) signaling play essential roles in the inflammatory response during AS, with recent studies also focusing on their involvement with ECs during the development of AS. TLRs are pattern recognition receptors that play a crucial role in innate immunity. They recognize microbial ligands, such as lipopolysaccharide (LPS), and activate transcription factors, including nuclear factor-kappa B (NF-κB), which subsequently induce antimicrobial activity [6]. The NF-κB pathway is a crucial regulator of inflammation and is involved in the pathogenesis of AS. Its activation leads to the release of inflammatory mediators, such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), which contribute to the development of AS [7].

MicroRNAs (miRNAs) are a class of small, single-stranded noncoding RNA that act as negative post-transcriptional regulators of gene expression [8]. Circular RNAs (circRNAs) are a novel class of endogenous non-coding RNAs with covalently closed-loop structures [9]. Both miRNA and circRNA play essential roles in coordinating cellular functions such as inflammation, differentiation, and apoptosis [10]. Recent studies have also focused on the involvement of miRNA and circRNA regulating TLR2/4 and NF-κB signaling in the inflammatory response of AS.

In this summary, we present the current state of knowledge with regard to TLR2/4 and NF-κB signaling in ECs during the development of AS. Moreover, we discuss the involvement of miRNA and circRNA in the AS inflammatory response, with the aim of providing insight into potentially novel therapeutic approaches.

2. Endothelial Cell Dysfunction and the Initiation of Atherosclerosis

ECs act as sensors and effectors that release biological agonists and antagonists [3]. They form a functional cell layer that lines the entire circulatory system and helps to maintain multi-organ health and homeostasis [11]. The functions of healthy endothelium include serving as a semi-permeable barrier, maintaining vascular tone, angiogenesis, vascular repair, regulating hemostasis, mediating mechanon-transduction, and regulating metabolism [12]. ECs synthesize and release endothelial-derived relaxing factors such as nitrous oxide (NO) and endothelium-derived hyperpolarization factor (EDHF), as well as metabolites of arachidonic acid and peptides (endothelin, reactive oxygen

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species [ROS], etc.) that regulate vasoconstriction and vasodilation [13]. NO is produced by the endothelial isoform of NO synthase (eNOS) and also affects the behavior of other cell types in the vascular system. For example, NO reduces the contractility of adjacent vascular smooth muscle cells (VSMCs), inhibits platelet activation and adhesion, and reduces leukocyte adhesion [5]. The single layer of ECs in the aortic intima of the normal aorta is a complete structure that encloses the elastic plate. Without a complete EC structure, or in the event of uneven hyperplasia within the EC layer, the inner elastic plates break and compress, thus allowing the accumulation of foam cells, lipid plaques, and VSMCs [14]. These processes begin due to endothelial dysfunction, which is defined as a variety of non-adaptive alterations in normal functional phenotypes [5]. ECs can switch from a quiescent phenotype to a host defense phenotype during the response to proinflammatory agents, such as endotoxins, modified lipoproteins, advanced glycosylation end products (AGE), and disturbed flow-derived IL-1 and TNF [15]. Such sustained maladaptive changes lead to endothelial dysfunction characterized by reduced eNOS activity and resulting in decreased bioavailability of NO. Other pathologic changes are subsequently triggered, including lipid-laden inflammation and oxidative stress [12]. Endothelial dysfunction occurs during sepsis [16], cardiovascular diseases such as atherosclerosis and hypertension [17,18], pulmonary hypertension [19], metabolic disorders such as type-2 diabetes and obesity [20], and autoimmune diseases such as autoimmune hepatitis [21] and rheumatoid arthritis [22].

While endothelial dysfunction is the initial step in AS pathology, an early event in the inflammatory response is activation of the NF-κB signaling axis. ECs change into a proinflammatory endothelial phenotype in response to proinflammatory agents [5]. This is characterized by the secretion of leukocyte adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), chemokines such as IL-1, MCP-1, CCL20, SDF-1 and CXCR4, and prothrombotic mediators via NF-κB signaling [3]. The expression of VCAM-1 and ICAM promotes macrophage adhesion and activation, thereby forming a critical link between endothelial dysfunction and inflammatory responses during AS [5]. ECs are the principal cell type that express proinflammatory cytokines such as TNF-α, whereas ROS, shear stress, low-density lipoprotein (LDL) and VCAM-1 disrupt EC junctions and remodel actin through their interaction with integrin. These events trigger leukocytes to migrate into the intimal space and to activate intracellular signaling in circulating leukocytes [23]. Upon secretion by ECs and leukocytes, ICAM-1 governs leukocyte trans-endothelial migration, including leukocyte rolling, sticky contacts with the artery wall, and the direction of transmigration [24]. Migration is also stimulated by secreted chemokines [3], and the NF-κB signaling axis can stimulate the transcription of leukocyte adhesion molecules as well as these chemokines.

3. Toll-Like Receptors 2 and 4 in Atherosclerosis

3.1 Overview of Toll-Like Receptors

TLRs are one of the vital pattern recognition receptors (PRRs) of the innate immune system. They are responsible for recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) during embryonic development [24]. TLRs are type 1 transmembrane glycoproteins that recognize PAMPs or DAMPs through their extracellular leucine-rich repeat (LRR) domain. PAMPs and DAMPs activate downstream signaling pathways through their cytoplasmic Toll/interleukin (IL)-1 receptor (TIR) domain [25]. TLR-dependent signaling leads to the activation of transcription factors and inflammatory responses [26].

To date, 13 TLRs have been characterized in mammals, of which TLRs 1-10 are functional in humans [27]. TLRs in mammals are synthesized in the endoplasmic reticulum (ER), potentially undergo sequential glycosylation in the Golgi complex, and are then transported to the membranes of plasma or intracellular vesicles, such as the endoplasmic reticulum and endosomes [28]. TLRs located in the plasma membrane include TLR1, TLR2, TLR4, TLR5, and TLR6, while those located in intracellular vesicle membranes include TLR3, TLR7, TLR8, and TLR9 [26]. Although endosomal TLRs require trafficking factors, TLRs localized in the plasma membrane are transported to the plasma membrane through conventional secretory pathways [28].

Activation of specific TLR signaling pathways in mammals is dependent upon both the agonist and the cell type [26]. For example, during the response to the prototype ligand for TLR4, LPS, ECs primarily express IL-6, IL-8, CSF2, CSF4, ICAM-1, and SELE, but little TNF-α or IL-1L. In contrast, macrophages express TNF-α and IL-1L in response to LPS, as well as IL-6 and IL-8 [24]. High mobility group protein B1 (HMGB1)-induced migration due to TLR4 signaling occurs in both human umbilical vein ECs (HUVECs) and human pulmonary arterial ECs (HPAECs). However, nuclear translocation of NF-κB occurs in HUVECs, whereas translocation of interferon regulatory factor 3 (IRF3) occurs in HPAECs [29].

TLRs are expressed in all innate immune cells, including monocytes, macrophages, dendritic cells (DCs), neutrophils and natural killer cells, as well as vascular cells like VSMCs and fibroblasts. However, the expression of specific TLR types varies between different cell types [30], as well as mediation of the innate immune response [24]. For example, ECs express TLR2, 4, 6, 11, 12 and 13, whereas macrophages express TLR5 in addition to these TLRs [30]. VSMCs have also been reported to express TLR2 and 4 [31,32].
3.2 Activation and Signaling from Toll-Like Receptors 2 and 4

Because of different ligand-binding structures [25], TLRs can recognize a variety of ligands including PAMPs and DAMPs. In response to PAMPs, TLR2 dimerizes with either TLR1 or TLR6. The resulting structure is able to detect lipopolysaccharides. Similarly, TLR3 detects double stranded and ribosomal RNA, while TLR4 can detect LPS when accompanied by the co-receptor MD-2. Moreover, TLR5 detects flagellin, while TLR7 and TLR8 bind ssRNA, and TLR9 detects DNA containing cytosine-phosphate-guanine (CpG) motifs [28]. In addition, TLR2 and TLR4 recognize intracellular DAMPs such as HMGB1, histones, heat shock proteins, and extracellular DAMPs such as short fragment hyaluronan and extracellular matrix [33]. Finally, TLR2 and TLR4 recognize oxidized lipoproteins within endogenous lifestyle-associated molecular patterns (LAMPs) [33], which is critical in the formation of atherosclerotic plaques.

TLR signal conduction is a three-step process [28]. First, agonistic ligands bind and mediate the dimerization of TLRs. Through interactions between ligand and TLR LRR domains, the TLR extracellular domain induces the formation of homodimers such as TLR3, 4, 5, 7, 8 and 9, or heterodimers such as TLR2/1 and TLR2/6, both of which possess a typical “m”-shaped structure [34]. Second, TLR dimers mediate assembly of the supramolecular organizing center (SMOC), which is widely considered as the subcellular site of signal initiation. Dimerization of the extracellular LRR domains results in dimerization of the intracellular TIR domains, which subsequently activates cell-extrinsic, cell-intrinsic, and cell-type-specific responses and TLR signaling pathways [28]. TIR domains are detected by the receptor-proximal membrane protein TIRAP/MAL, and are necessary for the initiation of TLR2, 4, 7, and 9 signaling. Alternatively, this action can occur through the peripheral membrane protein TRIF (TIR domain-containing adaptor-inducing IFN-beta)-related adaptor molecule (TRAM), which detects the dimerization of TLR4 and TLR3 and further stimulates the assembly of SMOC. SMOCs are essential in multiple innate immune pathways, including putative ribosomes that govern TLR signaling. Third, SMOCs mediate the activation of kinases, which then primarily drive transcription and glycolysis. The myddosome, an oligomeric complex formed by the interaction between myeloid differentiation primary response protein 88 (MyD88) and IL-1R-associated kinase (IRAK) family serine-threonine kinases, subsequently auto-phosphorylates and recruits the E3 ubiquitin ligase tumor necrosis factor receptor-associating factor 6 (TRAF6) [28]. TRAF6 then activates the kinase transforming growth factor b (TGF-b)-activated kinase 1 (TAK1) to stimulate transcription factors including IKB kinase, which activates NF-κB and mitogen-activated protein kinase (MAPK), thereby mediating the activation of activator protein-1 (AP-1). TRAF6 also stimulates glycolysis by recruiting the inhibitor of kappa B kinase (IKK)-related kinase TRAF family member associated NF-κB activator (TANK)-binding kinase 1 (TBK1) [35]. Unlike the myddosome, the triffosome which is composed of TRAM, TRIF, and TRAF3, not only stimulates the transcription response through IRF3, NF-κB, and AP-1 but also induces necroptosis by activating TBK1 [28].

TLR signaling pathways are generally classified as being MyD88-dependent. These are initiated by TIRAP/MAL and are governed by the myddosome. This signaling axis results mostly in the production of proinflammatory cytokines. Signaling by TRIF is initiated by TRAM and governed by the triffosome. This signaling axis is primarily responsible for the production of type I interferons [28].

TLR2 and TLR4 are both located on the plasma membrane and transmit signals through the MyD88-dependent axis to activate NF-κB and AP-1 [36]. When TLR4 undergoes endocytosis, it also transmits signals through TRIF-dependent signaling, thus further activating IRF3 [37].

3.3 Contribution of Toll-Like Receptors 2 and 4 to the Inflammatory Response in Atherosclerosis

TLRs in ECs serve as sensors for the innate immune system and initiate inflammatory responses that subsequently activate the adaptive immune system [26]. With the initiation and progression of AS, TLR2 and TLR4 expression increases within the endothelium, atherosclerotic plaques, and circulating blood cells [38]. Using laser scanning confocal microscopy (LSCM), Mullick et al. [39] found that vascular perturbations associated with a high fat diet (HFD) increased the expression of EC TLR2. Qu et al. [40] found that abnormal fluid flow increased the expression of TLR4 in ECs within the inner curvature of the aorta in vivo. This result was mimicked in vitro by HUVECs. Symptomatic plaques that are unstable and display vascular stenosis or blood flow blockage show higher expression of TLR4 in ECs than asymptomatic plaques [41]. Inhibition of TLR2 and TLR4 inhibits inflammatory response and AS progression. In Mullick et al. [39] study, they also found that TLR2/- mice showed reduced intimal leukocyte accumulation within the endothelial layer compared to wildtype mice, and exhibited less intimal lipid foam cell accumulation in AS lesions.

4. NF-κB Signaling in Endothelial Cells

4.1 Overview of NF-κB Signaling and Its Role in Transcriptional Regulation

NF-κB is a rapidly inducible transcription factor that governs cell survival, differentiation, and proliferation. It exerts its activity by occupying conserved NF-κB binding sites within the genome and promoting gene expression that controls various signaling pathways [42–44]. NF-κB interacts with the consensus sequence 5′-GGGRNWYYCC-3′ (N: any base; R: purine; W: adenine or thymine; Y: pyrimidine), which has been found in the enhancer and promoter
sequences of hundreds of genes [42]. Aberrant activation of NF-κB is associated with various disease states including autoimmune disease, malignancy, cardiovascular disease, and metabolic disorders [43]. Zhang et al. [42] integrated the monogenic disease data of clinical phenotypes with data from transcriptomic, proteomic and whole-genome analysis and concluded that NF-κB dysregulation arising from mutation resulted in immunodeficiency, inflammation, and embryonic lethal tumor development.

4.2 Canonical and Non-Canonical Pathways for NF-κB Activation

NF-κB pathways are classified as canonical or non-canonical depending on their mechanism of activation. The NF-κB family includes the NF-κB transcription factor family, the inhibitor of NF-κB (IκB) family, and the IκB kinase (IKK) complex. The five members of the NF-κB transcription factor family are p50/p105, p52/p100, p65 (RelA), RelB, and c-Rel. c-Rel contains an N-terminal Rel homology domain (RHD) that functions in DNA binding, dimerization, and binding to inhibitory proteins [42,43]. It is commonly accepted that RelA (p65) and p50 heterodimers are activated by the canonical pathway, while RelB and p52 heterodimers are activated through the non-canonical pathway. Further, RelA, RelB, and c-Rel contain transcriptional activation domains (TADs) that act as activators when dimerized, whereas homodimers of p50 or p52 function as repressors [44].

In unstimulated conditions, NF-κB activity is restrained by a member of the IκB family, which includes IκBα, IκBβ, IκBε, B cell leukemia-3 (BCL-3), IκBζ, and IκBNS. IκB antagonizes NF-κB activity by sequestering NF-κB in the cytoplasm [42]. When activated by various signals such as innate pattern-recognition receptors (PRRs) (e.g., TLRs), T-cell receptor (TCR), B-cell receptor (BCR), or proinflammatory cytokine receptors (e.g., TNF receptors), the IKK complex comprising IKKα, IKKβ, and non-catalytic IKKγ (NF-κB essential modifier, or NEMO) phosphorylates IκB. This phosphorylation event results in targeting of the IκB inhibitor for ubiquitin-mediated proteolysis, thus allowing NF-κB to move to the nucleus where it acts as a transcriptional modulator [42,45].

The non-canonical NF-κB signaling can promote autoimmunity and inflammatory pathologies depending on the cell type involved [46]. Such diseases include rheumatoid arthritis, systemic lupus erythematosus, primary glomerulonephritis kidney disease, and metabolic dysregulation. Non-canonical NF-κB signaling differs from the canonical pathway by acting through the TNF receptor superfamily (TNFRS). Activation of TNFRS stimulates the cIAP1-TRAF2-TRAF3 complex to stabilize NF-κB inducing kinase (NIK), which promotes the proteosomal processing of p100 into a functional p52 subunit. Subsequently, biologically active NF-κB p52/RelB heterodimers are able to enter the nucleus [46].

5. The Role of Toll-Like Receptors 2 and 4, and NF-κB Signaling in Atherosclerosis

5.1 Toll-Like Receptors 2 and 4 as Mediators of NF-κB Activation

The TLR family are essential members of innate PRRs. Their signals activate the canonical NF-κB pathway and subsequently the expression of cytokines, chemokines, and an array of additional genes involved in immune regulation [28]. TLR ligands mediate the extracellular dimerization of TLRs, resulting in the assembly of SMOC [28]. In the MyD88-dependent pathway, the MyD88 complex recruits the E3 ubiquitin ligase TRAF6, resulting in the auto-phosphorylation and activation of TAK1, which then mediates the activation of IKKs. This results in phosphorylation of IκB and the nuclear translocation of NF-κB p50/RelA heterodimers [45]. In the TRIF-dependent pathway, TRIF recruits TRAF6 and RIP1, which activate TRIF3 [45]. For example, in the study by Echavarria et al. [47], the TLR4 agonist LPS was found to increase the level of p65, increase NF-κB transcriptional activity, and decrease IκB protein levels. Furthermore, activation of MAPK was apparent through increased phosphorylation of the MAPKs, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and ERK1/2. Increased expression of adhesion molecules such as VCAM1, ICAM1, and E-selectin was found to occur in HUVECs, as well as proinflammatory cytokines such as TNFα, IL1.1, IL6, and IL8.

The TLR2,4/NF-κB signaling pathway induces the expression of various cytokines and chemokines at both the RNA and protein levels. LPS increases the expression of the TNF-α, IL-6 IL-9, IL-17A, IL-17F, IL-2, and IL-21 cytokines in ECs [48]. In response to chemokine release, increased expression of VCAM-1 was found to stimulate migration of leukocytes to the site of inflammation [49]. Similar observations were made for ICAM-1, which governs leukocyte trans-endothelial migration, and E-selectin, which is expressed only on activated endothelium and promotes leukocyte adhesion [47,50,51]. Also found to be upregulated were MCP-1, which recruits monocytes and T cells to the site of inflammation [52], CXC16, which mediates T-cell adhesion to the endothelium and promotes cell proliferation [53], and LOX-1, which controls the uptake of oxidized low-density lipoprotein (ox-LDL) and endothelial-monocyte adhesion [54]. TLR/NF-κB stimulation also activates the nod-like receptor protein 3 (NLRP3) pathway, a well-characterized sensor protein in the inflammasome pathway, leading to pyroptosis associated with AS [36,55]. TLR4/NF-κB may also participate in the proprotein convertase subtilisin/Kexin type 9 (PCSK9) pathway, which increases ox-LDL uptake and up-regulates inflammatory cytokine expression [36]. NF-κB signaling also regulates the expression of NF-κB-regulated miRNAs (e.g., miR-146a, miR-155, miR-21) which act as feedback effectors of this signaling. In addition, some miR-
The transmission of toll-like receptor (TLR) signaling follows three steps. First, agonistic ligands mediate the formation of TLR2 heterodimers and TLR4 homodimers in the plasma membrane. These include pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and lifestyle-associated molecular patterns (LAMPs). The TLR4 homodimers subsequently translocate from the cell surface to intracellular vesicles via endocytosis. Second, TLR2 heterodimers and TLR4 homodimers mediate assembly of the supramolecular organizing center (SMOC), including the Myddosome which governs MyD88-dependent signaling, and the Triffosome which governs TRIF-dependent signaling. TLR2 and TLR4 mediate assembly of the Myddosome, while TLR4 also mediates assembly of the Triffosome (black arrows). Third, SMOCs mediate the activation of kinases, which then activate the transcriptional response. The Myddosome activates the inhibitor of kappa B kinase (IKK)-related kinase Tumor necrosis factor receptor-associated factor (TRAF) family member associated Nuclear factor-κB (NF-κB) activator (TANK)-binding kinase 1 (TBK1), which in turn activates the kinase AKT (also known as PKB; protein kinase B). The kinase transforming growth factor b (TGF-b)-activated kinase 1 (TAK1) stimulates IKK-mediated activation of NF-κB and mitogen-activated protein kinase (MAPK)-mediated activation of activator protein-1 (AP-1). NF-κB not only stimulates the expression of additional immune genes, regulates downstream signal pathways (e.g., nod-like receptor protein 3 (NLRP3), proprotein convertase subtilisin/Kexin type 9 (PCSK9), induces cytokines and chemokines expression), but regulates microRNA (miRNA) expression. The Triffosome also activates TAK1 and mediates similar downstream responses, as well as activating TRAF3-dependent TBK1 which then activates interferon regulatory factor 4 (IRF4) leading to the interferon (IFN) response (green arrows). TLR signal transduction may be regulated by miRNAs at various levels (red arrows).

NAs that are activated by other signaling pathways could also modulate the NF-κB signaling in a cross-talk fashion \[56\] (Fig. 1).

5.2 Effect of TLR2 and 4 on NF-κB Signaling in ECs during Atherosclerotic Development

5.2.1 Lipid Accumulation

HFD that is rich for example in cholesterol and trans-fatty acids will increase the levels of plasma cholesterol and serum triglycerides. This induces ECs to switch to a maladaptive phenotype characterized by excessive inflammatory responses, insulin resistance, ROS production, and impaired vasorelaxation. Bhaskar et al. \[52\] reported that a hypercholesterolemic diet in mice enhanced the inflammatory response. This was indicated by elevated levels of serum C-reactive protein (CRP), cyclooxygenase (COX), 5-lipoxygenase (5-LOX), NOS and myeloperoxidase (MPO), as well as upregulating the mRNA level of IL-6. Kramer et al. \[57\] fed mice a long-term western diet characterized by a high fat and high carbohydrate content. This in-
increased the levels of fasting serum triglycerides and serum non-esterified free fatty acid (NEFA), resulting in glucose intolerance, insulin resistance, and increased arterial blood pressure. Kondo et al. [58] reported that 4 weeks of a high trans-fatty acid (TFA) diet led to the accumulation of plasma lipids and delayed catabolism of circulating TFA-containing lipids, while also triggering prothrombogenic activity in ECs.

Lipid accumulation upregulates the expression of TLR2 and TLR4, as well as downstream signaling components such as MyD88, TRAF6, and NF-κB. It also increases the expression of cytokines that suppress inhibition of TLRs and promote activation of TLR2/4 and NF-κB signaling. In a mouse model of atherosclerosis, it was found that ox-LDL increased the expression of TLR4, MyD88, and TRAF6 in thoracic aortas, while non-esterified fatty acid induced ROS production in human aortic endothelial cells (HAECs) [57]. These effects were also observed by a TLR4 inhibitor. The most potent lipid for inducing cytokine expression in ECs and macrophages appears to be ox-LDL [59]. In the aforementioned study by Bhaskar et al. [52], ox-LDL resulted in overexpression of adhesion molecules in ECs. This was observed by the upregulation of VCAM-1, ICAM-1 and MCP-1, and was accompanied by upregulation of mRNA expression for TLR2 and TLR4, as well as their downstream target NF-κB. Zhang et al. [60] reported that vascular ECs were damaged by ox-LDL through TLR4/TREM-1 signaling and increased levels of CD42b+/CD31+ endothelial microparticles (EMPs) and TNF-α, as well as decreased expression of the NO-related eNOS. Knockout of TLR4 reversed these deleterious effects and decreased expression of the TLR4 downstream gene TREM-1. Ox-LDL was also shown to significantly increase the protein levels of NLRP3 and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), leading to activation (cleavage) of Caspase-1 in HAECs [61].

Apart from ox-LDL, saturated and trans-fatty acids have also gained considerable attention for their ability to induce an inflammatory response and endothelial dysfunction through TLR signaling in ECs. Palmitate (PA) is the most abundant saturated fatty acid in the human bloodstream. Kim et al. [62] found that PA increased the expression of numerous proinflammatory genes in HAECs, including E-selectin, TNFα, IL-1β, and IL-6, but not MCP-1. This effect was inhibited by knockdown of TLR4. Shi et al. [63] also observed that PA induced an inflammatory response in HUVECs through TLR4-mediated NF-κB and MAPK pathways. Kondo et al. [58] reported that trans-fatty acid (TFA) increased the mRNA and protein expression of proinflammatory (TNF-α and transferin) and thrombogenic (PAI-1) molecules and decreased the expression of anti-thrombogenic molecules (thrombomodulin) and tissue factor pathway inhibitor. Of note, the knockdown of TLR2 and TLR4 was able to attenuate these effects.

Unlike ox-LDL which directly binds TLR2 or TLR4 dimers, PA induces an inflammatory response by altering cell metabolism, gene expression, lipid metabolism, and membrane lipid composition through TLR signaling [64]. Ren et al. [50] found that TLR activation induced by PA differs from canonical TLR agonists in several ways. First, it takes longer for PA-dependent E-selectin expression to plateau, and for peak phosphorylation of JNK and activation of NF-κB to occur. Second, acyl-CoA synthetases were found to be necessary for PA-stimulated inflammatory responses and insulin-induced vasodilation. This suggests that PA is required for metabolic reprogramming, and that intracellular fatty acyl-CoA could be a source for such metabolic reprogramming. Kim et al. [62] observed that PA also increased the splicing of XBP-1, an ER stress marker, through a TLR4-dependent mechanism in vitro. These investigators noted that a key feature of endothelial dysfunction in vivo, impaired insulin vasodilator action, could be reversed by silencing TLR4 or reducing ER stress. This observation indicates that ER stress, through TLR4 signaling, could be an essential part of the endothelial dysfunction induced by saturated fatty acids (SFAs). It should also be noted that SFAs induce the dimerization of TLR2 and TLR4 and recruit these molecules into lipid rafts [65]. However, it remains unclear whether TFAs bind directly to TLRs or whether they alter other fatty acid components of the membrane [58].

5.2.2 Infection

A number of infectious agents have been implicated in atherosclerotic pathogenesis, including Chlamydia pneumoniae [66,67], Porphyromonas gingivalis [68], Helicobacter pylori [69], cytomegalovirus (CMV), Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), herpes simplex virus-1 (HSV-1), HSV-2, and hepatitis C virus (HCV) [30]. Microbial cell surface components are detected by TLRs through PAMP-PRR interaction. These include LPS, lipoprotein and flagellin, as well as dsRNA, ssRNA and Cpg DNA of viral origin, and lipopolysaccharides, Cpg DNA, ssRNA and profilin from parasites [28]. After recognizing such pathologically-related factors and subsequently activating TLR signaling, ECs recruit immune cells through upregulation of the leucocyte adhesion molecules VCAM-1 and ICAM-1 [69], LOX-1 [54,70], and CXC16 [53] following adhesion of the immune cell to ECs [69]. Be-nagiano et al. [66] observed that C. pneumoniae phospholipase D (CpPLD) increased the mRNA and protein expression of various chemokines and adhesion molecules in HUVECs, including CCL-4, CCL-2, CCL-20, CXCL9, ICAM-1 and sVCAM-1. Echavarria et al. [47] noted that exposure of HUVECs to LPS for 4 hr induced significant increases in leucocyte adhesion, adhesion molecule expression, and proinflammatory cytokine production, but did not affect the expression of anti-inflammatory cytokines such as IL4 and IL10.
LPS is a well characterized component of the outer membrane of most gram-negative bacteria and is strongly recognized by the immune system during inflammatory responses [71], endothelial dysfunction, and apoptosis [72]. LPS is recognized by TLRs, of which TLR4 and myeloid differentiation factor 2 (MD2) are the primary receptor and activator of subsequent signaling [25]. Zhao et al. [54] found that LPS increased the expression of TLR4 in ECs but reported no change in TLR2 expression. The TLR4-MD2 heterodimer interacts with LPS at the cell surface and promotes translocation to lipid rafts. TLR4-MD2 has a strong affinity for TIRAP/MAL, thus promoting the assembly of myotomes and initiating MyD88-dependent signaling [28]. CD14 mediates a process by which TLR4 undergoes endocytosis to intracellular vesicles and subsequently engages with TRAM to activate TRIF-dependent signaling [37]. Thus, activation of TLR4 initiates both MyD88-dependent signaling at the cell surface and TRIF-dependent signaling in intracellular vesicles, both of which lead to increased expression of proinflammatory factors and subsequent inflammation. Qu et al. [40] reported that LPS-induced TLR4 activation is mediated by a MyD88-dependent mechanism, leading to phosphorylation of the NF-κB p65 subunit and markedly elevated transcription of proinflammatory markers including TNF-α, VCAM1, and CCL2. Zhao et al. [54] further noted that LPS activated TLR4 in the MyD88-dependent signaling and subsequently triggered ROS production mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and COX-2. These effects resulted in MAPK activation and the regulation of LOX-1 expression by NF-κB.

The inflammation that occurs during AS is chronic and mimics the signal pathway transmission and communication mechanisms observed in immune cells. The inflammatory response induced by LPS varies according to the dose, duration, and specific pathogen. Xiao et al. [53] noted that the viability of HUVECs decreased significantly after treatment with LPS for 24 hr compared with lower doses, and that viability rebounded within 24 hr when treated with 1 μg/mL LPS. Zhao et al. [54] found that high doses of LPS inhibited EC proliferation, and that LOX-1 expression increased significantly when the cells were treated for a prolonged time or with a higher concentration of LPS. Triantafillou et al. [68] observed that LPS from either H. pylori or P. gingivalis activated ECs through TLR2/NF-κB-dependent mechanisms. The action of LPS can range from a weak agonist to a potent antagonist of TLR4. This range of responses prevents bacteria from inducing rigorous TLR4 responses that could result in chronic endothelial inflammation.

TLR signaling induced by LPS is interactive in nature. Lipid rafts are regularly distributed in all cellular membranes and form unique microdomains that serve as platforms for signal transduction and receptor activation [73]. Huang et al. [69] noted that LPS-induced TLR4 translocated to lipid rafts, whereas nystatin, a lipid raft inhibitor, blocked the downstream upregulation of VCAM-1 and ICAM-1 expression. Other studies found that LPS from H. pylori and P. gingivalis activated ECs through TLR2/NF-κB-dependent signaling, as well as inducing receptor clustering of TLR2, TLR1, CD36, and CD11b/CD18 within lipid rafts [68]. Sikorski et al. [74] reported that signal transducer and activator of transcription 1 (STAT1) could be a point of convergence for the cross-talk between IFN-γ and TLR4. Moreover, Choy et al. [72] found that LPS-induced HUVEC inflammation, apoptosis, and endothelial dysfunction occurred independently through TLR4 and bone morphogenetic protein 4 (BMP4) pathways. Immune cell communication also participates in stimulation of the inflammatory response. Talepor et al. [48] studied co-cultures of HUVECs with peripheral blood mononuclear cells (PBMCs) obtained either from patients with high stenosis (HS) or from healthy controls (HCs). They then evaluated the production of various cytokines induced by LPS. The co-culture groups, either with HS or HC, induced higher levels of cytokine production compared with monocytes, strongly indicating cross-talk between these cell types.

5.2.3 Hyperglycemia

Diabetes and AS share a close relationship and both diseases impair the endothelium and cause endothelial dysfunction. Lu et al. [75] reported that HFD-induced type 2 diabetes increased the size of AS lesions and intimal lesions of AS plaques by increasing the accumulation of monocytes and macrophages, the expression of IL-6 and MMP-9 expression in AS plaques, and the collagen content of AS lesions. Using a streptozotocin-induced animal model of diabetes, Leng et al. [49] found that high glucose could impair vasodilation in response to acetylcholine (ACh), decreased levels of eNOS and NO, increased levels of IL-6 and TNF-α, and enhanced expression of ICAM-1 and VCAM-1. These investigators also found that high glucose could impair vasodilation. Their study of HUVECs also provided evidence that high glucose promotes endothelial dysfunction, leading to inhibition of cell proliferation, decreased expression of eNOS and NO, and increased expression of cytokines and chemokines.

Surprisingly, TLRs do not recognize glucose. However, TLR signaling participates in hyperglycemia-induced endothelial dysfunction. Leng et al. [49] found that diabetic atherosclerotic rings showed impaired vasodilation in response to acetylcholine (ACh), decreased levels of eNOS and NO, increased levels of IL-6 and TNF-α, and enhanced expression of ICAM-1 and VCAM-1. These investigators also found that high glucose conditions significantly increased the expression of TLR2 and the secretion of IL-6 and TNF-α in ECs. DAMPs such as HMGB1 that are associated with cell injury and necrosis are critical in hyperglycemia-induced endothelial dysfunction. Mudalier et al. [77] reported that high glucose concentrations increased the secretion of HMGB1 by
cultured human dermal microvascular HMEC-1 cells, with the HMGB1 subsequently activating NF-κB signaling and its DNA occupancy. Increased HMGB1 due to leakage from necrotic cells or secretion from immune cells such as ECs and macrophages are recognized by TLR2, TLR4, and the receptor for advanced glycation end products (RAGE). These events activate NF-κB signaling and establish a positive feedback loop for amplifying and sustaining inflammation [78]. Another study reported that low-dose LPS synergizes with high glucose induced TLR2 expression in ECs, whereas low-dose LPS alone could not [76]. This result provides further evidence that high glucose conditions can amplify the inflammatory response.

5.2.4 Hemodynamics

The endothelium is located between circulating blood and tissue. It senses changes in mechanical stress and in the concentration of metabolic factors and is responsible for activating signal transduction in response to such changes [3]. High-risk factors including hypercholesterolemia, hypertension, diabetes mellitus, and smoking [4] promote the formation of atherosclerotic lesions, which tend to occur in regions with low wall shear stress (WSS). WSS tends to occur at sites of disturbed flow (DF), such as vessel branch points and bifurcation [3]. ECs that are exposed to DF undergo changes in morphology and function, eventually resulting in cell turnover and senescence [5]. DF also alters TLR gene expression, and TLR signaling is activated by DAMPs such as fibronectin extra domain A (EDA), hyaluronan fragments, HMGB1, and serum amyloid A. Tobias et al. [2] found that murine ECs exposed to DF showed upregulation of TLR2 expression. Furthermore, en-face laser scanning confocal microscopy revealed that TLR2 was only observed in the DF area of murine aortic tissue, while hyperlipidemia could increase TLR expression [39]. Qu et al. [40] found that ECs reside in the human aortic arch. This area is constantly exposed to DF, is activated by the TLR4/MyD88/ NF-κB signaling axis and shows increased expression of VCAM1. HUVECs that are exposed to DF mimetics also upregulate TLR expression, and knockdown of TLR4 protects ECs from DF-induced inflammation. Furthermore, DF increased fibronectin extra-domain A (FnEDA) expression and the interaction between TLR4 and FnEDA, while knockdown of FnEDA reversed the activation of TLR4 and the inflammatory response. In contrast, high shear stress inhibited activation of the TLR4 signal pathway and decreased the resistin-induced expression of CCL19 in a shear time-dependent manner [79].

6. Noncoding RNAs in AS pathology

6.1 Noncoding RNAs Modulate the Inflammatory Response Induced by TLR2/4 in Atherosclerosis

Noncoding RNAs, including long noncoding RNA (lncRNA), microRNA (miRNA), long intergenic noncoding RNA (lincRNA), and circular RNA (circRNA), play essential roles in coordinating cellular functions such as inflammation, differentiation, and apoptosis [10]. Noncoding RNAs are also associated with many inflammation-related diseases, such as AS, diabetes mellitus, obesity, and osteoarthritis [10]. The level of these noncoding RNAs is commonly dysregulated in AS, and they participate in the stimulation of various signaling mechanisms including the TLR/NF-κB signaling axis. Liu et al. [80] reported that the serum level of lncRNA RP11-490M8.1 was lower in AS patients, and this reduced LPS-induced apoptosis via TLR4/NF-κB signaling. Further, Yang and Gao [81] observed that miR-590 expression was downregulated in the aortic tissue of hyperlipidemia mouse models, and this could inhibit TLR4 signaling.

miRNAs are defined as being non-coding, single-stranded RNA oligonucleotides with an average length of 22 nucleotide residues. Through binding to 3’ untranslated regions (3’ UTRs) of target genes, miRNAs can negatively regulate post-transcriptional gene expression by destabilizing their target mRNA or by inhibiting translation. miRNAs have been shown to regulate cell differentiation, migration, proliferation, and cell death [8]. They are also novel regulators of endothelial function and dysfunction through multiple aspects including control of endothelial inflammation, vascular tone, uncoupling of endothelial NOS, endothelial-to-mesenchymal transition, and apoptosis [12]. Reduction or loss of miRNA expression in ECs can lead to abnormal phenotypes such as increased release of NO, decreased EC growth, and cord formation in matrigel assays [8]. Moreover, some miRNAs like miR126 could also control inflammatory response, and some other miRNAs such as miR34a, miR-217, and miR-146a exacerbate EC senescence, thus affecting endothelial dysfunction [82]. miR-590 was reported to promote proliferation, inhibit HAEC apoptosis, and decrease PARP cleavage and cleaved-caspase-3 accumulation in response to ox-LDL treatment [80]. In addition, miRNAs are secreted from cells by small vesicles termed exosomes or micro-vesicles. This may be a form of cell-cell communication and could be applied for diagnostic and therapeutic purposes [8].

Three different mechanisms have been described regarding the regulation of TLR signaling by miRNAs [83]. First, miRNAs directly bind with a component of TLR signaling. Chen et al. [61] found that TLR4 and TXNIP were direct targets of miR20 due to the presence of an evolutionarily conserved miR-20a binding site within their 3’ UTR. Moreover, TXNIP 3’ UTR luciferase activity could be repressed by co-transfection with miR-20a mimics. Another study found that miR-21 binds to the 3’ UTR of TLR4, a miR-21 mimic suppressed TLR4 expression, and that a miRNA inhibitor upregulated TLR4 expression [84]. MiR-26a-5p was also reported to target TLR4 [85]. Second, the expression of certain miRNAs is stimulated by TLR signaling. Xiao et al. [53] observed that LPS upregulated miR-217 and this negatively regulated CXCL16 expression, sin-
Table 1. miRNAs involved in TLR signaling and their effects on endothelial cells.

<table>
<thead>
<tr>
<th>miR</th>
<th>Inducer</th>
<th>Expression level</th>
<th>Mechanism</th>
<th>Effects on ECs</th>
<th>Regulator</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-590</td>
<td>ox-LDL</td>
<td>↓</td>
<td>Inhibit TLR4/NF-κB</td>
<td>attenuate atherosclerotic lesion, promote proliferation, block ox-LDL-induced apoptosis</td>
<td></td>
<td>[81]</td>
</tr>
<tr>
<td>miR-204-5p</td>
<td>ox-LDL</td>
<td>↓</td>
<td>Inhibit TLR4</td>
<td>increase cell viability and inhibit cell apoptosis, inflammatory response, and oxidative stress</td>
<td></td>
<td>[87]</td>
</tr>
<tr>
<td>miR-217</td>
<td>ox-LDL</td>
<td>↓</td>
<td>Inhibit TLR4 PI3K/Akt/NF-κB</td>
<td>increase the viability of atherosclerotic ECs, inhibit their apoptosis, inflammatory response, and EndMT</td>
<td></td>
<td>[89]</td>
</tr>
<tr>
<td>miR-20a</td>
<td>ox-LDL</td>
<td>↓</td>
<td>Directly target TLR4 and TXNIP</td>
<td>inhibit TLR4-mediated inflammatory response and NLRP3 activation</td>
<td></td>
<td>[61]</td>
</tr>
<tr>
<td>miR-26a-5p</td>
<td>ox-LDL</td>
<td>↓</td>
<td>Directly bind TLR4 and downregulate</td>
<td>inactivate TLR4/NF-κB, reduce ox-LDL-induced EC apoptosis</td>
<td>Kaempferol</td>
<td>[85]</td>
</tr>
<tr>
<td>miR-20a</td>
<td>ox-LDL</td>
<td>↑</td>
<td>Inhibit TLR4/NF-κB</td>
<td>reduce LPS-induced CXCL16</td>
<td></td>
<td>[53]</td>
</tr>
<tr>
<td>miR-146a, miR-155</td>
<td>LPS</td>
<td>↑, ↓</td>
<td>TLR4/NF-κB</td>
<td>related to proinflammatory response potentially</td>
<td>Dapagliflozin</td>
<td>[88]</td>
</tr>
<tr>
<td>miRNA-146a</td>
<td>LPS</td>
<td>↑</td>
<td>Downregulate IRAK1 and TRAF-6</td>
<td>suppress the activation of NF-κB</td>
<td></td>
<td>[86]</td>
</tr>
<tr>
<td>miRNA-126</td>
<td>LPS</td>
<td>↑</td>
<td>Downregulate VCAM-1</td>
<td>suppress leukocyte adherence to endothelial cells</td>
<td></td>
<td>[47]</td>
</tr>
<tr>
<td>miR-146b-5p</td>
<td>LPS</td>
<td>/</td>
<td>Selectively reduce IRAK1 and TRAF6 expression</td>
<td>inhibits LPS-induced leucocyte adhesion, adhesion molecule expression, proinflammatory cytokine production, p38 and SAPK/JNK phosphorylation, and NF-κB activation</td>
<td>Ang-1</td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>LPS</td>
<td>↓</td>
<td>Directly target TLR4</td>
<td>downregulate TLR4 expression</td>
<td>propofol</td>
<td>[84]</td>
</tr>
<tr>
<td>miR-6835</td>
<td>LPS</td>
<td>/</td>
<td>Interaction between TLR-4 and AdipoR1 in lipid rafts</td>
<td>enhance LPS-induced inflammation process</td>
<td></td>
<td>[90]</td>
</tr>
</tbody>
</table>

↑, increase; ↓, decrease.

Abbreviations: ox-LDL, oxidized low-density lipoprotein; LPS, lipopolysaccharide; TLR, Toll-like receptor; NF-κB, Nuclear factor-κB; PI3K, Phosphoinositide 3-kinase; Akt/PKB, protein kinase B; EndMT, Endothelial-to-Mesenchymal Transformation; TXNIP, thioredoxin-interacting protein; NLRP3, nod-like receptor protein 3; CXCL16, chemokine ligand 16; IRAK-1, interferon regulatory factor 1; TRAF-6, Tumor necrosis factor receptor-associated factor 6; VCAM-1, vascular cell adhesion molecule 1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; AdipoR1, adiponectin receptor.
ce overexpression of miR-217 inhibited TLR4/NF-κB expression. Angel-Morales et al. [86] also found that miRNA-146a could be upregulated by NF-κB to negatively regulate the expression of IRAK-1 and TRAF6. Third, miRNA could act as a ligand for a TLR, such as TLR8. miRNA could also be the downstream target of signaling. For example, Lu et al. [87] observed that ox-LDL induced increased expression of X-inactive specific transcript (XIST), which acts as a competing endogenous RNA (ceRNA) for miR-204-5p following increased TLR4 expression (Table 1, Ref. [47,53,61,81,84–90]).

Many molecules that regulate the inflammatory response modulate TLR signaling in ECs through miRNA. Ang-1 was found to block TLR4 signaling by upregulating miR-146-5p, which selectively attenuates the TLR4 signal transducers IRAK1 and TRAF6 [47]. Propofol downregulated LPS-induced expression of TLR4 through the upregulation of miR21 [84]. Dapagliflozin was reported to increase the expression of anti-inflammatory miR-146a and to decrease the expression of pro-inflammatory miR-155 [88]. Similarly, kaempferol inactivated TLR4/ NF-κB signaling to reduce apoptosis of HAECS induced by ox-LDL through upregulation of miR-26a-5p [85] (Table 1).

6.2 CircRNA is Involved in Endothelial Dysfunction and Inflammation during AS

Circular RNA (circRNA) is a form of endogenous noncoding RNA characterized by a covalently closed loop structure. CircRNAs are formed through back-splicing, whereby a covalent bond is formed between a downstream 5′ splice site and an upstream 3′ splice site, resulting in a circular molecule [91]. Unlike linear RNAs, circRNAs resist exonuclease degradation and are highly stable, making them ideal candidates as biomarkers [92, 93]. CircRNAs have been found to play a role in various biological processes including gene transcription, post-transcriptional regulation, and protein translation. They do this by functioning as miRNA sponges, RNA-binding protein sequesters, and transcriptional regulators [94]. CircRNAs have also been implicated in the development and progression of various diseases, including cardiovascular disease [95], where they have been shown to regulate endothelial cell function, inflammation, and plaque destabilization. Although many of the functions of circRNAs have yet to be elucidated, they have emerged as essential regulators of gene expression and as potential biomarkers for various diseases.

There is some evidence to suggest that circRNAs play a role in the progression of AS, with several studies investigating the effects of different circRNAs on EC behavior, inflammation, and plaque destabilization. For example, circRNA-0006896 was found to be positively correlated with triglyceride, LDL-C and CRP levels in patients with unstable/vulnerable plaque AS, and negatively associated with albumin levels [96]. Another study found that knockdown of circRNA-PTPRA inhibited AS progression by repressing ox-LDL-induced EC injury by sponging miR-671-5p [97]. Additionally, circRNA R51F1 was found to regulate ox-LDL-induced EC inflammation in AS by modulating the miR-135b-5p/HDAC1 axis [98]. These studies suggest that circRNAs may regulate EC function and inflammation in AS.

To date, however, there is limited information on how circRNAs might affect the expression of genes related to EC dysfunction in AS. Some studies have shown that circRNAs can regulate autophagy, apoptosis, inflammation, oxidative stress, and proliferation of ECs and hence the development of AS. For example, circ_0068087 negatively regulated the level of miR-186-5p in HUVECs by interacting with this miRNA [99]. This promoted ox-LDL-induced injury in HUVECs by allowing upregulation of ROBO1 expression. Knockdown of hsa_circ_0005699 attenuated the inflammatory and apoptotic response induced by ox-LDL in HUVECs by regulating the miR-450b-5p/NFKB1 axis [100]. Taken together, these findings suggest that circRNAs may regulate gene expression associated with EC dysfunction in AS by enhancing the inflammatory response. However, more work is needed to fully understand the specific effects of circRNAs on the expression of genes associated with EC dysfunction in AS.

7. Conclusion

In conclusion, TLR2/4 and NF-κB signaling are important for the activation of inflammatory responses during endothelial dysfunction and initiation of AS. TLRs in ECs, especially TLR2 and TLR4, are essential PRRs of the innate immune system. As such, they serve as critical sensors for recognizing PAMPs, DAMPs, and LAMPS, as well as for activating downstream transcriptional responses. NF-κB activation in ECs plays a central role in the transcription of proinflammatory factors, leading to the chronic and minute inflammation observed in AS initiation and progression. During the development of AS, it has been shown that lipid accumulation, infection, hyperglycemia and WSS lead to an inflammatory response by ECs through TLR2/4 and NF-κB signaling. Ox-LDL and LPS can directly activate TLR2/4 and NF-κB signaling, while hyperglycemia and WSS can induce the formation of DAMPs to activate and amplify this signaling. Of note, during the initial stages of AS the TLR2/4 and NF-κB signaling can be modulated, and endothelial dysfunction is reversible. Non-coding RNAs have attracted considerable attention recently due to their potential applications in early diagnosis and safe personalized treatments. However, many questions remain unanswered and future studies should explore novel therapeutic targets and evaluate the safety and efficacy of existing drugs that modulate TLR2/4 and NF-κB signaling.

Author Contributions

BY and XY: Conceptualization, Writing-original draft, Writing-editing. XC, XH, BX: Conceptualization,
Writing-review. DL, JC, WL, YL: Conceptualization, Writing-review & editing. JY: Visualization. JL: Conceptualization, Writing-review & editing, Funding acquisition. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

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Conflict of Interest
The authors declare no conflict of interest.

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