

## STATE-OF-THE-ART REVIEW

# Innate Immune Receptors, Key Actors in Cardiovascular Diseases



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### HIGHLIGHTS

- The present review recapitulates relevant findings related to the role of the receptors of the innate immune system (TLRs and NLRs) in the progression of the most prevalent CVDs.
- TLRs and NLRs play a key role in the progression of atherosclerosis, acute myocardial infarction or heart failure.
- The development of new specific strategies to impair exacerbated TLR and NLR activation in CVDs are strong candidates for therapy and opens a new research field.

### SUMMARY

Cardiovascular diseases (CVDs) are the leading cause of death in the industrialized world. Most CVDs are associated with increased inflammation that arises mainly from innate immune system activation related to cardiac damage. Sustained activation of the innate immune system frequently results in maladaptive inflammatory responses that promote cardiovascular dysfunction and remodeling. Much research has focused on determining whether some mediators of the innate immune system are potential targets for CVD therapy. The innate immune system has specific receptors—termed pattern recognition receptors (PRRs)—that not only recognize pathogen-associated molecular patterns, but also sense danger-associated molecular signals. Activation of PRRs triggers the inflammatory response in different physiological systems, including the cardiovascular system. The classic PRRs, toll-like receptors (TLRs), and the more recently discovered nucleotide-binding oligomerization domain-like receptors (NLRs), have been recently proposed as key partners in the progression of several CVDs (e.g., atherosclerosis and heart failure). The present review discusses the key findings related to the involvement of TLRs and NLRs in the progression of several vascular and cardiac diseases, with a focus on whether some NLR subtypes (nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing receptor 3 and nucleotide-binding oligomerization domain-containing protein 1) can be candidates for the development of new therapeutic strategies for several CVDs.

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**ABBREVIATIONS  
AND ACRONYMS****AMI** = acute myocardial infarction**Ca<sup>2+</sup>** = calcium ion**CARD** = caspase activation and recruitment domain**CVD** = cardiovascular disease**DAMPs** = danger-associated molecular patterns**DAP** = D-glutamyl-meso-diaminopimelic acid**ER** = endoplasmic reticulum**HF** = heart failure**IL** = interleukin**I/R** = ischemia/reperfusion**MAPK** = mitogen-activated protein kinase**NF- $\kappa$ B** = nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells**NLR** = nucleotide-binding oligomerization domain-like receptors**NLRP** = nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing receptor**NOD** = Nucleotide-binding oligomerization domain-containing protein**PAMP** = pathogen-associated molecular pattern**ROS** = reactive oxygen species**SR** = sarcoplasmic reticulum**TLR** = toll-like receptor

The danger theory of immunology proposed by Polly Matzinger in 1994 (1) reshaped our understanding of the immune system. This theory posited that the innate immune system does not discriminate between self and nonself, but rather responds to “danger signals” that can be endogenous or exogenous in nature. Exogenous danger signals, often referred to as pathogen-associated molecular patterns (PAMPs), are highly conserved motifs in microbial pathogens, whereas endogenous danger signals, or danger-associated molecular patterns (DAMPs), are proteins, cytokines, chemokines, and other molecules from distressed and injured cells. Both PAMPs and DAMPs can activate the innate immune response through pattern recognition receptors (PRRs) on immune responsive cells, which activate different signaling cascades to ultimately trigger a wide array of responses against cell damage (2).

PRRs can be classified into 2 major groups based on their cellular location: 1) the transmembrane protein families—toll-like receptors (TLRs) and C-type lectin receptors; and 2) the cytoplasmic protein families—retinoic acid-inducible gene-like receptors, absent in melanoma 2-like receptors, cytosolic DNA receptors, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (3-9). Although innate immune response is orchestrated mainly by classic immune cells, including macrophages and dendritic cells, other nonprofessional cells

(e.g., endothelial cells, cardiomyocytes, and fibroblasts) also express these receptors and can actively contribute to immune response via PRR signaling (7,10).

Growing evidence supports the notion that innate immunity is closely related to the development of several CVDs, particularly when the heart responds to ischemia or mechanical stress (10-15). In this regard, there are extensive data that have documented a crucial role for some TLRs and NLRs, which are present on immune cardiac resident cells, cardiac fibroblasts and cardiomyocytes, in the

initiation, development, and maintenance of inflammatory response during heart failure (HF) or atherosclerosis (**Central Illustration**) (12,16-18). In this review, we summarize the key characteristics of TLRs and NLRs, with a particular focus of the role of TLRs and NLRs (nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing receptor [NLRP]3 and nucleotide-binding oligomerization domain-containing protein 1 [NOD1]) in the development of cardiovascular-related pathologies.

**TOLL-LIKE RECEPTORS**

The toll-like family of receptors is a key component of the innate immune system that constitute 13 different receptors that are evolutionarily conserved from plants to humans (19,20). The precise number of TLRs differs across mammalian species; 10 types have been described in humans, whereas TLR11 is not functional and TLR12 and TLR13 are not expressed (**Figure 1A**) (19). They are mainly present on immune cells, such as macrophages, dendritic cells, natural killer cells, and lymphocytes, but they are also found on nonimmune cells, including fibroblasts and even cardiovascular and nerve cells. The engagement of PAMPs and DAMPs with TLRs induces key molecular events that ultimately lead to innate immune responses and the development of antigen-specific acquired immunity (21,22).

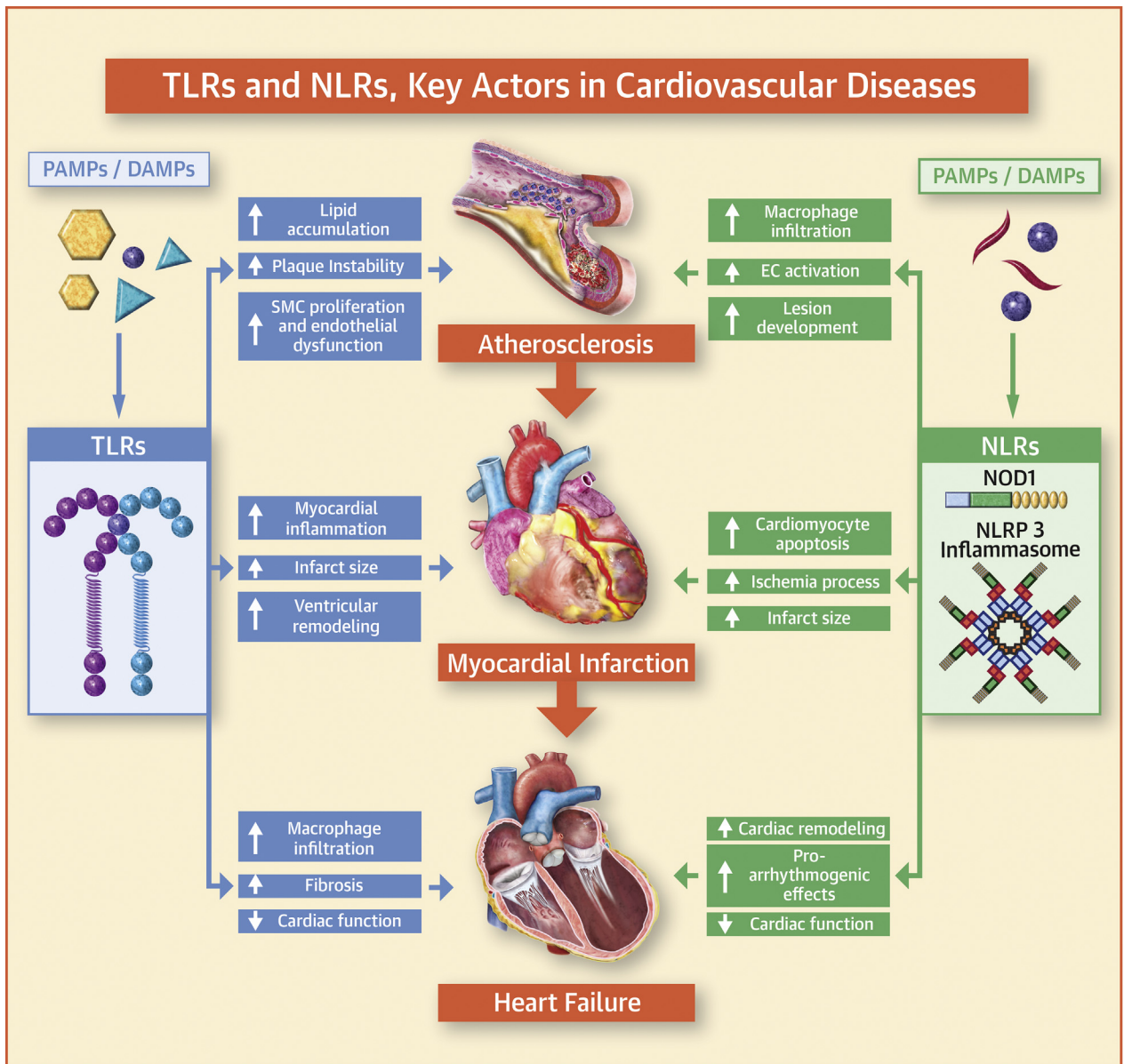
TLRs can be classified into 2 subfamilies based on their subcellular localization (23): plasma membrane TLRs include TLR1, TLR2, TLR4 to 6, and TLR10; and endosomal TLRs include TLR3 and TLR7 to 9. Cell surface TLRs mainly recognize microbial membrane components such as lipids, lipoproteins, and proteins, whereas intracellular TLRs recognize nucleic acids derived from bacteria and viruses, and also self-nucleic acids in disease (23). Key ligands of the TLRs are summarized in **Table 1**.

Each TLR is composed of an ectodomain with leucine-rich repeats that mediate PAMP recognition, a transmembrane domain, and a cytoplasmic toll/interleukin (IL)-1 receptor (TIR) domain that initiates downstream signaling (24,25). The interaction of TLRs with their cognate ligands triggers their

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**CENTRAL ILLUSTRATION** Innate Immune Receptors in Cardiovascular Diseases

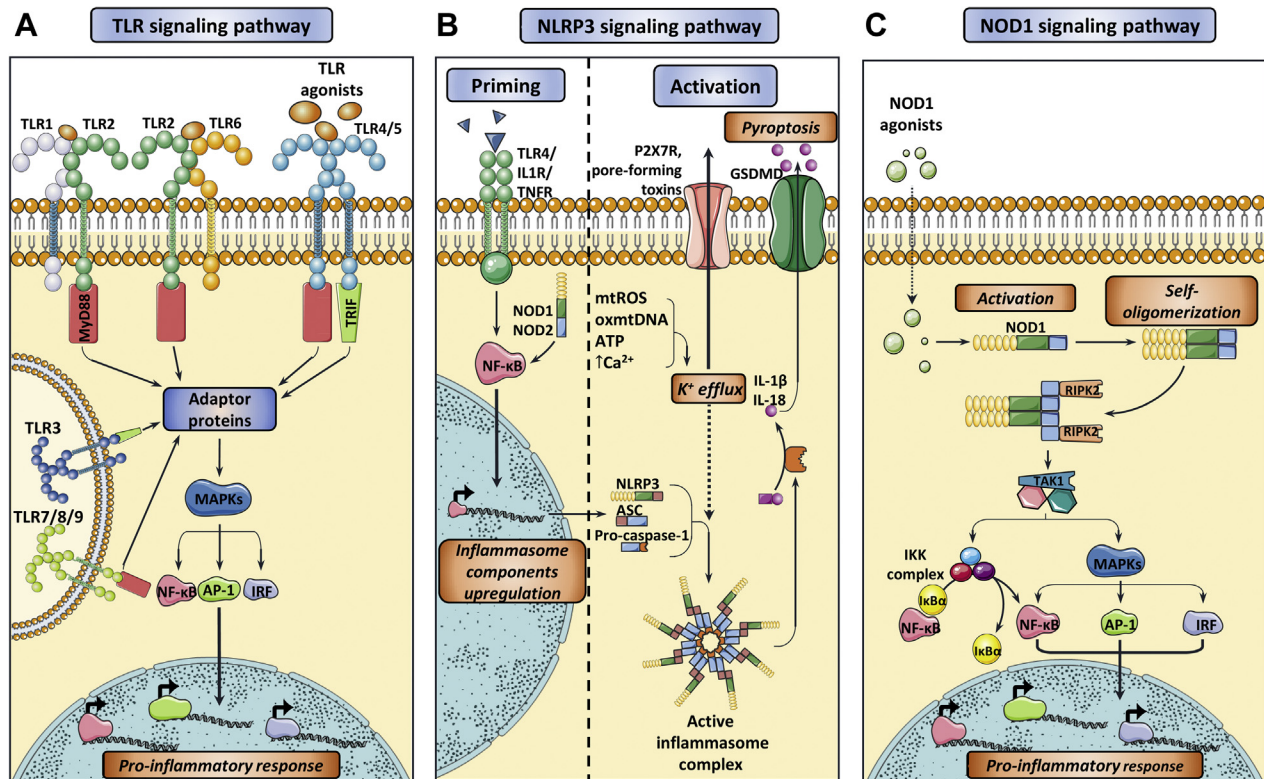


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Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) play a key role on the onset of cardiovascular diseases. This figure illustrates the potential detrimental action of the exacerbated activation of TLRs and NLRs in cardiovascular diseases, focusing on atherosclerosis, acute myocardial infarction, and heart failure as the most prevalent pathologies worldwide. DAMP = danger-associated molecular pattern; EC = excitation-contraction coupling; NLRP = nucleotide-binding oligomerization domain, leucine rich Repeat and pyrin domain-containing receptor; NOD 1 = nucleotide-binding oligomerization domain-containing protein 1; PAMP = pathogen-associated molecular pattern; SMC = smooth muscle cell.

dimerization. Subsequently, they can interact with several adaptor proteins, such as myeloid differentiation primary response protein 88 (MyD88) or TIR domain-containing adaptor protein-inducing

interferon (IFN)- $\beta$  (TRIF) (26), which leads to the downstream activation of mitogen-activated protein kinases (MAPKs) (27). The crucial endpoint of the cascade reaction is the activation of several

**FIGURE 1** Activation of the Main Innate Immune Receptors Involved in CVDs

**(A)** Toll-like receptor (TLR) signaling pathway. The interaction of TLR agonists with their corresponding TLRs induce a downstream signaling pathway that are mainly mediated by adaptor proteins that are capable of activating mitogen-activated protein kinase (MAPKs) and finally induce the nuclear translocation of the transcription factors nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B), Activator protein 1 (AP-1), and interferon regulatory factor (IRF), promoting the activation of pro-inflammatory molecules. Main effectors molecules upstream to this pathway are myeloid differentiation primary response protein 88 (MyD88) or TIR domain-containing adaptor protein inducing IFN- $\beta$  (TRIF). TLR4/TRIF binding is depicted to happen on the cell surface for simplicity reasons, although it is believed to occur in endosomes after receptor internalization. **(B)** Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing receptor 3 (NLRP3) inflammasome priming and activation signaling. Appropriate NLRP3 inflammasome activation requires an initial priming step triggered by several inflammatory cytokines, which, in turn, upregulates the transcription of the different inflammasome components. Next, a plethora of stimuli ultimately causing potassium ( $K^+$ ) efflux induce inflammasome assembly, which is then capable of activating interleukin (IL)-1 $\beta$  and IL-18 as well as gasdermin D (GSDMD), leading to pyroptosis and exacerbating the inflammatory response. **(C)** Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) signaling pathway. NOD1 agonists mainly consist of pathogen-associated molecular patterns (PAMPs) and/or danger-associated molecular patterns (DAMPs) that induce conformational changes on NOD1, leading to receptor self-oligomerization and recruitment of Receptor-interacting serine/threonine-protein kinase 2 (RIPK2). This adaptor protein then triggers a signaling cascade that subsequently activates NF- $\kappa$ B and MAPKs, eventually upregulating the transcription of pro-inflammatory genes. CVD = cardiovascular disease.

transcription factors, including nuclear factor- $\kappa$  light chain enhancer of activated B cells (NF- $\kappa$ B), activator protein-1 (AP-1), and interferon regulatory factors (IRFs). After their translocation into the nucleus, they activate multiple pro-inflammatory genes and enhance the levels of nitric oxide and interferons, which are directly toxic to invading micro-organisms (Figure 1). Moreover, TLR stimulation contributes to the maturation of dendritic cells, and, in some cases, induces the adaptive immune system.

Although PAMP recognition by TLRs is crucial for host defense responses to pathogen infection,

aberrant activation of TLR signaling by PAMPs and/or DAMPs and mutations in TLR signaling molecules are related to the development of several diseases (e.g., autoimmune, chronic inflammatory, and allergic diseases), as well as cancer and CVDs (28,29).

**ROLE OF TLRs IN THE PROGRESSION OF CVDs.** TLRs are expressed on most of the cells of the cardiovascular system, including endothelial cells, smooth muscle cells, and cardiomyocytes (30,31). Human heart tissue expresses all TLR isoforms, although the

**TABLE 1 Classification of the Identified Types of Toll-Like Receptors and Their Main Activators**

TLR	Main Ligands	Associated Pathogen
Location: plasma membrane		
TLR1	Triacylated Lipoproteins	Bacteria Mycobacteria
TLR2	Lipoproteins Zymosan Mannan Others	Bacteria Mycobacteria Fungi Protists Nematodes
TLR4	Lipopolysaccharide Heat-shock proteins Oligosaccharides	Bacteria Nematodes Viruses
TLR5	Flagellin	Bacteria
TLR6	Lipoproteins Zymosan	Bacteria Fungi
TLR10	Unknown	Unknown
Location: endosomes		
TLR3	dsRNA	Viruses
TLR7	ssRNA Synthetic compounds	Viruses
TLR8	ssRNA Synthetic compounds	Viruses
TLR9	Unmethylated CpG oligos	Viruses Bacteria

dsRNA = double-stranded RNA; ssRNA = single-stranded RNA; TLR = toll-like receptor.

relative mRNAs levels of TLR2, 3, and 4 are approximately 10-fold higher than other isoforms. Growing evidence indicates the involvement of innate immune activation mediated by myocardial TLRs in CVDs (25,32). Short-term activation of TLRs appears to have cytoprotective effects on the cardiovascular system, whereas prolonged or excessive activation of TLRs induces chronic low-grade inflammation, which leads to endothelial dysfunction, increased cell death, and adverse cardiac remodeling. In the following sections we discuss the role of the different TLRs in vascular and cardiac diseases.

**Involvement of TLRs in atherosclerosis and other vascular diseases.** Atherosclerosis is a chronic inflammatory disease that is triggered by accumulation of lipid particles (mainly oxidized low-density lipoproteins), endothelial cells, and macrophage-derived foam cells on the arterial wall, which creates an atheromatous plaque or atheroma (33). Atheromatous plaques may progress into unstable plaques, which are characterized by a highly inflammatory and necrotic core infiltrated with macrophages and lymphocytes and a thin fibrous cap that renders these plaques prone to rupture and destabilization.

Because of these processes, overall vascular homeostasis and hemodynamics are perturbed, which increases the risk of thrombosis and ultimately leads

to severe CVDs (e.g. coronary artery disease or acute myocardial infarction [AMI]) (34).

Because inflammation plays a crucial role in the atherogenic process (35), TLRs and their signaling cascades have emerged as important mediators in this pathology. Oxidized low-density lipoprotein has been found to be one of the most crucial TLR ligands during atherogenesis, influencing macrophage differentiation to foam cells and inducing the secretion of pro-inflammatory cytokines (36-38).

Most studies in the area of TLRs in atherosclerosis have focused on TLR4 and TLR2 because they are overexpressed in atheromas (39); certain atherosclerosis treatments like statins are able to reduce their expression (40,41). Knockdown of *Tlr4* or *Tlr2* in atherosclerosis-prone, apolipoprotein E-deficient (*Apoe*<sup>-/-</sup>) mice results in a reduction in foam cell accumulation and less severe atherosclerosis compared with control *Apoe*<sup>-/-</sup> mice (42,43). Similarly, genetic deletion of *Tlr4* has been found to reduce atherosclerotic lesions by 24% and macrophage infiltration by 65% (42). Stewart et al. (44) described that oxidized low-density lipoprotein could elicit inflammation through activation of a newly identified TLR4/TLR6 heterodimer by regulation of the CD36 scavenger receptor. Consequently, most results suggest that TLR4 is responsible for specific atherogenic processes, such as foam cell formation, lipid accumulation, plaque instability and rupture, and arterial remodeling, whereas TLR2 apparently does not contribute to atheroma destabilization and is involved in other processes (39).

The influence of TLR2 on atherosclerosis seems to be complex because it typically establishes a heterodimer with TLR1 or TLR6, and TLR2/1 and TLR2/6 heterodimers have been observed to be overexpressed in atheromas (39). Generally, TLR2 activation tends to stimulate atheroma development and thickening and to induce lipid accumulation (45), endothelial cell inflammation (39), and smooth muscle cell migration and proliferation (46,47). Accordingly, genetic deletion of *Tlr2* in atherosclerotic mice reduced the severity of disease and aggregation of foam cells in the aorta (48). *Tlr1*<sup>-/-</sup> and *Tlr6*<sup>-/-</sup> mice with atherosclerosis exhibited the same degree of disease severity as control atherosclerotic mice (49), which indicated that TLR2 might play a role in atherosclerosis as a heterodimer in contrast to TLR1 and TLR6, which do not seem to be atherogenic per se. In this context, it would be interesting to evaluate the consequences of the inhibition of both TLR1 and TLR6 to test whether a compensatory mechanism exists between the heterodimers.

Endosomal TLRs have also been associated with atherosclerosis, but published studies are contradictory or inconclusive as to whether they play protective or deleterious roles. For instance, TLR3 expression seems to be upregulated in atheromas, and its activation increases plaque size (50). However, *Tlr3*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice develop atherosclerosis earlier than *Tlr3*<sup>+/+</sup>*Apoe*<sup>-/-</sup> mice (24), and further stimulation of TLR3 in this model reduces the formation of scar tissue (51), which suggests a protective effect for this TLR. Likewise, TLR7 and TLR9 appear to play protective roles because lipid aggregation, macrophage infiltration and accumulation, and production of pro-inflammatory cytokines are exacerbated in both *Tlr7*<sup>-/-</sup>*Apoe*<sup>-/-</sup> and *Tlr9*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice (52-54). In contrast, other groups have reported that TLR7 or TLR9 blockade may have beneficial effects in other murine atherosclerosis models by reducing scar tissue, lipid accumulation, macrophage infiltration, and plaque instability (55-57).

Beyond atherosclerosis, TLRs have been associated with several vascular diseases, including hypertension (58) and abdominal aortic aneurysm (59). The involvement of TLRs in hypertension became evident from the observation that angiotensin II can activate TLR4 signaling, thus upregulating TLR4, myeloid differentiation primary response protein 88, and NF- $\kappa$ B (60). Several experimental models broadly described the contribution of TLR4 to hypertension progression (61,62). In addition to TLR4, TLR9 activation was found to increase blood pressure, leading to vascular alterations in normotensive rats (63) and triggering hypertension in pregnant rats (64). TLR9 inhibition was also found to reduce blood pressure in hypertensive rats (65).

TLR4 upregulation was also widely associated with abdominal aortic aneurysm; its inhibition prevented aortic dilation and aneurysm progression in several experimental models (59,66,67). Inhibition of TLR2 also ameliorated abdominal aortic aneurysm in mice (68), and its genetic deletion was found to prevent abdominal aortic aneurysm aggravated by periodontal bacterial infection (69). TLR3 also seems to be upregulated in aortic wall tissue during abdominal aortic aneurysm development; however, its specific involvement in this pathology remains unclear (70).

**ROLE OF TLRs IN CARDIAC DISEASES. Acute Myocardial Infarction.** Despite great advances in treatment strategies over the last several years, AMI, which is caused by a sudden obstruction of the coronary arteries from an acutely formed thrombus, remains a major cause of death and morbidity (71). Restoration of blood flow to ischemic myocardium is

associated with cardiac injury mainly due to the toxic effects of reactive oxygen intermediates that are generated during heart reperfusion (72). Although the cause of this injury is multifactorial, increasing experimental evidence suggests a pivotal role for the innate immune system in initiating the inflammatory cascade that leads to tissue injury. Thus, it has been described that in patients with unstable angina or with AMI, endogenous DAMPs (e.g., such as the heat shock proteins), high mobility group box 1 protein, and even genomic DNA or adenosine triphosphate (among other biomolecules) are released from damaged cardiac cells and signal through TLR receptors (73,74). In the same line, it has been reported that the increased presence of heat shock protein-60 on the surface of cardiomyocytes after ischemic heart failure can activate TLR4, leading to tumor necrosis factor- $\alpha$ -mediated apoptosis and increasing cardiac injury (75,76). Similarly, TLRs activated during myocardial ischemia and/or reperfusion (I/R) injury induce rapid NF- $\kappa$ B activation with the subsequent release of inflammatory cytokines, which mediate left ventricular dysfunction after injury (77).

Recent studies have revealed that TLR2 or TLR4 deficiency attenuates myocardial inflammation, reduces infarct size, and preserves ventricular function after transient ischemic injury in mice. This is supported by the findings that TLR2 (78) and TLR4 (79,80) antagonists have demonstrated efficacy in reducing NF- $\kappa$ B activation and infarct size and also improve cardiac function in mouse models of AMI. Using an AMI model of coronary artery ligation, Shishido et al. (81) reported that *Tlr2*<sup>-/-</sup> mice presented a significant reduction in mortality, left ventricular dysfunction and less ventricular remodeling compared with wild-type mice. These data indicate that in the context of cardiac ischemic injury, loss of cardiac TLR2 signaling is beneficial for cardiac function. Using a similar model, Timmers et al. (82) reported that *Tlr4*-defective mice showed reduced left ventricular remodeling and preserved left ventricular function after myocardial infarction. Other TLRs have been analyzed in this context. For example, TLR3-TIR domain-containing adaptor protein inducing IFN- $\beta$  signaling was found to represent an injurious pathway during cardiac I/R (83). TLR9 appeared to play a protective role against cardiac rupture in the early phase after AMI, but its stimulation at onset of ischemia did not alter infarct size (84).

Despite these results, it has been also reported in animal models that activation of TLR2, 4, and 9 before I/R can lead to reduced infarct size and improved cardiac function through pre-conditioning mechanisms (85). These protective effects are

**TABLE 2 Classification of the Identified Types of NOD-Like Receptors (NLRs) and Their Main Activators**

NLR	Main Activators
<b>NLR A</b>	
CIITA	IFN- $\gamma$
<b>NLR B</b>	
NAIP1/2	Injectisome
NAIP5/6	Flagellin
<b>NLR C</b>	
NOD1 (NLRC1)	Peptidoglycan iE-DAP
NOD2 (NLRC2)	Peptidoglycan Muramyl dipeptide ssRNA
NLRC3	Undetermined
IPAF (NLRC4)	Injectisome/flagellin (mediated by NAIPs)
NLRCS	IFN $\gamma$ LPS Poly I:C
NLRX1	LPS Viral RNA
<b>NLRP</b>	
NLRP1	Anthrax lethal toxin Toxoplasma gondii ATP depletion Muramyl dipeptide
NLRP2	LPS/TNF- $\alpha$
NLRP3	K <sup>+</sup> efflux Bacterial toxins and RNA Nigericin Aluminum hydroxide ATP Cholesterol and uric acid crystals Particulate matter Mitochondrial damage
NLRP4	Viral DNA/RNA
NLRP5/8/13/14	Undetermined
NLRP6	Viral RNA TLR ligands Lipotheicoic acid
NLRP7	Bacterial acylated lipoproteins Lysosomal proteases? K <sup>+</sup> flux?
NLRP9	dsRNA
NLRP10	NOD1 activation
NLRP11	TLR4 ligands
NLRP12	ATP TLR2 ligands Yersinia pestis

ATP = adenosine triphosphate; CIITA = major histocompatibility complex class II transactivator; iE-DAP =  $\gamma$ -D-glutamyl-meso-diaminopimelic acid; IFN = interferon; K = potassium; IPAF = interleukin-1 $\beta$  (IL-1 $\beta$ )-converting enzyme-protease-activating factor; LPS = lipopolysaccharide; NLR = nucleotide-binding oligomerization domain-like receptors; NAIP = Baculoviral IAP repeat-containing protein 1; NLRP = nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing receptor; NOD1 = Nucleotide-binding oligomerization domain-containing protein 1; TNF = tumor necrosis factor; other abbreviations as in Table 1.

mediated mainly by the phosphoinositide 3-kinase/protein kinase B pathway (PI3K/PKB), which prevents apoptotic signaling in cardiomyocytes and reduces the NF- $\kappa$ B inflammatory cardiac response (86). These results thus support an alternative therapy in AMI through a pre-conditioning approach.

**Heart Failure.** In many cases, patients with AMI ultimately develop HF, which is a complex clinical syndrome that occurs because of structural or functional impairment of ventricular filling or ejection of blood in the heart, and a failure to pump blood efficiently to meet the body's needs. HF is a leading cause of morbidity and mortality worldwide and increasingly affects millions of people (87).

Cardiac inflammation plays a critical role in the development of HF, and TLRs are actively involved in this process. TLR2 has been described to be upregulated in murine HF models, both in cardiomyocytes and in vascular endothelial cells, and HF progression in significantly reduced in *Tlr2*<sup>-/-</sup> mice (81). Inhibitory therapy using anti-TLR2 antibodies has been recently found to block angiotensin II-induced cardiac fibrosis by suppressing macrophage recruitment and inflammation in the heart (88). TLR4 is also upregulated in patients with chronic HF and induces a robust production of pro-inflammatory cytokines that favors the progression of myocardial dysfunction and fibrosis (89,90). Pharmacological blockade of TLR4 using different molecules has been found to attenuate myocardial I/R injury and the development of cardiac hypertrophy (79,91). There is also emerging evidence for the involvement of TLR9 in HF, which can be activated by endogenous DAMPs, including mitochondrial DNA, to modulate the progression of the disease (92). It has been reported that plasma levels of mitochondrial DNA are elevated in patients with HF and are associated with increased mortality (92).

Related to the role of TLRs in the modulation of cardiac function, chronic stimulation of TLRs can lead to cardiac dysfunction, at least in part through the modulation of cardiac ion channel activity. It is well known that cardiac function and calcium ion (Ca<sup>2+</sup>) homeostasis are closely coupled in the heart (93). Cardiac contraction is tightly regulated by changes in intracellular Ca<sup>2+</sup> levels, and myocyte mishandling of this ion is a central cause of both cardiac dysfunction and arrhythmias in the pathophysiological mechanisms of CVD. In this context, Ca<sup>2+</sup> is a key mediator of cardiac excitation-contraction coupling, the process from electrical excitation of the myocyte to contraction of the heart that is initiated by an action potential in cardiomyocytes (93). Specifically, the initial depolarization phase of the action potential activates sarcolemma L-type Ca<sup>2+</sup> channels, firing Ca<sup>2+</sup> entry into the cytosol and triggering a large release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR) by ryanodine receptors, which allows cell contraction. Relaxation is achieved by Ca<sup>2+</sup> removal from the

cytosol by SR-Ca<sup>2+</sup> adenosine triphosphatase 2a and the sodium–calcium exchanger (93).

In relation to these processes, the direct effect of TLR2 activation by heat shock protein-70 on contractile performance has been confirmed in vitro using a murine cardiomyocyte cell line (94). Likewise, cardiac TLR4 activation by lipopolysaccharide has been shown to increase both the Ca<sup>2+</sup> efflux via the sodium–calcium exchanger and action potential duration, which leads to arrhythmogenic events (95,96). In addition, the inflammatory cytokine high-mobility group box 1 protein can induce TLR4-dependent activation of nicotinamide adenine dinucleotide phosphate oxidase, which leads to reactive oxygen species (ROS) overproduction and oxidative stress (97,98). Importantly, oxidative stress activates ryanodine receptor type 2 during diastole, impairing both cardiac contraction and function. In contrast, TLR4 inhibition appears to prevent the occurrence of these abnormal events, restoring the contractile capacity of the heart and improving cardiac function (99). It has also been reported that *Tlr4*<sup>-/-</sup> mice are protected against lethal anthrax toxin–induced cardiac dysfunction and suppressed intracellular Ca<sup>2+</sup> mishandling (100,101).

#### NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN-LIKE RECEPTORS

The NLRs are modular cytosolic sensors of intracellular DAMPS and PAMPs and can be divided into 4 subfamilies based on their N-terminal domain configuration: NLRA, NLRB/ NAIP, NLRC, and NLRP. A conserved tripartite structure is common to the human NLR family, consisting of an amino-terminal, effector-binding domain, a central domain, and a carboxy-terminal, ligand-recognition domain, which mediates intracellular ligand sensing (102). The central NOD regulates its self-oligomerization, which is crucial for downstream effector activation. The amino-terminal effector-binding domain mediates binding to effector molecules, which determines the final activated signaling pathway and varies depending on whether the protein interaction is homo- or heterophilic. In this sense, the caspase-recruitment domain (CARD) and the pyrin domain mediate homophilic protein–protein interactions, which are associated with death effectors. Other NOD proteins have a heterophilic amino-terminal effector-binding domain, such as the neuronal apoptosis inhibitory protein or the major histocompatibility complex class II transactivator (103,104). The main activators of the different types of NLRs are listed in [Table 2](#).

NLRs regulate a broad range of molecular functions, including NF-κB signaling, retinoic acid–inducible gene-I–like receptor signaling, autophagy, major histocompatibility complex gene regulation, reproduction, and development (105). Several reports related to some NLRs, especially NLRP3 and NOD1, have been recently published and are discussed in the following sections.

**NLRP RECEPTORS.** NLRP receptors have recently garnered attention due to their association with inflammasomes. Inflammasomes are macroprotein complexes that mediate the inflammatory response upon DAMP and PAMP signaling, by processing and activating pro-caspase-1 to caspase-1. Caspase-1 further activates a series of pro-inflammatory cytokines, such as IL-1β, IL-18, and high-mobility group box 1 protein, unleashing a potent inflammatory reaction (106,107). In addition, recent data have shown that caspase-1 can also elicit pyroptosis, a specific pro-inflammatory type of cell death mediated by gasdermin D (108). Once cleaved by the inflammasome complex, gasdermin D is capable of forming nonselective large oligomeric pores within the cell membrane that induce cell death and the release of intracellular content, particularly IL-1β, which further enhances the inflammatory reaction ([Figure 1B](#)) (106,108,109).

Assembly of the inflammasome involves the oligomerization of 3 essential proteins: a sensor molecule consisting of a PRR that recognizes a specific signal; an apoptosis-associated, speck-like protein containing CARD that serves as a scaffold; and pro-caspase-1 (106,107). Thus far, 5 types of PRRs have been described to establish inflammasomes: NLRP1, NLRP3, NLRC1/NOD1, pyrin and absent in melanoma 2 (106). Other PRRs, including IFI16, NLRP6, NLRP7, NLRP12, and NLRP14, are also able to form inflammasomes; however, their structures are yet to be determined. Of these PRRs, research has mainly focused on NLRP3 due to its association with several diseases including Alzheimer's, asthma, rheumatoid arthritis, and CVDs (107).

**NLRP3 inflammasome.** Activation of the NLRP3 inflammasome has been described to occur in a 2-step process—an initial priming step followed by an activation step. During priming, PAMPs and endogenous cytokines signal through specific receptors and activate the NF-κB pathway, ultimately inducing expression of inflammasome components (NLRP3, an apoptosis-associated speck-like protein containing CARD, and pro-caspase-1) and targets (pro-IL-1β and pro-IL-18) (106). Priming also entails different post-translational modifications on NLRP3, including



deubiquitination (110) and phosphorylation (111), which trigger conformational changes that favor the formation of the inflammasome.

The activation step involves the assembly of the complex and activation of pro-caspase-1. Signaling is initiated by cellular indicators of instability and damage, including cation mobilization (potassium and  $\text{Ca}^{2+}$  efflux), adenosine triphosphate, pore-forming toxins, mitochondrial dysfunction, lysosomal rupture, and particulate matter (108). This plethora of stimuli ultimately induce potassium efflux, which is believed to be the pivotal step for NLRP3 inflammasome activation (Figure 1B) (112). New modulators of the activation step are still being discovered, such as NEK7, a never in mitosis A-related serine/threonine kinase that acts as a positive regulator of the NLRP3 inflammasome (113,114). NEK7 deficiency reduces IL-1 $\beta$  production and disease severity in murine models (106,114).

**Role of NLRP3 inflammasome in CVDs.** The first evidence of the involvement of the NLRP3 inflammasome in CVDs was described by Duewell et al. (115), who observed that cholesterol crystals can activate this complex. This discovery complemented previous evidence of the role of both IL-1 $\beta$  and IL-18 in cardiac inflammation (116-118) and fibrosis (119,120). Accordingly, the NLRP3 inflammasome and its targets represents one of the major mediators of the deleterious immune response responsible in part for many CVDs. They have been linked to hypertension (121), HF (122,123), atrial fibrillation (124), AMI (125), diabetes (126), and vascular diseases, including atherosclerosis (127).

**Involvement of NLRP3 in atherosclerosis and vascular diseases.** Great progress has been made in characterizing the involvement of NLRP3 in atherosclerosis because pro-atherogenic components such as free fatty acids, oxidized low-density lipoprotein, and cholesterol crystals can elicit NLRP3 inflammasome assembly (115,128). In particular, cholesterol crystals cannot be completely phagocytosed by macrophages, which causes lysosome destabilization and cathepsin B release, which, in turn, trigger inflammasome activation (115). In the same line, atherosclerosis is characterized by an increase in ROS, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction, all of which also contribute to NLRP3 inflammasome priming and activation (129).

The crucial role played by the NLRP3 inflammasome in atherosclerosis has been demonstrated with regard to both its components and its targets. For example, NLRP3, an apoptosis-associated speck-like protein containing CARD (Asc), caspase-1, IL-1 $\beta$ , and IL-18 levels have all been found to be elevated in the

plaques of patients with atherosclerosis (130,131), particularly in unstable plaques (132). This increase has been observed both in foam cells and in endothelial cells (132). In addition, NLRP3 appears to be upregulated in the aorta of patients with atherosclerosis (133). However, studies performed in atherosclerosis-prone mice yielded conflicting results as to the participation of the NLRP3 inflammasome. Although some studies reported that the deletion of *Nlrp3*, *Asc*, *Casp1*, or *Il1a/b* attenuated lesion development (116,134), diminished macrophage infiltration (135), and decreased the severity of the disease (136), another study failed to observe any significant change in atherosclerosis development or macrophage infiltration upon deletion of any of these genes (137). These findings seem to be highly dependent on the animal model chosen (*Ldlr*<sup>-/-</sup> or *Apoe*<sup>-/-</sup>) and the content of the high-fat diet used; thus, more research is needed to consider the implication of these 2 variables on inflammasome activation and atherogenesis. In the same line, studies with statins—a common medication for atherosclerosis and other CVDs—exhibited contradictory evidence in relation to NLRP3 function, with some studies describing inhibition (138,139), activation (140,141), or lack of effect (139) on the activation of the inflammasome complex and downstream cytokine production.

Regarding the pathogenesis of aortic aneurysm, aortas from patients showed increased *Nlrp3* and *Il1b* expression levels (142). Inhibition of this cytokine or its receptor in a murine model resulted in a preserved aorta structure and reduced inflammation (143). Interestingly, Erthart et al. (144) recently reported that patients with a more severe form of aortic aneurysm show attenuated inflammasome expression, possibly due to a change in the immune response as the disease progressed.

**NLRP3 inflammasome in cardiac diseases. Acute myocardial infarction.** Studies in models of myocardial I/R injury revealed that NLRP3, caspase-1 activity, IL-1 $\beta$ , and IL-18 were all upregulated in the ischemic heart (145). Moreover, knocking out or inhibiting NLRP3 reduced infarct size and the overall I/R injury (145-147), decreased macrophage and neutrophil infiltration (145), and diminished cardiac fibrosis and left ventricular dysfunction (146). Similar results were obtained in mice lacking *Asc* or *Casp1*, which was accompanied by a decrease in IL-1 $\beta$  levels (148). The activation of NLRP3 was proven to occur in both cardiac cell types (cardiomyocytes and cardiac fibroblasts) and in infiltrating cells (mainly macrophages and neutrophils) (148,149). Cardiomyocytes do not seem to produce consistently high levels of IL-1 $\beta$ , yet they are capable of cleaving

caspase-1 and inducing pyroptosis, indicating that cardiac fibroblasts might be the chief cardiac cell population involved in inflammasome activation in the context of myocardial infarction and subsequent cardiac remodeling (148).

Toldo et al. (150) hypothesized that mitochondrial damage generated during the ischemic episode served as the priming signal for inflammasome, whereas the increase in ROS during the subsequent reperfusion provided the activating signal. In this line, several studies that treated I/R injury with antioxidant compounds before or at reperfusion reported a reduction in infarct size, in NLRP3 levels, in active caspase-1, and in IL-1 $\beta$ , which supported the link between ROS production and inflammasome activation in AMI (151-153).

Based on the results of these pre-clinical studies, 2 clinical trials were designed to investigate targeting the NLRP3 inflammatory cascade in patients with AMI: 1) a trial on anakinra, which is a recombinant IL-1 $\beta$  receptor antagonist; and 2) a trial on canakinumab, which is a monoclonal antibody targeting IL-1 $\beta$ . Both trials are in phase II and phase III, respectively (116,154). Both trials reported a decrease in cardiovascular risk with treatment; however, further research is needed to determine effective doses and to ascertain and reduce the associated side effects (116,154).

**HEART FAILURE.** Research on the involvement of the NLRP3 inflammasome in HF is more scarce, with only a few studies describing an increase in both IL-1 $\beta$  (155,156) and IL-18 (157) in heart and plasma from patients with HF. Mallat et al. (157) also identified IL-18 expression in cardiomyocytes, macrophages, and endothelial cells in these patients. In the same line, Toldo et al. (158) demonstrated that inflammasomes were highly formed in leukocytes, cardiomyocytes, fibroblasts, and endothelial cells in heart biopsies from patients with acute myocarditis, especially in those with severe HF.

There is a general consensus that the NLRP3 inflammasome, mainly via the activation and release of IL-1 $\beta$  and IL-18, is strongly implicated in the adverse cardiac remodeling processes that provoke cardiac dysfunction and subsequent HF (159). Accordingly, the available evidence points to the inflammasome complex as a new potential target to treat CVDs and opens a door to the discovery of new mechanisms that regulate cardiac function.

As previously mentioned, both cardiac function and Ca<sup>2+</sup> homeostasis are closely coupled in the heart. In addition, Ca<sup>2+</sup> signaling has been suggested to be a key regulator in NLRP3 inflammasome activation (160). Administration of activators of the Ca<sup>2+</sup>-

sensing receptor, a member of the 7-transmembrane receptor super family (161) implicated in Ca<sup>2+</sup> release from ER stores and thus involved in NLRP3 activation, activates the phospholipase C signaling pathway in mouse bone marrow-derived macrophages. This leads to inositol phosphate accumulation and Ca<sup>2+</sup> release from ER stores, which increases intracellular Ca<sup>2+</sup> that activates NLRP3 (162). Also, the administration of a Ca<sup>2+</sup>-sensing receptor activator has been shown to induce myocardial fibrosis in Wistar rats (163). Supporting the notion that Ca<sup>2+</sup> signaling is involved in inflammasome activation, the constitutive activation of the NLRP3 inflammasome in cardiomyocytes enhances the expression of RyR2, which leads to increased pro-arrhythmogenic SR Ca<sup>2+</sup> release events (124). Conversely, genetic deletion of *Nlrp3* reduces the incidence of atrial fibrillation episodes (164).

**NUCLEOTIDE-BINDING OLIGOMERIZATION CONTAINING PROTEIN 1.** In the early 2000s, 2 NOD-containing molecules, NOD1 and NOD2, were discovered by a database search for a homologue for the apoptosis regulator Apaf-1 (165-167). Since then, the NOD family has widened; currently, it consists of >20 human proteins, together with a large number of proteins from animals, plants, bacteria, and fungi (167).

As alluded to earlier, *Nod1* encodes an intracellular scaffolding protein that consists of CARD, NOD, and leucine-rich repeat domains (Figure 1C). NOD1 exists as an inactive monomer in cytosol, and, upon ligand recognition, it undergoes a conformational change that promotes its activation. Once activated, NOD1 self-oligomerizes and recruits receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through homotypic CARD-CARD interactions (168). Receptor-interacting serine/threonine-protein kinase 2 subsequently mediates the recruitment and activation of the serine/threonine kinase TAK1 that, in turn, activates the I $\kappa$ B kinase complex and the MAPK pathway. I $\kappa$ B kinase then phosphorylates the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , which releases NF- $\kappa$ B, allowing it to translocate to the nucleus and modulate the expression of downstream target genes (Figure 1C) (169).

NOD1 mainly detects D-glutamyl-meso-diaminopimelic acid (DAP), which is a dipeptide present in peptidoglycan primarily found in Gram-negative bacteria, but is also found in specific groups of Gram-positive bacteria. However, NOD1 signal transduction can also be stimulated in the absence of direct cellular infection by a bacterial pathogen. Keestra-Gounder et al. (170) recently demonstrated that pro-inflammatory responses induced by ER stress are mediated through the NOD1/NOD2

pathway, which suggests a potential role for NOD1 in inflammatory diseases associated with this perturbation. The link between NOD1 and ER stress appears to be the unfolded protein response, as inhibiting IRE1 $\alpha$ , a kinase implicated in this pathway, attenuates the NOD1-associated inflammatory reaction (170). Some studies have associated ER stress with an imbalance in cellular Ca<sup>2+</sup> and the activation of NOD1 signaling (171,172). A recent study revealed that NOD1 activation both by bacterial pathogens and the NOD ligand C12-iE-DAP induced unfolded protein response activation through the ER kinase PERK, as well as Ca<sup>2+</sup> flux from the ER membrane Ca<sup>2+</sup> channel IP3R, which exacerbated the inflammatory response via NOD1 signaling (171). In the same line, Molinaro et al. (172) demonstrated that the increase in intracellular Ca<sup>2+</sup> concentration in intestinal epithelial cells triggered NOD-dependent inflammatory signaling, including NF- $\kappa$ B. All these data supported a link between the activation of NOD1 and the perturbation of the homeostasis of cellular Ca<sup>2+</sup>, which suggested that the NOD1 inflammatory pathway could be responsible for several cardiac pathologies, in part through Ca<sup>2+</sup> signaling.

**Role of NOD1 in atherosclerosis and other vascular diseases.** The endothelium represents the first barrier against blood-borne bacterial PAMPs and is therefore an important component of the innate immune system response to pathogens. In this regard, Moreno et al. (173) found that NOD1 is selectively expressed in vascular smooth muscle and its activation triggers the expression of the inflammatory mediators NOS2 and COX2. Similarly, specific NOD1 ligands can activate inflammatory responses in intact human vessels in vitro (174) and induce a significant number of pro-inflammatory responses in endothelial cells, which points to NOD1 as a crucial partner in vascular diseases (174).

The migration and proliferation of vascular smooth muscle cells that occurs in atherosclerosis contributes to vascular inflammation through the release of pro-inflammatory cytokines. The pathogenesis of diabetes mellitus accelerates these atherosclerotic vascular events, which have been related to NOD-mediated innate immunity signaling pathways. Human aortic vascular smooth muscle cells treated with insulin or iE-DAP showed a significant increase in Nod1 expression, together with increased levels of IL-8 and IL-1 $\beta$  (175).

In relation to the role of NOD1 in atherosclerosis, Kanno et al. (176) demonstrated, for the first time, that long-term oral administration of a synthetic NOD1 ligand accelerated the development and progression of atherosclerosis in *Apoe*<sup>-/-</sup> mice. In addition, the

complete loss of NOD1 significantly decreased the size of atherosclerotic lesions, providing robust evidence of a relationship between NOD1 and atherosclerosis progression (176). Also, NOD1 expression was increased in endothelial cells of human and mouse atheromatous plaques, and the endothelial expression of the adhesion molecule VCAM-1 was reduced in atheroma lesions in *Nod1*<sup>-/-</sup> *Apoe*<sup>-/-</sup> mice compared with *Apoe*<sup>-/-</sup> mice (14). Furthermore, treatment of endothelial cells from *Apoe*<sup>-/-</sup> mice with a pharmacological inhibitor of NOD1 prevented the increased expression of VCAM-1 (17), which suggested a crucial role of NOD1 in the onset of atherosclerosis.

**NOD1 involvement in cardiac diseases: acute myocardial infarction and heart Failure.**

NOD1 has been associated with several cardiovascular-related diseases, such as HF (16) and diabetic cardiomyopathy (177). Recently, Yang et al. (178) demonstrated that activation of NOD1 with DAP (a synthetic activator of NOD1) significantly aggravated cardiac I/R injury in mice and enhanced cardiomyocyte apoptosis and inflammation. In a similar line, Delgado et al. (179) demonstrated that NOD1 activation in mice promoted a significant decrease in cardiac function. NOD1 is functional in cardiomyocytes, and isolated cardiomyocytes from iE-DAP-treated mice showed diminished I<sub>CaL</sub> density, depressed Ca<sup>2+</sup> transients, and a slower time decay of intracellular Ca<sup>2+</sup> transients, which correlated with the reduced expression of SR-Ca<sup>2+</sup> adenosine triphosphatase 2a (179), features that are common in CVDs. In addition, systolic Ca<sup>2+</sup> mishandling was accompanied by diastolic release events, such as increasing Ca<sup>2+</sup> sparks frequency and occurrence of Ca<sup>2+</sup> waves. Supporting these results, iE-DAP treatment of *Nod1*<sup>-/-</sup> mice failed to modify the cardiac excitation–contraction coupling parameters (179).

*Nod1* genetic deletion or pharmacological inhibition also prevents Ca<sup>2+</sup> mishandling associated with HF in ventricular cells. In this context, Val-Blasco et al. (16) demonstrated that *Nod1* deletion in a murine HF model improved systolic Ca<sup>2+</sup> release and SR-Ca<sup>2+</sup> load and prevented the occurrence of pro-arrhythmic events. Moreover, *Nod1* deletion also prevented the  $\beta$ -adrenergic response in failing cardiac murine cardiomyocytes (180).

In light of these studies, NOD1 emerges as an important regulator of Ca<sup>2+</sup> dynamics in cardiac excitation–contraction coupling and can be the starting point for a new research line for Ca<sup>2+</sup> handling impairment in specific CVDs, such as dilated cardiomyopathy or I/R injury, where NLR activation has already been described (178,179).

## CONCLUSIONS

The present review summarizes relevant findings related to the role of the classic and the more recently discovered PRRs in the progression of the most prevalent cardiac and vascular diseases. In this regard, both TLRs and NLRs are clearly involved in the progression of some CVD pathologies, such as atherosclerosis, AMI, or HF. All or most of these actions are mediated by immune cells, but also by cardiomyocytes, fibroblasts, as well as endothelial and vascular cells, pointing to cardiovascular cells as new targets for TLR and NLR actions. The sustained activation of TLR2/4, NLRP3, or NOD1 are widely related to cardiac remodeling because they contribute to promotion of the progression of pathological processes, mainly by the induction of pro-inflammatory

mediators. In contrast, the specific inhibition or the genetic deletion of these PRRs prevents cardiovascular damage in most CVDs. Accordingly, the development of new specific strategies to impair exacerbated PRR activation in these pathologies arise as strong candidates for therapy and opens a new field in CVD research.

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**KEY WORDS** cardiovascular disease, innate immune system, NLRP3, NOD1, nucleotide-binding oligomerization domain-like receptors, toll-like receptors

