This Review is the first in a new thematic series on NF-κB Signaling in the Cardiovascular System, which includes the following articles:

Introduction: Multiple Facets of NF-κB in the Heart: To Be or Not to NF-κB [Circ Res. 2011;108:1122–1132]

The Emerging Role of Innate Immunity in the Heart and Vascular System: For Whom the Cell Tolls

TNF and NF-κB Signaling in Cardiac Remodeling and Failure

Lorrie Kirschenbaum, Guest Editor

The Emerging Role of Innate Immunity in the Heart and Vascular System
For Whom the Cell Tolls

Douglas L. Mann

Abstract: Recent studies suggest that the heart possesses an innate immune system that is intended to delimit tissue injury, as well as orchestrate homoeostatic responses, within the heart. The extant literature suggests that this intrinsic stress response system is mediated, at least in part, by a family of pattern recognition receptors, most notably the Toll-like receptors. Although the innate immune system provides a short-term adaptive response to tissue injury, the beneficial effects of this phylogenetically ancient system may be lost if innate immune signaling becomes sustained and/or excessive; in which case, the salutary effects of activation of these pathways are contravened by the known deleterious effects of inflammatory signaling. Herein, the biology of innate immune signaling in the heart is reviewed, as well as the literature suggesting that the innate immune system is involved in the pathogenesis of atherosclerosis, acute coronary syndromes, stroke, viral myocarditis, sepsis, ischemia/reperfusion injury, and heart failure. The review concludes by discussing new therapies that are being developed to modulate the innate immune system. (Circ Res. 2011;108:1133-1145.)

Key Words: innate immunity ■ homeostasis ■ heart failure

The adult heart responds to tissue injury by synthesizing an ensemble of proteins that promote homeostasis, either by activating mechanisms that facilitate tissue repair or, alternatively, by upregulating mechanisms that confer cytoprotective responses within the heart. The extant literature suggests that proinflammatory cytokines serve as the downstream “effectors” of the innate immune system by facilitating tissue repair within the heart. What has been less well understood, until recently, is how these myocardial innate immune responses are coordinated following tissue injury. The relatively recent discovery of a family of receptors termed Toll-like receptors (TLRs) and NOD-like receptors (NLRs) has greatly increased our understanding of the “upstream” molecular components that regulate the innate immune response.1 Moreover, recent studies have also identified an important role for the complement system an essential component of the innate immune system in the heart.2 As will be discussed in the following focused review in this series, recent insights into the role of TLRs have provided important new insights with respect to our understanding of the role inflammation in health and disease.

TLRs serve as pattern recognition receptors that recognize conserved motifs on pathogens, so called pathogen-associated molecular patterns. Typical examples of pathogen-associated molecular patterns include the lipopolysaccharides (LPSs) of Gram-negative organisms, the teichoic acids of Gram-
positive organisms, the glycolipids of mycobacterium, the zymosans of yeast, and the double-stranded RNAs of viruses (see Figure 1). These pathogen-associated molecular patterns are unique to these pathogens and, in some cases, are required for their virulence. Thus, among the quintessential features of the innate immune system is that it serves as an “early warning system” that enables the host to accurately and rapidly discriminate self from nonself. More recently, it has become clear that TLRs also recognize molecular patterns of endogenous host material that is released during cellular injury or death, so-called damage-associated molecular patterns (DAMPS).\(^3,4\) As shown in Figure 1, DAMPs can be derived from dying or injured cells, damaged extracellular matrix proteins, or circulating oxidized proteins. This latter observation has provided a potentially important link between tissue injury, activation of proinflammatory mediators, and the resulting myocardial response to stress. The similarity of the biological response following TLR activation secondary to tissue injury and bacterial/viral infection likely represents a phylogenetically conserved host response, insofar as infection and tissue injury commonly occur together. Nonetheless, although the brisk and sometimes overwhelming inflammatory response at the time of tissue injury may have been the most effective and efficient means for the host to clear microorganisms at the site of tissue injury, it often leads to excessive and/or unwanted tissue damage. The explanation for why evolutionary pressure would select and/or retain an “early warning” system that contains both adaptive and maladaptive components is not known, but may relate to the fact that the innate immune system developed in organisms that reproduced early and had relatively short life spans (ie, weeks to months). Thus, the long-term detrimental effects of innate immune activation were not subjected to evolutionary selection pressure because they did not present a problem for hosts with short life spans.

### Expression and Regulation of Toll-Like Receptors in the Adult Mammalian Heart

Although the Toll receptor was originally discovered as a protein that was responsible for dorsal–ventral polarity in the fly, the subsequent pioneering work of Ruslan Medzhitov and Charles Janeway demonstrated that a human homolog of the *Drosophila* toll protein activated both NF-κB and NF-κB–dependent genes in mammalian cells.\(^3\) At the time of this writing, 13 mammalian TLR paralogs have been identified, of which 10 functional TLRs have been identified in humans (functional TLRs 11 to 13 are only expressed in mice). TLRs 1 to 6 are expressed on the cell surface of mammalian cells, whereas TLRs 3, 7, and 9 are expressed in intracellular compartments, primarily endosomes and the endoplasmic reticulum, with the ligand binding domains facing the lumen of the vesicle. TLR10 is the most recent member of the human TLR receptor family discovered; however, its function and direct ligand are still unknown. Humans also encode a TLR11 gene, but it contains several stop codons and the protein is not expressed.

mRNA for TLRs 1 to 10 has been identified in the human heart.\(^6\) The relative expression levels for TLR mRNA in the human heart is: TLR4>TLR2>TLR3>TLR5>TLR10>TLR6>TLR7>TLR8>TLR9. Of note, the relative expression levels of mRNA for TLRs 2, 3, 4 is approximately 10-fold higher than TLRs 1, 5 to 10.\(^6\) Although expression levels of TLRs have not been identified in human myocytes, TLR2, -3, -4, -6 mRNA has been identified in cardiac myocytes from...
neonatal rats. At the time of this writing, very little nothing is known with regard to the regulation of TLR expression within the heart, although TLR4 appears to be upregulated in the failing human heart.

Toll-Like Receptor Signaling Pathways

As shown in Figure 2A, TLRs are type 1 membrane-spanning receptors that have a leucine-rich repeat extracellular motif and an intracellular signaling motif that is similar to interleukin (IL)-1. With the exception of TLR3, all TLRs interact with an adaptor protein termed MyD88 (myeloid differentiation factor 88) via their Toll interleukin receptor (TIR) domains (Figure 2B). MyD88-dependent signaling through TLR2 and TLR4 requires an adaptor protein termed TIRAP (TIR domain-containing adaptor protein) to initiate signaling. When stimulated, MyD88 sequentially recruits interleukin (IL)-1 receptor associated kinases 4, 1 and 2 (IRAK4, IRAK1 and IRAK2) to the receptor complex. Phosphorylation of IRAK1 on serine/threonine residues by IRAK4 results in recruitment of tumor necrosis receptor associated factor (TRAF)6 to the complex, which is responsible for early responses in response to TLR signaling. More recent studies have suggested an important role for phosphorylation of IRAK2 by IRAK4 in terms of mediating late responses to TLR signaling. Phosphorylated IRAK1 and TRAF6 dissociate from the receptor and form a complex at the plasma membrane with transforming growth factor activated kinase (TAK)1, a mitogen-activated protein kinase kinase (MAPK), as well as TAB1-biding protein (TAB1) and TAK1-binding proteins 2 or 3 (TAB2 or TAB3), resulting in the phosphorylation of TAB2/3 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1, and TAB2 or TAB3) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1 subsequently phosphorylates IKKα/IKKβ/IKKγ (also known as IKK1, IKK2, and NF-κB essential modulator [NEMO], respectively) and mitogen-activated protein kinase kinase 6 (MP2K6, MKK6, MEK6). The IKK complex then phosphorylates IkB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes.

TLR4 can also signal through a MyD88 independent pathway by recruiting the adapter proteins TRAM (TRIF-related adaptor molecule) and TRIF (TIR domain-containing adaptor inducing interferon-β) to the receptor complex (Figure 2C). TRIF recruits the noncanonical IKKs, the serine/threonine–protein kinase TANK (TRAF-family member associated NF-κB activator)-binding kinase (TBK)1 and IKKe, which phosphorylate the transcription factor IRF3 (interferon-regulatory factor 3), thereby inducing interferon (IFN)-β and costimulatory IFN-inducible genes. TRIF also recruits TRAF6 and RIP-1, which leads to activation of mitogen-activated protein kinases and IKKα/IKKβ. These class-specific TLR signaling cascades allow different TLRs to trigger distinct signaling pathways and elicit distinct actions in a cell-specific manner. For example, endothelial cells lack TRAM, thereby restricting TLR4 signaling to the MyD88-dependent pathway in these cells.

TLRs signal by forming homo- or heterodimers which allows for approximation of the TIR domains, creating “docking” platforms for recruitment of adaptor proteins and kinases that activate downstream signaling cascades. TLR2 and TLR6 are capable of forming heterodimers or homodimers, whereas TLR3 and -4 signal by forming homodimers. Three general categories of TLR ligands have been identified, including proteins (signal through TLR5), nucleic acids (signal through TLR3, TLRs 7 to 9) and lipid-based elements (signal through TLR2, TLR4, TLR6, TLR2/TLR6). Although Gram-negative and Gram-positive bacteria have been shown, respectively, to signal through TLR4 and TLR2 in the heart, the exact ligands that activate TLR signaling in the heart following tissue injury are not known. As noted previously, TLR receptors are activated by proteins released by injured and/or dying cells, as well as by fragments of the extracellular matrix (see Figure 1). For example, both heat shock protein 60 and 70, and/or fibronectin, are sufficient to activate innate immune responses in the heart through TLR2 and TLR4 signaling. Once these damage associated molecular patterns are recognized by their cognate pattern recognition receptors, they activate NF-κB–dependent genes that regulate the expression of cell adhesion molecules and chemokines, which in turn recruit macrophages and neutrophils to the myocardium to facilitate tissue repair.

Given the importance TLR signaling, it is not surprising that nature has evolved multiple pathways to negatively regulate TLR signaling. As illustrated in Figure 3, TLR-signaling pathways are negatively regulated by several molecules that are induced following stimulation of TLRs, including IRAK-M (IL-1-receptor-associated kinase M), a nonfunctional IRAK decoy that inhibits the dissociation of the IRAK1-IRAK4 complex from MyD88, thus preventing the formation of IRAK-TRAF6 complexes; SOCS1 (suppressor of cytokine signaling 1), which associates with IRAK1 and negatively modulates TLR signaling; and SHIP-1 (Src homology 2 domain-containing inositol 5-phosphatase 1), a phosphatase that hydrolyzes the 5’ phosphate of PI-3,4-P2, which inhibits PI3 kinase–dependent TLR-MyD88 interactions and NF-kB activation and thus negatively regulates TLR signaling. TRIM30α destabilizes the TAK1 complex by promoting the degradation of TAB2 and TAB3, whereas MyDD88s (myeloid differentiation primary-response protein 88 short), an alternatively spliced variant of MyD88, blocks the association of IRAK4 with MyD88. SARM (sterile-alpha and armadillo motif-containing protein) is a novel adaptor protein that specifically blocks TRIF-dependent but not MyD88-dependent signaling. TOLLIP (Toll interacting protein) is thought to maintain immune cells in a quiescent state and/or terminate TLR-mediated signaling, by interacting with the cytoplasmic TIR domains of TLR2 and TLR4 and suppressing IRAK1 phosphorylation. Finally, the TIR (Toll/IL-1 receptor) domain–containing receptors SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and ST2 have also been shown to negatively regulate TLR signaling.
Figure 2. TLR structure and signaling. A, TLRs and interleukin-1 receptors have a conserved cytoplasmic domain that is known as the Toll/IL-1 R domain. The TIR domain is characterized by the presence of 3 highly homologous regions (known as boxes 1, 2, and 3). Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLRs have tandem repeats of leucine-rich regions (known as leucine rich repeats [LRR]), whereas IL-1 receptors have 3 immunoglobulin (Ig)-like domains. B, Stimulation of TLRs triggers the association of MyD88, which in turn recruits IRAK4, thereby allowing the association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1. TRAF6 is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1, TAB1, and TAB2 at the plasma membrane (not shown), which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1, and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitination of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex, which consists of IKK-α, IKK-β, and IKK-γ (also known as IKK1, IKK2, NEMO, respectively). The IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes. C, The MyD88-dependent pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9. TIRAP, a second TIR domain–containing adaptor protein, is involved in the MyD88-dependent signaling pathway through TLR2 and TLR4. By contrast, TLR3- and TLR4-mediated activation of IRF3 (IFN-regulatory factor 3) and the induction of IFN-β occur in a MyD88-independent manner. As shown, a third TIR domain–containing adaptor, TRIF, is essential for the MyD88-independent pathway through TLR3 and TLR4. TRAM, a fourth TIR domain–containing adaptor, is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent pathway. TRIF mediates the activation of the noncanonical IKKs, IKK-ε, and TBK1, as well as MAP kinase. Note TLR3 is predominately located within endosomes (not illustrated). Modified from Akira and Takeda.12
SIGRR interacts transiently with TLR4, IRAK4, and TRAF6, negatively regulating TLR signaling, whereas ST2 sequesters MyD88 and TIRAP, thereby inhibiting NF-κB activation.

Another highly conserved mechanism for regulating innate immunity is being revealed for microRNAs, so-called immuno-miRs, that regulate innate immune gene expression by preventing mRNA translation or by promoting mRNA degradation. As illustrated in Figure 4, several microRNAs negatively regulate TLR signaling, whereas others positively regulate TLR signaling. For example, miR-146 targets TRAF6 and IRAK1, and is upregulated following LPS stimulation, thereby negatively regulating mRNA levels of TRAF6 and IRAK1.21 miR-155 is also upregulated in response to LPS and targets SHIP-1, which negatively regulates NF-κB signaling by inhibiting PIP3, phosphatidylinositol-3,4, 5-triphosphate)-dependent signaling.22 In contrast, LPS increases miR-21 levels, which targets PDCD4, a transcriptional inhibitor of the antiinflammatory cytokine IL-10, leading to increased levels of IL-10.23 Thus, increased expression of miR-146 or miR-21 negatively regulate innate immune signaling, whereas increased expression of miR-155 positively regulates innate immune signaling. It bears emphasis that these immuno-miRs are expressed in the heart and are differentially regulated in heart failure, with increases in miR-21, miR-146, and miR-155 reported in some, but not all, studies.24

Toll Receptors and the Vascular System

Innate immunity has been implicated in angiogenesis (reviewed elsewhere11), as well as in the development of atherosclerosis and acute coronary syndromes, which will be discussed in more detail below.

Role of TLRs in Atherosclerosis

Three lines of evidence implicate TLR signaling in the development of the inflammation in atherosclerosis. First, TLRs 1, 2, 4, and 5 are expressed in atherosclerotic plaques by resident cells and leukocytes that migrate into the arterial wall. Moreover, TLR4 is upregulated and is concentrated in the shoulder region of the plaque, which is the area that is most sensitive to undergoing plaque rupture.25 TLR signaling upregulates proinflammatory cytokines, cell adhesion molecules by vascular endothelial cells, and enhances the release of matrix metalloproteinase by macrophages, all of which could contribute to progressive atherosclerosis and/or plaque rupture. Oxidized low-density lipoprotein has also been shown to upregulate the expression of TLR4.26 Loss-of-function approaches have shown that TLRs play an important role in the development of atherosclerosis. For example, when TLR4 or TLR2 knockout mice were crossed with the atherosclerosis-prone apolipoprotein E (ApoE) knockout mice or LDL receptor (Ldlr) knockout mice, respectively, the double homozygous progeny had reduced atherosclerosis when compared with ApoE or Ldlr knockout controls, even though serum cholesterol levels did not differ.27,28 Mice lacking Myd88 also had reduced plaque burden when compared with appropriate controls.27 TLR signaling has also been implicated in inappropriate arterial remodeling, which contributes to the pathobiology of atherosclerosis and restenosis.29 Second, epidemiological studies have demonstrated an association between bacterial infection and atherosclerotic disease, suggesting a possible link between the development of atherosclerosis and activation of proinflammatory TLR signaling.30 Indeed, infectious agents such as Chlamydia pneumoniae have been detected within atherosclerotic lesions, where they may act as TLR ligands.11 Although drug intervention trials for the treatment of acute coronary syndromes with gatifloxacin,31 and trials with azithromycin for the secondary prevention of coronary events32 have failed to show benefit in clinical trials, these negative outcomes do not necessarily exclude the potential involvement of other infectious agents in the pathogenesis of atherosclerosis. A third line of evidence in support of the role of TLR signaling in the pathogenesis of atherosclerosis is based on the identification of polymorphisms in the genes encoding TLRs, which have implicated a causal link between TLR signaling and athero-
sclerosis (Table). Of all the TLR mutations identified thus far, the Asp299Gly single-nucleotide polymorphism of TLR4 has been studied the most. The Asp299Gly polymorphism affects the composition and structure of the extracellular domain of TLR4. Importantly, the 299Gly allele has been associated with a blunted proinflammatory response to Gram-negative bacteria, and has been associated with decreased risk of atherosclerosis and/or cardiovascular in some, but not all studies (Table). Indeed, the nested case-control PRIME (Prospective Epidemiological Study of Myocardial Infarction) study showed that there was no association between the Asp299Gly polymorphism and the risk of coronary atherosclerosis (coronary artery disease), or inflammation.33 In contrast to aforementioned studies which imply a detrimental role for TLR2 and TLR4, a recent study suggests that TLR3 activation prevented neointimal formation in response to arterial injury, and that genetic deletion of TLR3 enhanced damage of the elastic lamina damage after arterial injury, as well and accelerated the onset of atherosclerosis in hypercholesterolemic ApoE−/− mice, suggesting a beneficial role forTLR3 signaling.34 Thus, the role of TLRs in vascular disease not as simple as was previously supposed.

**Role of TLRs in Acute Coronary Syndromes**

In addition to promoting atherosclerosis, studies have suggested that TLR-induced inflammation may influence atherosclerotic plaque stability, and hence may contribute to the development of acute coronary syndromes in patients with coronary artery disease.35 Indeed, studies have suggested microbial products within atherosclerotic lesions may promote plaque growth and/or rupture by activating inflammatory cells within the plaque.11,35 However, as shown in the Table, clinical studies that have attempted to link the risk of acute myocardial infarction with TLR polymorphisms have yielded conflicting results. The REGRESS (Regression Growth Evaluation Statin Study) study group36 showed that 299Gly carriers had a lower risk of cardiovascular events, defined as fatal myocardial infarction, cardiac death, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, stroke, and transient ischemic attacks. Furthermore, carriers of the polymorphism had significantly more benefit from statin treatment.36,37 However, the largest study of nearly 5000 individuals found no association between the TLR4 Asp299Gly polymorphism and myocardial infarction.38 One of the limitations of all of these studies is the wide range of allelic frequencies in the control groups, which makes replication studies more difficult. Accordingly, larger prospective studies will be required to clarify the role of TLRs in acute coronary syndromes.

**Role of TLRs in Myocardial Disease**

Deciphering the role that the innate immune system plays in myocardial disease has been challenging, insofar as it has been difficult to reconcile 2 sets of conflicting observations in ischemic injury, one of which suggests that TLR signaling is beneficial, and the other of which suggests that TLR signaling is deleterious. Fortunately, recent “reductionist” studies that have been performed ex vivo or that have used chimeric TLR-deficient mice that harbor wild-type bone marrow cells have allowed for a clearer understanding of the central (ie, myocardial) and peripheral (ie, bone marrow–derived) effects of the innate immune system following ischemic injury. As will be discussed, the aggregate data suggest that short-term activation of TLR signaling confers cytoprotective responses within the
whereas longer-term TLR signaling is maladaptive and results in the upregulation of proinflammatory cytokines and cell adhesion molecules, which leads to activation and recruitment of the “ peripheral” neutrophils, monocytes, and dendritic cells to the myocardium, resulting in increased cell death and adverse cardiac remodeling.

Support for the beneficial role of innate immune signaling in the heart stems from a large body of evidence which suggests that proinflammatory cytokines confer short-term cytoprotective responses in the heart (reviewed elsewhere). Subsequent studies linked TLR signaling with cytoprotection, and demonstrated that LPS activation of TLR4 protected the myocardium from myocardial ischemia/reperfusion (I/R) injury (reviewed elsewhere). Isolated rat hearts that were pretreated with a low dose of LPS 24 hours before terminal euthanasia had preserved LV function after I/R injury compared with the saline treated control hearts. The cytoprotective effects of LPS were observed after 12 to 24 hours and were sensitive to inhibition with cycloheximide, analogous to the biology of “late preconditioning.” Moreover, the cytoprotective effects of LPS used signaling pathways that were shown to be important in ischemic preconditioning, namely NOS2 and Akt.

<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>No. of Participants</th>
<th>Study Design</th>
<th>TLR Gene</th>
<th>Polymorphism(s)</th>
<th>Odds Ratio</th>
<th>Outcome</th>
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<td>Asp299Gly</td>
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<td>Polymorphisms showed no association with intimal-media thickness</td>
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<td>Labrum et al (2007)</td>
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<td>Prospective</td>
<td>TLR4</td>
<td>Asp299Gly Thr399Ile</td>
<td>NR</td>
<td>Polymorphisms showed no association with intimal-media thickness</td>
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<td>Lin et al (2005)</td>
<td>457</td>
<td>Retrospective, Chinese population</td>
<td>TLR4</td>
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<td>Retrospective</td>
<td>TLR4</td>
<td>Asp299Gly</td>
<td>0.53</td>
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<td>REGRESS trial (2003)</td>
<td>885</td>
<td>Prospective, subgroup analysis†</td>
<td>TLR4</td>
<td>Asp299Gly</td>
<td>0.74</td>
<td>Polymorphism had no association with CAD progression; however, the Asp299Gly polymorphism was associated with a lower risk of cardiovascular events in patients on statin treatment</td>
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<td>Polymorphism showed no association with CAD progression</td>
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<td>1.24</td>
<td>Polymorphisms increased the risk of MI in men, but not women</td>
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<td>1.94</td>
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<td>TLR2</td>
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<td>Polymorphism associated with decreased risk of MI in subgroup of patients receiving statin treatment</td>
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<td>0.86</td>
<td>Polymorphisms showed no association with MI</td>
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*A total of 293 of the participants had familial hypercholesterolemia. †Individuals randomized to pravastatin or placebo. CAD indicates coronary artery disease; MI, myocardial infarction; NR, not reported. Modified from Frantz et al.11
TLR Signaling in I/R Injury and Myocardial Infarction

As noted above, TLR-mediated signaling contributes to myocardial damage and adverse cardiac remodeling following I/R injury and/or myocardial infarction. Traditional “loss of function studies” in experimental heart failure models in mice and rats suggest that sustained TLR activation is maladaptive and can contribute to LV dysfunction and adverse cardiac remodeling (see Table I in the Online Data Supplement, available at http://circres.ahajournals.org). Mice with a missense mutation of TLR4 or targeted disruption of TLR4, TLR2, or MyD88 have reduced infarct sizes when compared to wild-type controls. Moreover, mice pretreated with a TLR4 antagonist (Eritoran), had reduced nuclear translocation of NF-κB, decreased the expression of proinflammatory cytokines (eg, IL-1, IL-6, TNF) and smaller infarct sizes when compared to vehicle-treated animals. Mortality and LV remodeling are reduced in mice with targeted disruption of TLR4 or TLR2. Studies performed ex vivo in TLR2 deficient mice suggest that the LV dysfunction that supervenes following I/R is mediated through TLR2-TRAP mediated upregulation of TNF.

Although the mechanism(s) for the deleterious effects of TLR signaling following I/R injury and/or myocardial infarction have not been elucidated, a recent study showed that the decrease in infarct size in TLR2 deficient mice following I/R injury was abrogated in chimeric TLR2 deficient mice that underwent bone marrow transplantation with wild-type bone marrow cells. This study was consistent with a prior study ex vivo, which showed that the LV dysfunction that supervened following I/R injury was mediated through a TLR2-TIRAP dependent pathway, but that infarct size was not different between TLR2-deficient and wild-type mice. Viewed together, these ex vivo and in vivo studies suggest that TLR2 activation within the heart leads to upregulation of proinflammatory cytokines, which may be beneficial in the short-term through mitochondrial stabilization, as well as through conservation of energy secondary to the development of left ventricular dysfunction (reversible), but that sustained TLR mediated signaling leads to activation and homing of leukocytes to the myocardium, with a resultant increase in tissue destruction (Figure 5). In this regard, it is worth noting that similar observations with regard to the role of central versus peripheral activation on innate immune signaling through TLR4 have been made in model of systemic sepsis.

What remains to be determined is how TLR signaling is activated in the periphery following myocardial injury, and why the resulting innate immune response is more destructive following tissue injury than it is following systemic sepsis, wherein the LV dysfunction and cardiac remodeling are largely reversible.

Septic Cardiomyopathy

Although the full spectrum of endogenous molecules that lead to myocardial depression in sepsis has not been defined completely, recent studies have shown that mice deficient in TLR4 or IRAK1 are protected from LPS-induced mortality and cardiac dysfunction. Importantly, intramyocardial TNF and IL-1β protein levels, as well as NOS2 and NO

Support for a cytoprotective role of TLR was provided by a recent study ex vivo in mice, which showed that ischemic preconditioning was mediated via a TLR2-TIRAP dependent signaling pathway. This study further showed that TIRAP mediated upregulation of TNF. Viewed together, these ex vivo and in vivo studies suggest that TLR2 activation within the heart leads to upregulation of proinflammatory cytokines, which may be beneficial in the short-term through mitochondrial stabilization, as well as through conservation of energy secondary to the development of left ventricular dysfunction (reversible), but that sustained TLR mediated signaling leads to activation and homing of leukocytes to the myocardium, with a resultant increase in tissue destruction (Figure 5). In this regard, it is worth noting that similar observations with regard to the role of central versus peripheral activation on innate immune signaling through TLR4 have been made in model of systemic sepsis.

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production were blunted and delayed after LPS challenge in TLR4 deficient mice. The effect of LPS on myocardial function is complex and is mediated directly by activation of TLR4 signaling in cardiac myocytes, with resultant elaboration of TNF, and indirectly by immune cells as well, insofar as chimeric mice with a deficiency of TLR4 in their leukocytes have a blunted cardiac response to LPS challenge. In addition, other TLR types have since been linked to pathogen-mediated cardiac dysfunction. For example TLR2 knockout mice are protected from Staphylococcus aureus-induced myocardial dysfunction, whereas TLR9 knockout mice are protected from synthetic bacterial DNA (CpG-ODN).

Viral Myocarditis

TLR signaling is activated by a variety of ligands that are associated with viral infections. By way of review, positive-strand RNA is released from viral capsid proteins after viral entry into the cell. The viral genome then replicates using the positive-strand RNA as its template, resulting in the formation of dsRNA intermediates. Accordingly, both single-strand RNA and dsRNA are present in virally infected cells. TLR3 and TLR7/8 signaling are activated by double-stranded RNA (dsRNA) and single-stranded RNA, respectively, whereas TLR9 recognizes bacterial and viral CpG DNA motifs. Thus viral infection can activate innate immune signaling in the heart through Myd88 dependent (TLR 7, 8) and MyD88 independent pathways (TLR 3). Infection of TLR3 knockout mice with encephalomyocarditis virus (EMCV), a positive single-strand RNA virus, resulted in earlier mortality in TLR3 knockout mice that was associated with increased viral replication and myocardial injury when compared with wild-type mice. Similar observations have been reported for Coxsackievirus group B serotype 3 (CVB3) in TLR3 deficient mice. Taken together, these results suggest that TLR3-mediated recognition of viral infection, with subsequent activation of antiviral mechanisms (eg, type I IFN response) are important in terms of minimizing viral replication in the heart. A recent study showed that the severity of cytomegalovirus (CMV)-induced myocardial inflammation was worse in TLR9 deficient and MyD88 deficient mice when compared with wild-type mice, and that viral titers were significantly higher in TLR9 deficient and MyD88 deficient mice. These results are in contrast to studies performed in CVB3 infected MyD88 deficient mice, which showed that mice lacking MyD88 signaling had improved survival compared with CVB3 infected wild-type mice. Moreover, pathological examination of the hearts revealed that there was a significant decrease in CVB3 titers and cardiac inflammation in the MyD88 deficient mice when compared with wild-type infected mice. The reasons for these discrepant findings with respect to the differences in outcomes in the above studies are not known, but may be related to the MyD88 independent antiviral mechanisms that have not yet been elucidated. Nonetheless, the data reviewed above using TLR knockout mice suggest that TLR-mediated viral-sensing mechanisms in the heart play an important role in the pathogenesis of myocarditis.

Functional Role of TLR Signaling in Human Heart Failure

The experimental literature reviewed above suggests that sustained activation of TLR signaling following cardiac injury is maladaptive and can lead to a heart failure phenotype. Unfortunately very little is known with respect to the role of the innate immune system in the failing human heart. To date, 2 studies have shown that TLR4 expression is increased in the hearts of patients with advanced heart failure. To further clarify the role of innate immunity in the failing heart, we examined the expression profiles of 59 innate immune genes using gene arrays that were from explanted hearts from patients with ischemic cardiomyopathy (ICM), idiopathic dilated cardiomyopathy (DCM), and viral cardiomyopathy (VCM); gene arrays from nonfailing hearts were used as the appropriate controls (data obtained from Cardiogenomics Consortium database [http://www.cardiogenomics.med.harvard.edu]). Expression data for innate immune signaling genes were analyzed by hierarchical clustering, as well principal component analysis (PCA), a mathematical modeling procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables that are termed principal components. There are 2 important findings illustrated in the PCA plot shown in Figure 6. The first is that the numeric values for the PCA plots for nonfailing hearts clustered differently than the numeric values for the PCA plots for the ICM, DCM, VCM hearts, which tended to cluster together (best observed in Figure 6A), suggesting that expression of innate immune genes is different in the failing heart. A second important finding is that the PCA profiles for the ICM patients and DCM patients clustered differently, raising the intriguing possibility that that the innate immune system is activated differentially in response to the nature of the pathological tissue injury pattern. Inspection of Figure 6A shows that the PCA plots for VCM patients overlapped those observed in DCM, which is of interest insofar as occult and/or persistent viral myocarditis has been suggested as a potential etiology for idiopathic dilated cardiomyopathy. An unsupervised hierarchical clustering analysis of genes that were expressed differently in DCM, ICM, VCM, and nonfailing human hearts confirmed the results of the PCA plots, and showed that there were distinct gene expression profiles for innate immune genes in failing and nonfailing hearts, and that there were distinct gene expression profiles for innate immune genes in ICM and DCM hearts. Although these provisional studies suggest that the innate immune system is activated in human heart failure, it will be important to more precisely determine the expression levels of the different components of the innate immune system, as well as link activation of the innate immune system to the development and progression of heart failure.

Translation Potential of TLR Signaling in Cardiovascular Disease

There has been significant interest in developing TLR antagonists as novel therapeutics in diseases such as sepsis, systemic lupus erythematosus and rheumatoid arthritis, wherein the immune system and inflammatory mediators are inappropriately overactive. Currently, there are a variety of
novel antagonists that are being developed for TLR 2, 4, 7, and 9 (reviewed elsewhere13). Given the focus of the present review on innate immunity and the heart, we will focus on TLR2 and TL4 antagonism (see Online Table II), for which there is the most direct evidence of TLR involvement during I/R injury. The role of TLR agonists for viral infections will be mentioned briefly.

**Toll-Like Receptor 2**

OPN-305 is a fully humanized anti-TLR2-specific monoclonal antibody that is a potent inhibitor of TLR2-mediated proinflammatory cytokine production. OPN-305 was granted orphan status for the prevention of the ischemia and reperfusion injury associated with organ transplantation, and planning for the first human trials as a potential treatment of inflammatory diseases are underway. OPN-301 is a related mouse IgG1 monoclonal antibody against TLR2 that reduces infarct size and prevents adverse cardiac remodeling in mice when given immediately before reperfusion injury.54 Given the role of TLR2 in mediating I/R injury, OPN-305 may represent an attractive target for I/R in the heart. AP177 is a TLR binding aptamer that was identified through a novel screening technology termed SELEX (systematic evolution of ligands by exponential enrichment).68 AP177 competitively antagonizes TLR2 ligand binding, and inhibits NF-κB activity and proinflammatory cytokine production. Although AP177 is not under clinical development at time of this writing, SELEX screening could be used to identify other novel TLR antagonists.

**Toll-Like Receptor 4**

There are a number of strategies that have been undertaken to inhibit TLR4 activation.13 Eritoran (E5564), which reduces the binding of lipid-A (the biologically active part of the LPS molecule), reduced mortality by 6.4% compared with the placebo group in a phase II sepsis trial, and is currently undergoing evaluation in phase III sepsis trials (NCT00334828). Given that the pharmacodynamic profile of Eritoran requires administration as a continuous infusion or by repeated intravenous injections, it may be most useful in acute coronary syndromes or I/R injury. Alternative approaches to TLR4 antagonism have exploited modifications of Lipid-A, which is the biologically active part of the LPS molecule that binds TLR4. The variations of lipid-A, including CRX-527 and lipid-IVα, have reduced agonist activity and presumably antagonize TLR4 by competing for binding to MD-2, which is a cofactor for LPS binding. TAK-242 as another TLR4 antagonist, although the precise target is not known. The development of TAK-242 was discontinued during a phase III sepsis clinical trial because the profile of the drug did not meet the criteria required to support continued development, and not because of drug safety issues (NCT00633477). Ibudilast (AV411) is another novel TLR4 antagonist that suppresses proinflammatory cytokines such as TNF and IL-6, which may also induce the antiinflammatory cytokine IL-10. Ibudilast is undergoing phase II trials for opioid dependence (NCT00723177).

TLR agonists are also being developed as empirical immunostimulatory molecules for cancer and some viral diseases (reviewed elsewhere13). Although immunostimulatory agents have not yet been applied in the setting of viral myocarditis, the literature reviewed herein suggests an important role for TLR3 signaling in the setting of myocarditis. Rintatolimod (poly I:C) is a dsRNA molecule that acts as a TLR3 agonist and is purported to regulate RNAse L levels, which is an enzyme that is important for the antiviral host response. Rintalomid is being developed for the treatment of hepatitis B and hepatitis C infection, HIV and influenza. Further studies will be necessary to determine whether TLRs are viable therapeutic targets in the setting of acute viral myocarditis.

**Summary and Future Directions**

As discussed herein, the innate immune system has been implicated in the pathogenesis of atherosclerosis, acute cor-
effects of acute activation of TLR signaling, whereas targeting TLRs makes less sense in chronic injury where the upstream signaling events have already occurred. In this latter scenario, targeting the downstream components of TLR signaling may be more effective. It is important to note that all of the known signals downstream from TLRs 1 to 10 converge on 4 TIR motif adaptor proteins in the neck of the hourglass, namely MyD88, TIRAP, TRAM, and TRIF, which may facilitate attempts to antagonize downstream TLR signaling therapeutically. It is also quite likely that in some settings wherein “sterile inflammation” occurs that innate immune signaling may be accomplishing beneficial homeostatic functions, in which case targeted stimulation of various TLRs may be therapeutically desirable. Although the growth of information regarding the role of innate immunity in the heart has been exponential since the original description of TLR4 in the heart, with the possible exception of I/R injury, our knowledge of how and when to modulate TLR signaling in the heart is incomplete. This statement notwithstanding, research in the role of innate immunity in the cardiovascular system will continue to benefit from the explosive growth in knowledge in this area, as well as the burgeoning interest in developing immunomodulatory strategies that target TLR signaling.

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None.

References


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## SUPPLEMENTARY TABLES FOR “THE EMERGING ROLE OF INNATE IMMUNITY IN THE MAMMALIAN HEART: FOR WHOM THE CELL TOLLS”

Table 1: TLR signaling Modulation of Myocardial Ischemia Reperfusion Injury and Cardiac Remodeling

<table>
<thead>
<tr>
<th>Mice</th>
<th>Infarct Models</th>
<th>Effects in Knockout Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLR2 signaling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>I/R (30' I/60R)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>sizes, reduced neutrophil recruitment, reduced ROS and cytokines</td>
</tr>
<tr>
<td>TLR2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Permanent coronary ligation&lt;sup&gt;2&lt;/sup&gt;</td>
<td>val rate, attenuated remodeling, but same infarct sizes at 4 wk</td>
</tr>
<tr>
<td><strong>TLR4 signaling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 BL/10 ScCr</td>
<td>I/R (60' I/24 h R)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>sizes, reduced MPO activity and complement 3 deposition</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>I/R (60' I/120' R)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>sizes, decreased cardiac expression of TNF, MCP-1, and ILs</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>I/R (60' I/24 h R)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>sizes, but no gain in LV function</td>
</tr>
<tr>
<td>WT with etorinan</td>
<td>I/R (30' I/120' R)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>sizes, reduced pJNK, reduced cytokine expression</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>Permanent coronary ligation&lt;sup&gt;7&lt;/sup&gt;</td>
<td>modeling, improved systolic function, reduced cytokine expression</td>
</tr>
<tr>
<td>C57 BL/10 ScCr</td>
<td>Permanent coronary ligation&lt;sup&gt;8&lt;/sup&gt;</td>
<td>action on day 6 after infarction, improved survival rate, reduced LV remodeling and apoptosis at 4 wk.</td>
</tr>
<tr>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>I/R (30' I/24 h R)&lt;sup&gt;9&lt;/sup&gt;</td>
<td>sizes, improved LV function, and attenuated cytokine expression and neutrophil recruitment</td>
</tr>
</tbody>
</table>

Key: TLR, Toll-like receptor; ROS, reactive oxygen species; MPO, myeloperoxidase; MCP-1, monocyte chemoattractant protein-1; pJNK, phosphorylated JNK; MyD88, myeloid differentiation primary-response gene 88. (Modified from Chao Am. J. Heart Circ Physiol 296: H1-H12, 2009 and Topkara VK et al, Therapeutic targeting of innate immunity in the failing heart. In Press J Mol Cell Cardiol ).
### Table II: Development Status of TLR2 and TLR4 antagonists

<table>
<thead>
<tr>
<th>Compound</th>
<th>Indications</th>
<th>Target</th>
<th>Drug Class</th>
<th>Clinical Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN-305</td>
<td>Inflammation, autoimmunity, ischemia/reperfusion</td>
<td>TLR2 antagonist</td>
<td>Antibody</td>
<td>Orphan status for prevention of ischemia reperfusion injury</td>
</tr>
<tr>
<td>OPN-401</td>
<td>IBD, rheumatoid arthritis</td>
<td>TLR2/TLR4 antagonist</td>
<td>Viral-derived peptide</td>
<td>Preclinical</td>
</tr>
<tr>
<td>AP177</td>
<td>NS</td>
<td>TLR2 antagonist</td>
<td>DNA aptamer</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Eritoran</td>
<td>Sepsis</td>
<td>TLR4 antagonist</td>
<td>Synthetic lipodisaccharide</td>
<td>Phase III</td>
</tr>
<tr>
<td>(E5564)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lidid-IVa</td>
<td>NS</td>
<td>TLR4 antagonist</td>
<td>Lipid A partial mimetic</td>
<td>Preclinical</td>
</tr>
<tr>
<td>TAK-242</td>
<td>Sepsis</td>
<td>TLR4 antagonist</td>
<td>Small molecule inhibitor</td>
<td>Suspended in phase III</td>
</tr>
<tr>
<td>1A6</td>
<td>Colitis</td>
<td>TLR4 antagonist</td>
<td>Antibody</td>
<td>Preclinical</td>
</tr>
<tr>
<td>CPG-52364</td>
<td>SLE</td>
<td>PolyTLR antagonist</td>
<td>Quinazoline derivative</td>
<td>Phase I</td>
</tr>
<tr>
<td>Ibudilast</td>
<td>Pain management, withdrawal</td>
<td>TLR4 antagonist</td>
<td>Small-molecule phosphodiesterase inhibitor</td>
<td>Phase II</td>
</tr>
<tr>
<td>(AV411)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Key: TLR = Toll-like receptor. (Modified from Hennessy et al., Nat. Rev Drug Discov. 9, 293-307; 2010 and Topkara VK et al, Therapeutic targeting of innate immunity in the failing heart. In Press J Mol Cell Cardiol )
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