Toll-like receptor signaling: a critical modulator of cell survival and ischemic injury in the heart

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First published November 14, 2008; doi:10.1152/ajpheart.00995.2008.—Toll-like receptors (TLRs) represent the first line of host defense against microbial infection and play a pivotal role in both innate and adaptive immunity. TLRs recognize invading pathogens through molecular pattern recognition, transduce signals via distinct intracellular pathways involving a unique set of adaptor proteins and kinases, and ultimately lead to the activation of transcription factors and inflammatory responses. Among 10 TLRs identified in humans, at least two exist in the heart, i.e., TLR2 and TLR4. In addition to the critical role of these in mediating cardiac dysfunction in septic conditions, emerging evidence suggests that the TLRs can also recognize endogenous ligands and may play an important role in modulating cardiomyocyte survival and in ischemic myocardial injury. In animal models of ischemia-reperfusion injury or in hypoxic cardiomyocytes in vitro, the administration of a sublethal dose of lipopolysaccharide, which signals through TLR4, reduces subsequent myocardial infarction, improves cardiac functions, and attenuates cardiomyocyte apoptosis. By contrast, a systemic deficiency of TLR2, TLR4, or myeloid differentiation primary-response gene 88, an adaptor critical for all TLR signaling, except TLR3, leads to an attenuated myocardial inflammation, a smaller infarction size, a better preserved ventricular function, and a reduced ventricular remodeling after ischemic injury. These loss-of-function studies suggest that both TLRs contribute to myocardial inflammation and ischemic injury in the heart although the exact contribution of cardiac (vs. circulatory cell) TLRs remains to be defined. These recent studies demonstrate an emerging role for TLRs as a critical modulator in both cell survival and tissue injury in the heart. Apoptosis; cardiomyocytes; inflammation; innate immune; interleukin-1 receptor-associated kinase; ischemia-reperfusion; myeloid differentiation primary-response gene 88 adapter-like protein; myocardial infarction; nuclear factor-κB; remodeling.

Innate immune systems such as those mediated via Toll-like receptors (TLRs) represent the first line of defense against invading microbial pathogens. These receptors play a critical role in both innate and adaptive immunity (1, 102, 141). There are at least 10 TLRs identified so far in humans, and they recognize and specifically bind to a variety of pathogenic agonists such as lipopeptide (via TLR2), double-stranded RNA (via TLR3), lipopolysaccharide (LPS) (via TLR4), flagellin (via TLR5), and deoxyctydylate-phosphate-deoxyguanylate DNA (via TLR9) by molecular pattern recognition (1, 73). The stimulation of these TLRs leads to, through their specific intracellular signaling pathways, the activation of various downstream transcription factors and the ultimate production of inflammatory cytokines in host immune cells. In addition to their pivotal role in host immune defense against invading pathogens, TLRs, demonstrated by emerging evidence from the past 5–10 years, appear capable of responding to stress and modulating inflammation and tissue damage following noninfectious insults such as hypoxia and ischemia in various tissues (107), such as the lung (68), liver (121), brain (84, 144), and heart (28, 36, 118, 136, 149).

In mice, the heart expresses at least six receptors involved in TLR signaling, namely TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 (17). A pathogenic ligand stimulation of TLR2, TLR4, TLR5, and TLR9 can lead to the activation of the NF-κB pathway and cardiomyocyte contractile dysfunction (17, 81, 169). The two most-studied TLRs in the heart are TLR2 and TLR4 (41, 79, 80, 109, 169). Animal studies have indicated that these receptors are in part responsible for cardiac dysfunction in certain pathological conditions characterized in either gram-negative or gram-positive bacterial infection, such as endotoxemia (79, 109), peptidoglycan-associated lipoprotein (169), and staphylococcus aureus (80). In the absence of a pathogen, an insult induced by transient tissue hypoxia and ischemia can induce a dramatic innate immune response in the myocardium, which has an adverse impact on cardiac anatomy and function. Probably for this very reason, the role of TLR signaling in ischemic myocardial injury and in other cardiovascular diseases has been intensely studied in both human
and animals (39). However, population-based studies designed to determine the impact of TLR4 polymorphism on the risk of myocardial infarction (MI) are so far inconclusive, and the data seem conflicting. Some studies suggest that individuals with the single nucleotide polymorphism of TLR4 Asp299Gly, who have an impaired host immune response toward LPS stimulation, have a lower risk of MI (9, 15, 54), whereas others suggest an increased (32) or the same level of risk (83) of MI in the polymorphism compared with the control population. In animal models of ischemic cardiac injury, the role of TLRs is incompletely defined. For example, the systemic administration of a sublethal dose of LPS, which signals through TLR4, reduced the subsequent ischemic MI and improved cardiac functions both in vivo and in isolated hearts (13, 18, 89, 99, 106, 138, 155, 166, 167). The activation of TLR4-myeloid differentiation primary-response gene 88 (MyD88) signaling also protects cardiomyocytes against apoptosis (26, 170). By contrast, in the absence of systemic TLR4 stimulation, mice deficient for TLR4 exhibited a reduced myocardial inflammation and infarction compared with wild-type (WT) mice in an in vivo model of ischemia-reperfusion (I/R) injury, suggesting that TLR4 mediates ischemic injury in the heart (28, 118). These studies demonstrate an emerging role for TLRs as a critical modulator in both cell survival and tissue injury in the heart. This article reviews the experimental evidence that demonstrates 1) the role of TLR signaling in modulating cardiomyocyte apoptosis, 2) LPS preconditioning against ischemic myocardial injury, and 3) the emerging role of TLR signaling in ischemic myocardial injury as well as inflammation as demonstrated by the loss-of-function studies in mice.

**Discovery of Toll and TLR**

Toll, meaning “amazing” and “wonderful” in German, was first described by Christiane Nüsslein-Volhard and colleagues in 1985 when they were studying the genetic mechanisms that control early embryonic development in the fruit fly *Drosophila* (4, 5). They discovered that *Drosophila* females that lacked Toll gene activity produced dorsalized embryos, in which all embryonic cells behaved like the dorsal cells of the WT embryo. As a result, the Toll-deficient embryos lacked any dorsal-ventral polarity. Together with Edward B. Lewis and Eric Wieschaus, Nüsslein-Volhard won the Nobel Prize in Physiology or Medicine in 1995 for their discoveries concerning the genetic control of early embryonic development (http://nobelprize.org/nobel_prizes/medicine/laureates/1995/). Ten years after Toll was discovered, investigators found that Toll also played an important role in *Drosophila* host immunity against bacteria and fungi (85). A fly deficient of Toll died quickly of bacterial or fungus infection. In 1997, a group of investigators led by P. Medzhitov and C. A. Janeway. Jr., discovered that a human homolog of the *Drosophila* Toll protein was a transmembrane protein with an extracellular domain consisting of a leucine-rich repeat domain and cytoplasmic domain homologous to the cytoplasmic domain of human interleukin-1 (IL-1) receptor (103). The expression of a constitutively active mutant of human Toll or Toll-like protein in the cell line activated the NF-κB pathway and the expression of NF-κB-mediated proinflammatory cytokines IL-1, IL-6, and IL-8. Between 1998 and 1999, two groups of investigators independently found that mice with mutated *Lps* gene, either a missense point (Pro→His, C3H/HeJ strain) or null mutation (C57BL/10 ScCr strain), conferred a natural hyporesponsiveness to endotoxin (120, 123). Through targeted gene (*Lps*) disruption, Hoshino and colleagues (55) generated TLR4-deficient mice that were resistant to LPS. These pioneering studies firmly established TLR4 as the receptor for LPS, a wall component of gram-negative bacteria.

**TLR-Signaling Pathways**

There are 10 TLRs identified in humans (12, 101). Upon binding to its specific ligand via pattern recognition, TLRs recruit and activate various downstream kinases such as IL-1 receptor-associated kinase (IRAK)-1, IRAK-4, and TNF receptor-associated factor (TRAF)-family member-associated NF-κB activator-binding kinase 1 (TBK1) via a specific set of adaptors (Fig. 1). There are five Toll/IL-1 receptor (TIR) domain-containing adaptors, namely MyD88, MyD88 adaptor-like protein (Mal), TIR-domain-containing adaptor protein inducing IFN-β-mediated transcription factor (TRIF), TRIF-related adaptor molecule (TRAM), and a sterile α- and armadillo-motif-containing protein (SARM) (112). TLRs interact with their respective adaptor(s) via the homologous binding of their unique TIR domains present in both the receptors and the adaptor molecules. Based on the specific adaptors recruited to TLRs, TLR signaling can be divided to two general pathways, namely, MyD88- and Trif-dependent (or MyD88-independent) pathways. The two distinct signaling pathways lead to the production of proinflammatory cytokines and type 1 IFN, respectively (112). As illustrated in Fig. 1, all TLRs, with the exception of TLR3, signal through MyD88-dependent pathways. In TLR2 (and TLR1 and TLR6) and TLR4 signaling, Mal is required for recruiting MyD88 to the receptors (37). By contrast, in TLR3 and TLR4 signaling, a MyD88-independent pathway is initiated and another adaptor, Trif, is proven critical (53, 162).

The first and best known TLR is TLR4 (55, 120, 123). For LPS recognition, three additional proteins are required, including LPS binding protein (161), CD14 (160), and MD-2 (135). TLR4 signals via the two distinct pathways, MyD88-dependent and Trif-dependent pathways (112). In MyD88-dependent pathway, activated TLR4 recruits downstream IRAKs through the adapter proteins Mal and MyD88. Following a cascade of kinase activation as described below, this pathway ultimately leads to the activation of NF-κB and production of proinflammatory cytokine such as TNF and ILs (Fig. 1). In Trif-dependent pathway, TLR4 signals through TRAM-Trif that results in TBK1 activation and a downstream stimulation of IRF3 and production of IFN (Fig. 1). In TLR2 signaling, TLR2 dimerizes with either TLR1 or TLR6. The heterodimers recruit and activate IRAK4/1 via a Mal/MyD88-dependent mechanism and ultimately lead to the induction of cytokines. TLR2 signaling does not induce a production of IFN (Fig. 1).

As stated above, MyD88 is one of the five TIR domain-containing adaptors in TLR signaling (112) and plays a critical role in the signaling of all TLRs except TLR3 (64, 104, 108) (Fig. 1). It was originally isolated as one of the 12 myeloid differentiation primary response genes (91). It has a NH2-terminal death domain (DD) and a COOH-terminal TIR domain. MyD88 binds to the TLR complex via the TIR-TIR domains and in turn recruits the downstream kinase IRAKs via the activation of TIR-TIR and IRAKs.
their DD-DD interaction. Like TLR4 mice (55), MyD88 mice (72) lack the ability to respond to LPS although MyD88-independent mechanisms in TLR4 signaling exist (53, 74, 162) (Fig. 1). First described as a signal transducer for the proinflammatory cytokine IL-1 (20), IRAK-1 was later implicated in the signal transduction of other members of the TLR/IL-1R family. Four different IRAK-like molecules have been identified: two active kinases, IRAK-1 and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-like molecule (IRAK-M) (63). All IRAKs are multidomain proteins, consisting of a conserved NH2-terminal DD and a central kinase domain (63). The DD is a protein interaction motif implicated in the binding of IRAKs to the upstream adaptor protein MyD88. The recruited IRAK-1 becomes phosphorylated by activated IRAK-4. Phosphorylated IRAK-1 binds to TRAF-6 (21, 158). The IRAK-1-TRAF-6 complex then activates transforming growth factor-β-activated protein kinase 1 (TAK1) through a process involving the cytosol translocation of TAK1- and two TAK1-binding proteins from membrane to cytosol and the ubiquitination of TRAF-6 (69, 140). Activated TAK1 then phosphorylates IKK-α/β as well as JNK/p38 kinases, leading to the activation of NF-κB and activator protein-1, respectively (153). It is noteworthy that several other signaling molecules (not shown in Fig. 1) are involved in the feedback regulation of TLR signaling. These include IRAK-M (82), MyD88s (19, 65), an alternative splice product of MyD88, and SARM (22). These negative regulators may play an important role in LPS tolerance, a transient state of LPS refractoriness after the initial, sublethal exposure to LPS. Several mechanisms, among others, have been proposed to be responsible for endotoxin tolerance, including 1) the downregulation of the surface expression of TLR4 (111, 132); 2) the inhibition of TLR signaling by IRAK-M, which prevents the dissociation of IRAK-1/4 from MyD88 and the formation of IRAK-TRAF-6 complexes (82) and by MyD88s, which block IRAK-4 binding to MyD88, as the negative regulators of TLR signaling (19); and 3) SARM, an adaptor protein that specifically blocks TRIF-dependent and MyD88-independent NF-κB activation and gene induction (22).

**TLR Signaling Modulates Cardiomyocyte Apoptosis**

Functional relevance of apoptosis in cardiac diseases. Although ischemia with associated hypoxia is the main initial event leading to ischemic MI, the molecular mechanisms involved in the consequent cardiomyocyte death are not completely defined and have been studied extensively. Several lines of evidence suggest that apoptosis, or programmed cell death, and the caspase proteases central to the apoptotic process could play a role in the pathogenesis of cardiac disease including ischemic myocardial injury. Cardiomyocyte apopto-
sis has been found in the injured myocardium of patients who had died of MI (62, 116, 131) and in animal models of infarction (43, 71, 130). Cardiomyocyte apoptosis is particularly prominent in reperfusion injury (43) and mainly localized in the border zone of the histological infarction area and a few in remote noninfarcted myocardiums (131). In animal models, the transgenic cardiac expression and conditional activation of procaspase-8 led to a significant cardiomyocyte apoptosis and a lethal-dilated cardiomyopathy; both can be prevented by an administration of a broad-spectrum caspase inhibitor (157). In a mouse model of chronic pressure overload, the conditional deletion of cardiac gp-130 cytokine receptor led to the rapid onset of dilated cardiomyopathy and a massive induction of cardiomyocyte apoptosis versus that in the control mice which exhibit compensatory hypertrophy, thus suggesting that cardiomyocyte apoptosis is a critical element in the transition between compensatory cardiac hypertrophy and heart failure (52). In a rat model of I/R injury, an intravenous administration of benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, a broad caspase inhibitor, resulted in a modest but significant reduction in infarct size (33.4%) and cardiomyocyte apoptosis (72.1%) (163), demonstrating a link between cardiomyocyte apoptosis and ischemic myocardial injury. In a similar model of I/R injury, the adenovirus-mediated gene expression of a constitutive active Akt (PKB) or human insulin-like growth factor I was sufficient to attenuate cardiomyocyte apoptosis and reduce infarct size (23, 42, 97, 98). Moreover, the transgene expression of the dominant-negative (dn) mutant of Fas-associated DD protein (FADD), an adaptor protein critical for the death receptor (DR) pathway of apoptosis, inhibited apoptosis of hypoxic cardiomyocytes and reduced MI after I/R injury (24, 25). Thus the modulation of at least some targets in the apoptotic signaling pathways appears capable of mediating a meaningful anatomic and functional rescue following pressure overload or I/R injury.

**Signaling pathways of cardiomyocyte apoptosis.** The execution of apoptosis is a tightly regulated and energy-dependent process that requires specialized cellular machinery (7, 8, 45). The central component of this machinery is the proteolytic system involving a family of proteases called cystein aspartic acid-specific proteases (caspases) (147, 148). The biochemical and cellular hallmarks of apoptosis is characterized by nuclear and DNA fragmentation and condensation, membrane blebbing, and cellular shrinkage. Although a fully comprehensive model of apoptosis has not been assembled, two general pathways have been delineated (45, 139, 148, 165), namely, the intrinsic or mitochondrial pathway and the extrinsic or DR pathway. In the intrinsic pathway, apoptosis occurs when specific stimuli lead to mitochondrial release of cytochrome c. Cytochrome c binds apoptotic protease-activating factor-1 in the cytosol and induces its oligomerization and the subsequent recruitment and activation of procaspase-9. In the DR pathway, a ligand such as CD95L/Fas ligand binding to DR leads to the activation of caspase-8 through specific adaptor molecules such as FADD. Both caspase-8 and -9 can lead to the activation of caspase-3 and to ultimate cell death. The two pathways coexist in many circumstance (29, 30, 87) and in cardiomyocytes (14, 67, 93, 143, 159).

**TLR signaling modulates cardiomyocyte apoptosis.** TLR4. TLR4 has been linked to both parallel proapoptotic and survival pathways. LPS induces apoptosis in endothelial cells (27) and hepatocytes (49), whereas it has an antiapoptotic effect in monocytes (44, 94), neutrophils (156), macrophages (90), and cardiomyocytes (26, 170). In the cells protected by LPS treatment, the activation of TLR4 triggers the expression of cell survival and inflammatory genes via NF-κB-dependent mechanisms. In the heart, an early report indicated that prolonged LPS treatment in rats activated proapoptotic and survival pathways and induced very modest cardiomyocyte apoptosis (100). However, the low levels of apoptosis appeared insufficient to account for the LPS-induced cardiomyocyte dysfunction, and its significance is uncertain. The data from a septic animal model (cecal ligation and puncture) indicated a minimal level of apoptosis in the heart, whereas a significant level of apoptosis was found in lymphocytes and in parenchymal cells of ileum, colon, lung, and skeletal muscle (33). Autopsy studies from patients who died of sepsis revealed minor apoptotic cell death in the heart, whereas there was profound lymphocyte and gastrointestinal epithelial cell death (56–58). In fact, a recent study indicated that an in vivo administration of LPS actually reduced myocardial apoptosis induced by I/R injury (48). Moreover, in cell types such as endothelial cells, the induction of LPS-induced apoptosis did not occur unless the production of endogenous survival proteins was blocked (10, 11), again suggesting a parallel survival pathways in these cells. In an in vitro model of apoptosis, recent studies have demonstrated that LPS leads to a time- and dose-dependent antiapoptotic effect and phosphorylation of Akt and ERK in isolated cardiomyocytes (26). Both TLR4 and its adaptor MyD88 seem to mediate the survival benefit in the LPS-treated cardiomyocytes (170). The LPS-induced antiapoptotic effect can be blocked by phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, MEK1 inhibitor, and by dnIKK-β, suggesting that all three kinases, i.e., Akt, ERK, and IKK-β, contribute to the LPS-induced survival effect in cardiomyocytes (26). Moreover, TLR4 mediates a robust inducible nitric oxide (NO) synthase (iNOS) induction both in mouse hearts and in isolated cardiomyocytes subjected to LPS. In that study (170), the antiapoptotic effect of LPS was almost abolished in cardiomyocytes deficient of iNOS, suggesting that iNOS was essential for the TLR4-induced antiapoptotic effect (26). It is unclear and of great interest as to how endogenous iNOS regulates, in response to TLR4 stimulation, apoptosis signaling. It is noteworthy that iNOS also appears essential for the other form of cardioprotection, i.e., ischemic preconditioning (47), and that NO, derived from NO donors, has been shown to inhibit caspase-3 activity through S-nitrosylation and to reduce cardiomyocyte apoptosis (92, 129). However, the role of endogenous iNOS-NO in response to TLR4 activation in regulating caspase activity in cardiomyocytes has yet to be determined.

**TLR2.** An early study indicated that the synthetic lipopeptide or *Escherichia coli* lipoprotein induced NF-κB activation and apoptosis in 293 cells (human embryonic kidney cells) that were stably transfected with human TLR2 but not in parental 293 cells which did not express TLR2, suggesting that TLR2 mediated a proapoptosis signaling (2). TLR2 also induces apoptosis in a human acute monocytic leukemia cell line THP-1 and in epithelial cells (2, 3). By contrast, other investigators have suggested that TLR2 signaling mediates an antiapoptotic effect and a proinflammatory pathway in cardiomyocytes subjected to hydrogen peroxide (40). These investigators found that oxidative stress-induced cytotoxicity in neonatal rat car-
diomyocytes was enhanced by blocking TLR2 signaling with a specific antagonistic antibody. The reason for the discrepancy is unclear but likely multifactorial. The final phenotypic outcome of TLR2 activation with respect to cell survival may well be dependent on cell types (cell lines vs. primary cultures) and the type of apoptosis models and agents used.

**MYD88.** In the transfected 293 cell line, the expression of high levels of exogenous MYD88 mediated TLR2-induced apoptosis, possible through FADD-caspase-8-dependent pathway (3). However, using a genetically engineered mice (MyD88\(-/-\)) and in an acute lung injury model, Jiang et al. (68) have recently demonstrated that MyD88 mediates an antiapoptotic signal in lung epithelial cells and plays a critical role in pulmonary tissue repair and inflammation during the bleomycin-induced lung injury. Similarly, MyD88 appears essential for TLR4-activated antiapoptotic signaling in mouse cardiomyocytes (170) and in macrophages (90). Moreover, the adenovirus-mediated expression of MyD88 modulates TLR2-induced cytokine production in mouse cardiomyocytes (169) but is not sufficient to affect cardiomyocyte apoptosis in vitro. By contrast, others reported that an overexpression of dnMyD88 led to reduced cardiomyocyte apoptosis in an in vivo model of I/R injury (59).

Interestingly, myocardial IRAK-1, a kinase critical for TLR signaling, quickly becomes activated in response to ischemic injury (26). It is unclear, however, as to how the kinase activation is initiated upon myocardial ischemia and the biological significance that it may confer. Importantly, transgene expression of IRAK-1, but not its kinase-deficient mutant, appears sufficient to protect cardiomyocytes against apoptosis in vitro (26).

**LPS Preconditioning Against I/R Injury**

Evidence from several lines of investigation suggests that the activation of systemic TLR4 by LPS protects myocardium against I/R injury (Table 1). In animal models of I/R injury (13, 18, 48, 88, 89, 99, 106, 138, 155, 166, 167), in both in vivo (13, 48, 99, 138, 155, 166, 167) and ex vivo (18, 88, 106), a prior systemic administration of a sublethal dose of LPS reduces subsequent MI and improved cardiac functions. For example, hearts isolated from rats pretreated with a low dose of LPS (0.5 mg/kg) 24 h before had a better preserved myocardial function after I/R compared with the saline-treated control hearts (18, 105). Similar cardiac protection in LPS-treated animals was observed in vivo and in different animal models of I/R injury, such as rabbit (13, 99), rat (18, 88, 106, 138, 155, 166, 167), and mice (48). The cardioprotective effect of LPS usually occurs between 12–24 h after the administration of LPS and is abolished by cycloheximide (106), suggesting a mechanism involving the de novo synthesis of cardioprotective proteins. LPS administration induces a robust induction of iNOS in the heart (154, 170), a process mediated via TLR4 (170). Similar to ischemic preconditioning (16), the cardioprotection conferred by LPS seems to be mediated by iNOS (155) and by Akt (48). Ha and colleagues (48) recently demonstrated that the cardiac benefit of LPS against I/R injury was abolished by an intraperitoneal administration of a PI3-kinase inhibitor or in transgenic mice expressing the inactive Akt mutant. These data suggest that LPS exhibits its cardioprotection via PI3-kinase/Akt pathway and appear to be consistent with the in vitro observation that Akt, among other survival pathways, mediates a TLR4-mediated antiapoptotic benefit (26, 170). The aforementioned studies are important since they demonstrated a clear link between the activation of systemic TLR4 innate immune system and cardioprotection against ischemic myocardial injury and are consistent with the in vitro findings in isolated cardiomyocytes that demonstrate the antiapoptotic property of TLR4 signaling. However, given the multiple systemic responses following an in vivo administration of LPS, it is unclear whether the observed cardiac benefits are the direct results of TLR4 signaling or due to other events secondary to systemic TLR4 activation. Therefore, the critical role of TLR4, particularly that of cardiac origin, in the cardioprotection in vivo remains to be defined.

**TLR Signaling Mediates Myocardial I/R Injury**

Innate immune response during myocardial I/R. The innate immune response to I/R is, by far, the most common cause of myocardial inflammation. Acute inflammation is a complex response of soluble and cellular factors that together serve as the effector mechanisms of innate immunity. Molecular and cellular mechanisms underlying I/R injury are complex (164), involving the activation of endothelial cells and complement, an increased vascular permeability, and a rapid accumulation of neutrophils (38, 110, 119). Several hours after the onset of myocardial reperfusion, neutrophils accumulate in the infarcted myocardial tissue in response to the release of chemoattractants: reactive oxygen species (ROS), cytokines, and the

<table>
<thead>
<tr>
<th>Cardiac I/R Models</th>
<th>Species</th>
<th>Effects on the Heart</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vivo I/R</td>
<td>Rat</td>
<td>Increased LVDP</td>
<td>18, 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased ( +dP/dt_{\text{max}} )</td>
<td></td>
</tr>
<tr>
<td>Neutrophil-mediated I/R</td>
<td>Rat</td>
<td>Increased LVDP</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased ( +dP/dt_{\text{max}} )</td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>Rat</td>
<td>Reduced ventricular arrhythmias and infarct size</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Reduced infarct sizes; decreased cardiac troponin T</td>
<td>166, 167</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Reduced infarct sizes via iNOS induction</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Reduced infarct sizes</td>
<td>13, 99</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Reduced infarct sizes via PI3-kinase/Akt</td>
<td>48</td>
</tr>
</tbody>
</table>

I/R, ischemia-reperfusion; LVDP, left ventricular (LV) developed pressure; \( +dP/dt_{\text{max}} \), maximum first derivative of developed LV pressure; \( -dP/dt_{\text{max}} \), minimum first derivative of developed LV pressure; iNOS, inducible nitric oxide synthase; PI3-kinase, phosphatidylinositol 3-kinase.
activated complements. The upregulated cell-adhesion molecules (ICAM-1, VCAM-1, and P-selectin), likely mediated by NF-κB activation (86) in response to myocardial ischemia, facilitate the migration of neutrophils into the myocardial tissue. Neutrophils mediate cardiomyocyte death by causing vascular plugging, releasing degradative enzymes, and ROS (152), which causes myocardial injury by inducing a mitochondrial permeability transition pore opening and a subsequent ATP depletion and cell death (50). Although many of the downstream events leading to I/R injury as mentioned above have been identified, the proximal signaling mechanisms that control these critical events during I/R remain incompletely defined.

**Inflammation contributes to I/R injury.** Evidence from several lines of investigation suggests that inflammation is an important functional contributor to the pathogenesis of ischemic myocardial injury. For example, in animal models, neutrophil depletion with antibodies (128) or physical filtering (34, 61), as well as the inhibition of neutrophil adhesion with anti-CD18 monoclonal antibody (142), all substantially reduce injury after reperfusion. In addition, interventions targeted at a variety of specific inflammatory mediators have demonstrated benefits in I/R injury, including complement depletion (96) or lipooxygenase inhibitors (133), or antibodies to the proinflammatory cytokine IL-1 (60). However, some anti-inflammatory intervention, notably corticosteroids, have yielded disappointing results and actually increased infarct size and its complications (126). Interestingly, some investigators have suggested that inflammation may actually play a beneficial role in the healing process after infarction (77). It is likely that inflammation may play multiple roles in injury and recovery after I/R injury. For example, early neutrophil infiltration may contribute to injury but later, predominantly mononuclear leukocyte recruitment may represent an important part of the healing process.

It is noteworthy that I/R injury can also occur experimentally in isolated hearts perfused with crystalloid solutions lacking cellular blood components and other systemic contributors (51) (e.g., in Langendorff perfusion system), as well as in the heart perfused with whole blood (e.g., in vivo I/R injury models). The common feature under both conditions is the reintroduction of molecular oxygen, which contributes to the production of oxygen free radicals that cause tissue injury. Under conditions of whole blood reperfusion, there is the added influence of inflammatory cells such as neutrophils, as well as other blood-borne components. In addition, whole blood reperfusion in vivo also involves the activation of the coagulation cascade and the formation of the thrombolic occlusion of vessels and heterogeneity in the distribution of blood flow within the area of risk.

**TLRs mediate myocardial inflammation and injury during I/R.** Frantz and colleagues (41) first documented that there was an enhanced TLR4 expression in remodeling murine myocardium remote from sites of ischemic injury and in heart tissue from patients with idiopathic dilated cardiomyopathy. What is unclear, however, is the functional significance of TLR4 upregulation under the cardiac conditions. During the past five years, evidence has emerged that clearly indicates that in addition to its role in the host immunity against invading pathogens, TLR signaling may also play a critical role in modulating cell survival and tissue injury (or repair) in “noninfectious” injury models in several organs, such as lung (68), liver (121), brain (84, 144), and heart (28, 118, 136, 149), although, to make things even more complicated, TLR signaling seems to have different roles in different injury models. Of note, all of these investigations have been carried out in mouse models where genetic modifications of target genes are readily available. For example, in the bleomycin-induced acute lung injury model (68), TLR2 and TLR4 seemed to mediate proinflammatory and prosurvival signaling. Hyaluronan, produced in response to the acute lung injury, induces a proinflammatory and an antiapoptotic effect in lung epithelial cells via both TLR2- and TLR4-MyD88-dependent mechanisms (68). Similarly, in a hyperoxic lung injury model, the lack of TLR4 led to an increase in lung injury, apoptosis, and mortality (168), whereas the induced, active TLR4 (CD4hTLR4) transgenic expression in the lung confers resistance to hyperoxia-induced pulmonary apoptosis (124). By contrast, mice with the inactive TLR4 mutant or genetically deficient for TLR4 (28, 75, 118), TLR2 (35), or MyD88 (36) or pretreated intravenously with a TLR4 antagonist (Eritoran) (134) exhibited reduced MI sizes compared with WT or vehicle-treated animals, respectively, suggesting that TLR2 and TLR4 signaling contributed to ischemic injury in the heart (Table 2). However, the in vivo studies may have been complicated by the systemic deficiency/inhibition of TLR signaling. The lack of TLR signaling in extra-cardiac sources such as inflammatory cells could have contributed to the reduction of myocardial inflammation and thus injury after I/R. Recent studies have supported the notion that TLR4 of the extracardiac source (i.e., bone marrow-derived hematopoietic cells) plays an important role in mediating cardiac dysfunction under certain pathological conditions (145). In fact, in all of these studies mentioned above, a systemic TLR deficiency also leads to a significant reduction in the level of myocardial inflammation as measured by neutrophil recruitment, an NF-κB-dependent expression of cytokines and chemokines, and a complement deposition in the heart after I/R. Therefore, it seems possible that the reduction in myocardial inflammation may have contributed to reduced MI sizes in these TLR-deficient animals. Thus the exact role of cardiac (vs. bone marrow-derived inflammatory cell) TLRs in ischemic myocardial injury remains to be investigated.

One of the key questions with respect to the role of TLR signaling in myocardial I/R injury is if and how TLRs become activated in response to ischemia and reperfusion. The observation that myocardial IRAK-1 quickly becomes activated following coronary artery ligation seems to suggest that TLRs (or IL-1) respond to ischemic insult, although the upstream signaling remains unclear (26). Over the past 10 years, using the cells derived from TLR2−/− or TLR4 mutant mice or cells transfected with TLRs, investigators have identified a numbers of endogenous mediators that induce proinflammatory cytokine production through TLR2- and/or TLR4-dependent manners (Table 3). For example, in addition to LPS, TLR4 recognizes heat shock proteins (Hsp-22, Hsp-60, and Hsp-70) (6, 31, 113, 127, 150, 151), fibrinogen (137), fibronectin containing repeat extraneous A (114), surfactant protein-A (46), and soluble heparan sulfate (70). TLR2 is known to recognize Hsp-60 and Hsp-70 (6, 150, 151) and hyaluronan (68). Some of these endogenous molecules such as Hsp-60 and Hsp-70 are produced in response to myocardial ischemia (78, 95) and confer potent antiapoptotic and cardioprotective effects in the
heart (66, 68, 76, 115). It remains unclear, however, and would be of great importance to investigate whether or not TLRs mediate the cardiac benefit induced by these molecular chaperons and how to reconcile these findings with the suggested role of TLRs in I/R injury in the aforementioned loss-of-function studies.

MyD88, a key adaptor, contributes to myocardial I/R injury. As illustrated in Fig. 1, MyD88 is a key adaptor protein that is critical for transducing signals from all TLR family members (64, 73, 104, 108), except TLR3, and IL-1 receptor family members. Recent studies, employing adenovirus-mediated transgene expression and genetically modified mice, have demonstrated that MyD88 may play an important role in the pathogenesis of I/R injury in the heart. When overexpressed in the heart, dnMyD88 led to a reduction in MI size and apoptosis in a rat model of I/R injury (59). It is of interest that the transgene expression of dnMyD88 also led to a decrease in myocardial inflammation given the well-characterized host innate immune response to adenoviral vectors in the heart (122). In a mouse model of I/R injury, Feng and colleagues (36) recently found that mice deficient of MyD88 had a reduced neutrophil recruitment and attenuated cytokine/adhesion molecule production (keratinocyte chemoattractant, monocyte chemoattractant protein-1, and ICAM-1), smaller infarct sizes, and much better preserved ventricular function as demonstrated by serial transthoracic echocardiographic and invasive hemodynamic measurements. In an effort to determine the potential impact of MyD88 deficiency in circulatory

<table>
<thead>
<tr>
<th>Mice</th>
<th>Infarct Models</th>
<th>Effects in Knockout Mice</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2−/−</td>
<td>I/R (30’ I/60 R’)</td>
<td>Smaller infarct sizes, reduced neutrophil recruitment, reduced ROS and cytokines</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Permanent coronary ligation</td>
<td>Improved survival rate, attenuated remodeling, but same infarct sizes at 4 wk</td>
<td>136</td>
</tr>
<tr>
<td>TLR4</td>
<td>C57 Bl/10 ScCr</td>
<td>Smaller infarct sizes, reduced MPO activity and complement 3 deposition</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>C3H/HeJ</td>
<td>Smaller infarct sizes, decreased cardiac expression of TNF, MCP-1, and ILs</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>I/R (60’ I/24 h R)</td>
<td>Smaller infarct sizes, but no gain in LV function</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>C3H/HeJ</td>
<td>Smaller infarct sizes, reduced pJNK, reduced cytokine expression</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>WT with eritoran</td>
<td>Reduced LV remodeling, improved systolic function, reduced cytokine expression</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>I/R (30’ I/120’ R)</td>
<td>Improved LV function on day 6 after infarction, improved survival rate, reduced LV remodeling and apoptosis at 4 wk.</td>
<td>125</td>
</tr>
<tr>
<td>C57 Bl/10 ScCr</td>
<td>Permanent coronary ligation</td>
<td>Smaller infarct sizes, improved LV function, and attenuated cytokine expression and neutrophil recruitment</td>
<td>36</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>I/R (30’ I/24 h R)</td>
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</table>

Table 2. TLR signaling modulates myocardial I/R injury and remodeling

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Endogenous Ligands</th>
<th>Cells</th>
<th>End Points</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Hsp-60</td>
<td>Fibroblasts</td>
<td>P38, JNK1/2, ERK1/2, IKK</td>
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</tr>
<tr>
<td></td>
<td>Hsp-70</td>
<td>HEK293</td>
<td>NF-κB</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Hyaluronan</td>
<td>Fibroblasts</td>
<td>NF-κB</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Macrophages</td>
<td>MIP-2, TNF, KC</td>
<td>68</td>
</tr>
<tr>
<td>TLR4</td>
<td>Hsp-22</td>
<td>Macrophages</td>
<td>IL-6</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Hsp-60</td>
<td>Fibroblasts</td>
<td>P38, JNK1/2, ERK1/2, IKK</td>
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<td></td>
<td>Hsp-70</td>
<td>HEK293</td>
<td>NF-κB</td>
<td>6</td>
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<tr>
<td></td>
<td></td>
<td>Fibroblasts</td>
<td>NF-κB</td>
<td>151</td>
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<tr>
<td></td>
<td></td>
<td>Macrophage</td>
<td>TNF</td>
<td>31</td>
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<tr>
<td></td>
<td></td>
<td>HEK293</td>
<td>NF-κB</td>
<td>114</td>
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<td></td>
<td></td>
<td>Fibronectin (extra domain A)</td>
<td>HEK293</td>
<td>114</td>
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<td></td>
<td>Fibrinogen</td>
<td>Macrophages</td>
<td>MCP-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyaluronic acid (oligosaccharides)</td>
<td>Dendritic cells</td>
<td>TNF, pMAPK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NF-κB</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparan sulfate (polysaccharide)</td>
<td>Dendritic cells</td>
<td>DC maturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyaluronan</td>
<td>Macrophages</td>
<td>MIP-2, TNF, KC</td>
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<tr>
<td></td>
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<td></td>
<td>NF-κB activity</td>
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<tr>
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<td></td>
<td>Lung surfactant protein A</td>
<td>Macrophages</td>
<td>TNF, IL-10</td>
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</table>

Hsp, heat shock protein; KC, keratinocyte chemoattractant; NO, nitric oxide; MCP-1, monocyte chemoattractant protein-1; pJNK, phosphorylated JNK; MyD88, myeloid differentiation primary-response gene 88.
components on cardiac injury associated with the in vivo model of I/R, they tested the impact of cardiac MyD88 deficiency on myocardial injury in isolated mouse hearts. Surprisingly, they found that in isolated hearts subjected to global I/R, MyD88 deficiency had no effect on MI and left ventricular (LV) function following I/R (36). This finding suggests that in an in vivo condition, MyD88 may play a pivotal role in mediating myocardial inflammation that is critical for I/R injury and is consistent with the hypothesis that the cardiac benefits observed in MyD88−/− mice in vivo may require circulating blood components during I/R.

**TLR signaling modulates LV remodeling after ischemic injury.** LV remodeling represents myocardial structural changes in the LV in response to chronic alterations in loading conditions and contribute to morbidity and mortality after ischemic injury (117). LV remodeling can be induced by either pressure overload or volume overload, which results in a concentric remodeling or an eccentric hypertrophy, respectively. After MI, a stretched and dilated infarcted tissue increases LV volume with a combined volume and pressure load on noninfarcted areas. In a permanent coronary artery ligation model, Shishido and colleagues (136) demonstrated that TLR2-deficient mice had less LV remodeling at 4 wk, improved LV function at 1 and 4 wk, and a significantly higher survival rate at 4 wk compared with WT mice (Table 2). The reduced LV remodeling was demonstrated by a decreased myocardial fibrosis in the noninfarcted areas, a reduced transforming growth factor-β, a collagen type 1 mRNA expression, and small LV dimensions at end diastole. Interestingly, the infarct size (at 4 wk) and level of inflammation (at 3–7 days) were similar between the two groups of animals in this model of MI. It is noteworthy, however, that the investigators in that study did not measure the infarct sizes during the early stage (e.g., 24–48 h) of ischemic injury. Therefore, the possibility that a potential MI size reduction in TLR2−/− mice compared with WT mice may contribute to the attenuated myocardial remodeling in TLR2−/− animals needs to be ruled out. In a similar model of postinfarction remodeling, TLR4 was found to modulate survival as well as LV remodeling after ischemic injury (125, 149). Although an enhanced JNK expression has been implicated as a possible intracellular mechanism for the attenuated LV remodeling in TLR4−/− mice (125), the exact mechanisms as to how TLR2 and TLR4 signaling is initiated during myocardial ischemia and how they modulate subsequent ventricular remodeling remain elusive.

**Summary**

During the past decade, emerging evidence has indicated that in addition to their critical role in host immunity against microbial infection, TLRs can also function as a sensor responding to tissue stress such as hypoxia and ischemia and modulate cell survival and tissue injury. Several lines of investigation have demonstrated the importance of cardiomyocyte apoptosis in myocardial I/R injury. Existing data suggest that TLR2 and TLR4 may mediate both pro- and antiapoptotic signaling, and the final phenotypic outcome probably depends on the cell types and the model of apoptosis used, although recent studies, both in vitro and in vivo, appear to support the notion that a direct activation of TLR4 leads to an antiapoptotic signaling in cardiomyocytes. A large body of evidence in several animal models demonstrates that LPS, at sublethal doses, has a preconditioning-like effect protecting the heart against subsequent I/R injury. However, given the multiple systemic reactions in response to an in vivo administration of LPS in these studies, it is unclear whether the observed cardiac benefits are the direct result of TLR4 stimulation or due to other events secondary to systemic TLR4 innate immune activation. Therefore, the critical role of TLR4, particularly that of cardiac origin, in the cardioprotection in vivo is still unclear. By contrast and somewhat paradoxically, studies from several laboratories using knockout mouse models suggest that TLR2, TLR4, and MyD88 may all contribute to myocardial inflammation and infarction after I/R. It is noteworthy, however, that in these models, TLR and MyD88 deficiency are systemic and, therefore, the exact contribution of TLR signaling of cardiac (vs. circulatory) origin is unknown. Nevertheless, the disparate findings illustrate the complexity and difficulties in defining the emerging role of TLRs as a critical modulator in both tissue inflammation and injury. Defining the role of innate immune signaling in ischemic myocardial injury may have important therapeutic implications.

**GRANTS**

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Review

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