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FORUM REVIEW ARTICLE

Toll-Like Receptors: New Players in Myocardial Ischemia/Reperfusion Injury

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Abstract

Innate immune and inflammatory responses have been implicated in myocardial ischemia/reperfusion (I/R) injury. However, the mechanisms by which innate immunity and inflammatory response are involved in myocardial I/R have not been elucidated completely. Recent studies highlight the role of Toll-like receptors (TLRs) in the induction of innate immune and inflammatory responses. Growing evidence has demonstrated that TLRs play a critical role in myocardial I/R injury. Specifically, deficiency of TLR4 protects the myocardium from ischemic injury, whereas modulation of TLR2 induces cardioprotection against ischemic insult. Importantly, cardioprotection induced by modulation of TLRs involves activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, suggesting that there is a crosstalk between TLRs and PI3K/Akt signaling pathways. In addition, TLRs also associate with other coreceptors, such as macrophage scavenger receptors in the recognition of their ligands. TLRs are also involved in the induction of angiogenesis, modulation of stem cell function, and expression of microRNA, which are currently important topic areas in myocardial I/R. Understanding how TLRs contribute to myocardial I/R injury could provide basic scientific knowledge for the development of new therapeutic approaches for the treatment and management of patients with heart attack. *Antioxid. Redox Signal*. 15, 1875–1893.

The Innate Immune Response in Myocardial Ischemia/Reperfusion Injury

THERE IS COMPELLING EVIDENCE that the innate immune response plays an important role in myocardial ischemia/reperfusion (I/R) injury (17, 18, 22, 25, 46, 47, 92, 93, 98, 110) and congestive heart failure (CHF) (40, 82, 85, 103, 121, 135, 172). Since a myocardial depressant factor was found in the sera of septic patients and experimental animals (100) 40 years ago, inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6 have been implicated as important factors in cardiac dysfunction (139, 140). Numerous studies have shown that I/R significantly increases inflammatory cytokine gene expression, that is, TNF-α, IL-1β, IL-6, IL-8, interferon-gamma (IFN-γ), and intercellular adhesion molecule-1, in the myocardium (68, 94, 95, 185). These proinflammatory and immunoregulatory cytokines appear to be directly involved in the progression of myocardial I/R injury, myocardial dysfunction, vascular wall remodeling, heart failure, and cardiac hypertrophy (87, 114, 130, 133). Elevated levels of these inflammatory cytokines and adhesion molecules in plasma and myocardium have also been observed in patients with CHF (40, 82, 85, 103, 118, 121, 135, 156, 172). However, it is unclear how the immune response is activated in ischemic myocardial diseases.

Interestingly, cardiac myocytes exhibit properties that are reminiscent of the innate immune response. For example, in response to I/R, or a systemic inflammatory insult such as sepsis/septic shock cardiac myocytes express inflammatory cytokines which initially activate a local inflammatory response. Cardiac myocytes also express cell surface molecules and chemokines, resulting in attraction of inflammatory cells into the myocardium. These observations suggest that cardiac myocytes are involved in innate immune and inflammatory responses, including release of inflammatory cytokines, chemokines, and expression of cell surface molecules which attract inflammatory cell infiltration. It has been demonstrated that these responses are involved in cardiac dysfunction. One important question is how I/R insult could trigger the innate immune and inflammatory responses of cardiac myocytes. Recent evidence demonstrated that Toll-like receptors (TLRs) play a critical role in the induction of innate immune and inflammatory responses (2, 7, 23, 124, 147, 182, 192).

TLRs are one of the pattern recognition receptors (PPRs) that recognize microbial molecular motifs called pathogen-associated molecular patterns (PAMPs) and initiate a

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defensive response to microbial invasion (67, 80, 166). The physiological functions of PRRs include to discriminate "self" and "non-self" molecules and initially activate innate immune and inflammatory responses to the non-self molecules; to regulate adaptive immune responses; to sense endogenous molecules or danger signals that are released from injured tissues; and to maintain tissue homeostasis by regulating tissue repair and regeneration. At present, three families of PRRs have been identified: TLRs, nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid inducible-gene-1 (RIG)-like receptors (RLRs) (67, 80, 166). TLRs were the first described and are the most widely studied. Increasing evidence suggests that TLRs are involved in many pathophysiological processes in human disease (67, 80), including cancer, allergic diseases (asthma), autoimmune disease (lupus), viral infection disease, infectious disease (sepsis/septic shock), inflammatory bowel disease, and cardiovascular disease (atherosclerosis, stroke). Recently published data have shown that TLRs are central players in myocardial I/R injury (10, 13, 21, 34, 41, 48, 136, 150, 159, 169). Further, modulation of TLR-mediated signaling pathways induce cardioprotection against myocardial I/R injury.

There have been several excellent reviews published recently regarding the role of TLRs in cardiovascular disease (14, 29, 54, 84, 165), In this review, we summarize recent progress in defining the role of TLRs during myocardial I/R and introduce new evidence showing the interactions between TLRs and important intracellular signaling pathways during myocardial I/R. Specifically, we discuss the role of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in modulation of TLR-induced cardioprotection. We also discuss the interactions between TLRs and other coreceptors, such as macrophage scavenger receptors (SRs), in the recognition of PAMPs. In addition, we summarize recent data showing that TLRs are involved in microRNA (miRNA) expression, induction of angiogenesis, and modulation of stem cell function. These new data will result in a better understanding of the mechanisms of TLRs in myocardial I/R injury and promote the development of new approaches for the treatment of patients with heart attack.

TLRs Are Critical for the Induction of Innate Immune and Inflammatory Responses

Toll-like receptors

Toll receptors are an ancient and evolutionarily conserved receptor family that regulate antimicrobial host defense in plants, invertebrates, and mammals (2, 7, 23, 124, 147, 182, 192). There are many review articles on TLRs (2, 4, 20, 67, 80, 86, 122, 166, 192); therefore, we will only briefly introduce TLRs in this review. Toll protein was originally identified in *Drosophila* as an essential component of the pathway that determines the dorsal–ventral axis in early embryogenesis in 1986 (8, 9, 102). Ten years later, Toll protein was demonstrated to play a critical role in the production of the anti-fungal peptide in *Drosophila melanogaster* immunity (102). Subsequently, human homologues of Toll, designated as TLRs, were discovered in 1997 (124). Toll protein is a type I transmembrane receptor whose extracellular region contains leucine-rich repeat (LRR) motifs. The cytoplasmic domain of Toll is similar to the mammalian IL-1 receptor (IL-1R) family, designated as the Toll/IL-1R (TIR) homology domain (124). At present, 10 TLRs have been identified in humans (35, 39). Mammalian TLRs are also characterized by extracellular LRR motifs and a cytoplasmic TIR homology domain, which is similar to that of the IL-1R family proteins. The IL-1R/TLR family shares a common signaling pathway leading to the activation of nuclear factor kappaB (NF-κB). TLR expression has been observed in various cells, including cardiac myocytes and endothelial cells (2, 7, 124, 147, 192).

TLRs recognize PAMPs

Innate immunity is the first line of defense against pathogens. Innate immune recognition is mediated by germline-encoded receptors/signal transduction molecules that recognize highly conserved macromolecular structures that are present in the cell wall of most pathogenic microorganisms, but are not present in higher species (122, 123). These structures are referred to PAMPs and the receptors that recognize PAMPs are called pattern-recognition receptors (PRRs). The best known PAMPs are lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acid (LTA) from gram-positive bacteria, mannans and glucans from fungal cell walls, as well as cytidine phosphate guanosine (CpG)-DNA from bacteria and double- or single-stranded RNA (ssRNA) from viruses (5, 20, 166). Recognition of PAMPs by PRRs results in the activation of intracellular signaling cascades that stimulate the expression of various genes including immune response and inflammation. The TLR family is one of the best characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (20, 86, 166). Recent studies have highlighted the critical role of TLRs in the recognition of PAMPs and their subsequent stimulation of intracellular signaling (2, 7, 23, 38, 124, 147, 182, 192).

Based on their subcellular localization, TLRs are divided into cell surface TLRs and intracellular TLRs. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, and TLR6, which recognize structures unique to bacteria or fungi (Fig. 1). TLR2 recognizes various lipoproteins from bacteria, mycoplasma, and fungi. TLR2 recognizes its ligands by forming a heterodimer with either TLR1 (TLR1/TLR2 to recognize triacyl lipoproteins) or TLR6 (TLR2/TLR6 to sense diacyl lipoproteins). TLR4 recognizes LPS derived from the outer membrane of gram-negative bacteria. This recognition is mediated with myeloid differentiation factor 2 (MD2) on the cell surface. TLR4 is also involved in recognition of viral envelope proteins. TLR5 is highly expressed by dendritic cells of the lamina propria in the small intestine and recognizes flagellin from flagellated bacteria.

Intracellular TLRs include TLR3, TLR7, TLR8, and TLR9 and are localized in intracellular compartments. Intracellular TLRs recognize nucleic acids derived from viruses and bacteria, as well as endogenous nucleic acids. TLR3 detects viral double-stranded RNA (dsRNA) in the endolysosome and recognizes a synthetic dsRNA, polyinosinic-polycytidylic acid (poly I:C). Mouse TLR7 and human TLR7/8 recognize ssRNA from RNA viruses. TLR9 senses unmethylated DNA with CpG motifs derived from bacteria and viruses (Fig. 1).

TLR Signal Transduction and Activation of NF-κB and IFN Regulatory Factor 3

Recognition of PAMPs by TLRs leads to activation of signaling pathways, resulting in upregulating the expression of

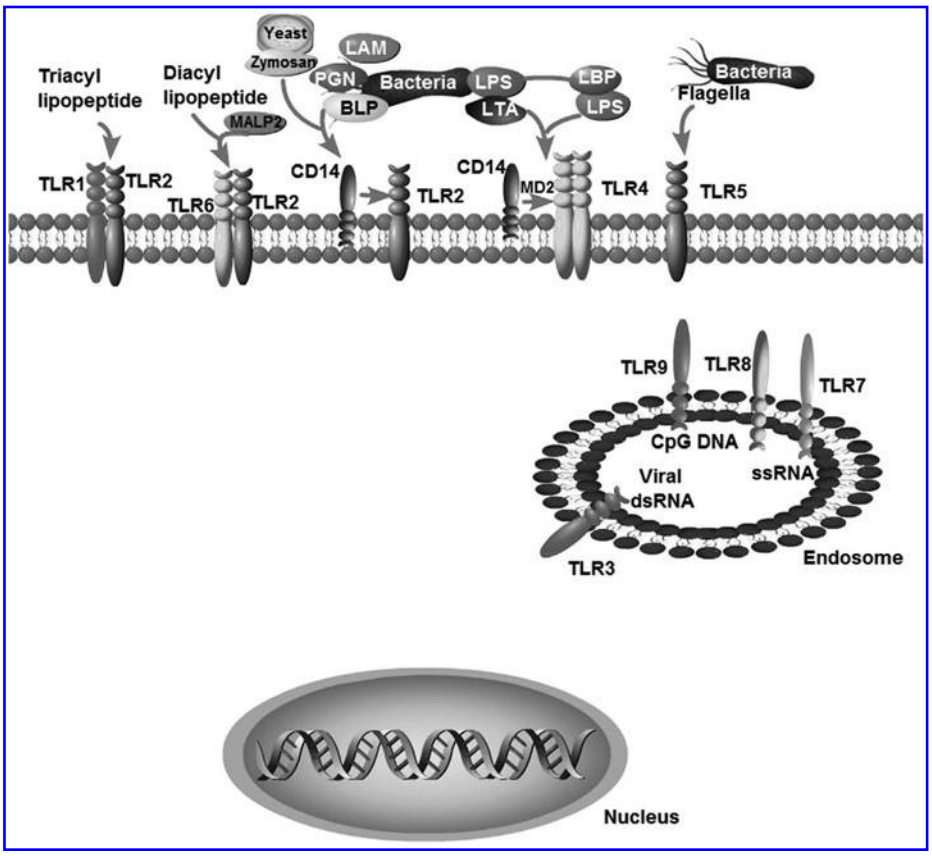

FIG. 1. Toll-like receptor (TLR) localization and their ligands. TLRs can be divided as cell surface TLRs and intracellular TLRs. Cell surface TLRs are TLR1, TLR2, TLR4, TLR5, and TLR6. They recognize pathogen-associated molecular patterns (PAMPs) from bacteria and fungi. TLR1/TLR2 recognize triacyl lipoproteins, whereas TLR6/TLR2 recognize diacyl lipoproteins and macrophage-activating lipopeptide 2 (MALP-2). TLR2 can sense zymosan from fungi, lipoarabinomannan (LAM) from mycobacteria, peptidoglycan (PGN) from gram-positive bacteria, and bacterial lipoprotein (BLP). TLR4 recognizes lipopolysaccharide (LPS) and lipoteichoic acid (LTA). LPS interacts with LPS binding protein (LBP) to form a complex that is recognized by CD14. CD14 is a glycosylphosphatidylinositol-linked, leucine-rich repeat-containing protein that binds LBP and delivers LPS-LBP to the TLR4-myeloid differentiation factor 2 (MD2) complex. TLR5 recognizes the flagellin protein component of bacterial flagella. Intracellular TLRs contain TLR3, TLR7, TLR8, and TLR9. TLR3 recognizes double-stranded RNA (dsRNA) and synthetic polyinosinic-polycytidylic acid (poly I:C). TLR7 recognizes single-stranded RNA (ssRNA) derived from RNA viruses. TLR8 is phylogenetically most similar to TLR7. Human TLR8 mediates the recognition of R-848 and viral ssRNA. TLR9 recognizes unmethylated 2′-deoxyribo(cytidine-phosphate guanosine) (CpG) DNA motifs that are frequently present in bacteria and viruses.

distinct genes. The differences between individual TLR-activated signaling cascades could be due to different TIR domain-containing adaptor molecules that are recruited to TLRs. There are five TIR domain-containing adaptors: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain containing adaptor protein (TIRAP)/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM). Based on the usage of distinct adaptor molecules, TLR signaling is roughly divided into two distinct pathways: MyD88-dependent and TRIF-dependent signaling pathways (Fig. 2).

The MyD88-dependent NF-κB signaling pathway

MyD88 is essential for the downstream signaling of various TLRs, with the exception of TLR3. TLR2- and TLR4-mediated signaling requires TIRAP/Mal as a bridge connecting between TLR and MyD88 (Fig. 2). MyD88 interacts with IL-1R-associated kinase 4 (IRAK-4), a serine/threonine kinase with an N-terminal death domain. IRAK-4 activates other IRAK family members, IRAK-1 and IRAK-2. The IRAKs then dissociate from MyD88 and interact with TNF receptor (TNFR)-associated factor 6 (TRAF6). TRAF6 acts as an E3 ubiquitin protein ligase. Together with an E2 ubiquitin-conjugating enzyme complex comprising Ubc13 and Uev1A, TRAF6 catalyzes the formation of a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself, resulting in generation of an unconjugated free polyubiquitin chain. The unconjugated free K63 polyubiquitin chain activates a complex composed of transforming growth factor-beta (TGF-β)-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3, which phosphorylates inhibitor of kappa B (IκB) kinase (IKK)-β and mitogen-activated protein kinase (MAPK) kinase 6. Subsequently, the IKK complex (IKK-α, IKK-β, and NF-κB essential modulator [NEMO]) phosphorylates IκB alpha (IκBα), which is an NF-κB inhibitory protein. Phosphorylated IκB undergoes degradation by the ubiquitin–proteasome system and the released NF-κB translocates into the nucleus and activates expression of proinflammatory cytokine genes. Activation of the MAPK signaling pathway is responsible for activation of the transcription factor complex (AP-1), which targets cytokine genes. In addition to activation of the MyD88-dependent NF-κB pathway, TLR7 and TLR9 signaling also induces the production of the type I IFNs through a TRIF-dependent pathway.

The TRIF-dependent signaling pathway

The TRIF-dependent pathway mediates the activation of both IFN regulatory factor 3 (IRF3) and NF-κB signaling pathways (Fig. 2). Stimulation of TLR3 and TLR4 can result in

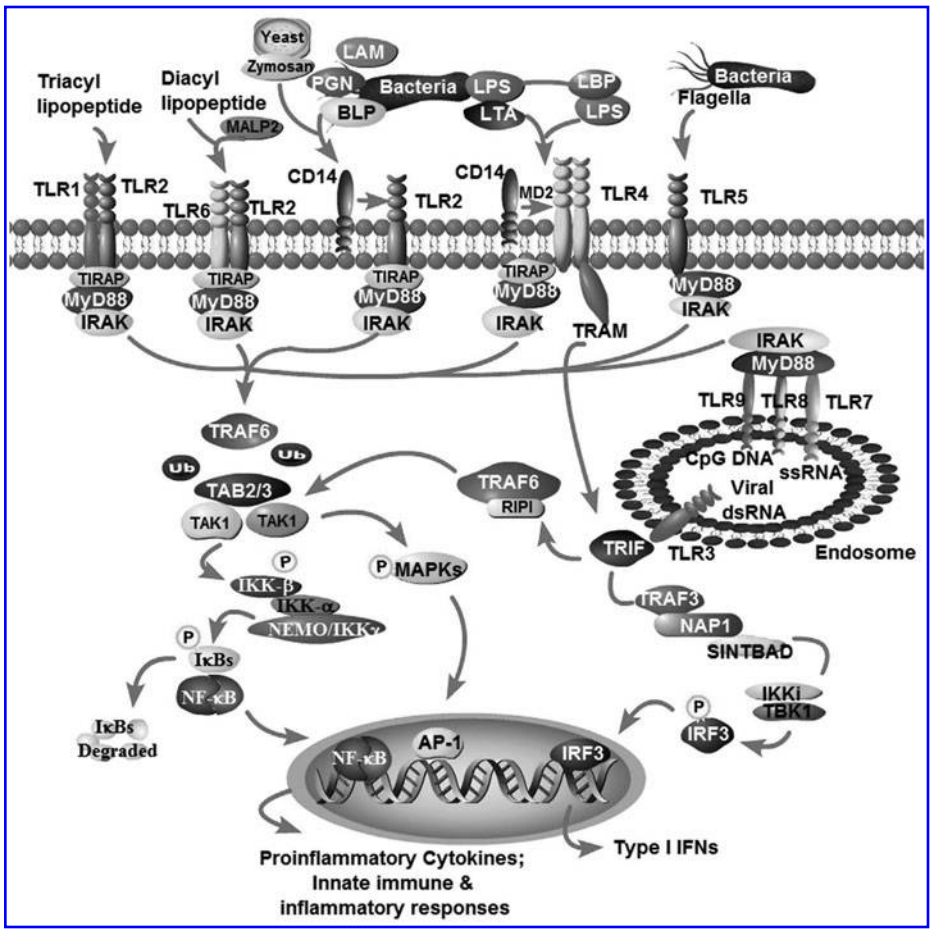

FIG. 2. TLR-mediated myeloid differentiation primary response gene 88 (MyD88)-dependent and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF)-dependent signaling pathways. Except for TLR3, other TLRs mediate cellular signaling through an MyD88-dependent pathway. MyD88 interacts with interleukin-1 receptor-associated kinases (IRAKs), which then associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which activates a complex composed of transforming growth factor-β-activated kinase 1 (TAK1) and TAK1 binding protein 1 (TAB1), TAB2, and TAB3. The complex leads to phosphorylation of inhibitor of kappa B (IκB) kinases (IKK-α, IKK-β) and mitogen-activated protein kinases (MAPKs). The IKK complex (IKK-α, IKK-β, and nuclear factor-kappaB [NF-κB] essential modulator [NEMO]) phosphorylates alpha IκB alpha (IκBα), which undergoes degradation by the ubiquitin-proteasome system. The released NF-κB translocates into the nucleus and activates expression of proinflammatory cytokine genes. Activation of the MAPK signaling pathway is responsible for activation of the transcription factor complex (AP-1), which stimulates the expression of proinflammatory cytokine genes. The TRIF-dependent pathway mediates the activation of both IFN regulatory factor 3 (IRF3) and NF-κB signaling pathways. Stimulation of TLR3 and TLR4 activates the transcription factor IRF3 and subsequent induction of IFN-β and IFN-inducible genes. TLR3 directly recruits TRIF. TRIF interacts with the IKK-related kinases, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), and IKK-i, which leads to IRF3 phosphorylation, nuclear translocation, and DNA binding. Stimulation of TLR4 requires TRIF-related adaptor molecule (TRAM) adaptor protein for activating TRIF. TRIF-dependent signaling also activates the NF-κB pathway. TRIF recruits TRAF6 and activates TAK1, which in turn activates the NF-κB and MAPK pathways.

the activation of the transcription factor IRF3 and subsequent induction of IFN-β and IFN-inducible genes (3, 38, 77, 127, 174). The adaptor protein TRIF is a key regulator of the IRF3-mediated IFN-β pathway. TLR3 directly recruits TRIF, whereas TLR4 requires another adaptor TRAM for activating TRIF. TRIF interacts with the IKK-related kinases, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKK-i, which stimulates IRF3 phosphorylation, nuclear translocation, and DNA binding.

TRIF-dependent signaling also activates the NF-κB pathway. TRIF recruits TRAF6 and activates TAK1, *via* ubiquitination-dependent mechanisms that are similar to those of the MyD88-dependent pathway. TRIF also recruits the adaptor receptor-interacting protein 1 (RIP1) through the distinct RIP homotypic interaction motif. RIP1 undergoes k63-linked polyubiquitination and binds with the adaptor TNFR-associated death domain (TRAFDD), an essential adaptor for TNFR signaling. TRAFDD forms a complex with Fas-associated death domain (FADD)-containing proteins, RIP1, and Pellino-1. Pellino-1 is a member of the Pellino family of RING-like domain containing E3 ubiquitin ligases. Current data suggests that TRIF forms a multiprotein signaling complex along with TRAF6, TRAFDD, Pellino-1, and RIP1. The complex activates TAK1, which in turn activates the NF-κB and MAPK pathways. Thus, TRIF is likely to use TBK1 for IRF3 activation and to interact with RIP1 for NF-κB activation, respectively.

TLR-Mediated Signaling Pathways Interact (Crosstalk) with the PI3K/Akt Signaling Pathway

The PI3Ks are a conserved family of signal transduction enzymes that are involved in regulating cellular proliferation and survival (26, 49). PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate PI(4,5)P2 (PIP2) to PI(3,4,5)P3 (PIP3). Signaling proteins with pleckstrin homology domains bind to PIP3. These signaling proteins include phosphoinositide-dependent kinase-1 (PDK1), PDK2, and Akt (protein kinase B [PKB]). PDK activates Akt by

phosphorylation of Ser473 and Thr308 (26). Akt is an important physiologic mediator of the PI3K pathway. Phosphorylation of Akt activates the enzyme, which modulates cell cycle entry, growth, and survival (26). Activated Akt phosphorylates several downstream targets of the PI3K pathway, including glycogen synthase kinase-3 (GSK-3) (79, 116). GSK-3β is a crucial regulator of many cellular functions, including cell survival and apoptosis (79). Recent evidence suggests that stimulation of TLRs leads to activation of the PI3K/Akt signaling pathway (11, 115, 132) (Fig. 3). The cytosolic domain of TLR2 contains a PI3K binding motif (YXXM), which binds the phosphorylated form of the p85 subunit of PI3K (11). Stimulation of TLR2 causes the recruitment of active Ras-related C3 botulinium toxin substrate 1 (Rac1) and PI3K to the TLR2 cytosolic domain, resulting in activation of the PI3K/Akt pathway (11). Mal is an adaptor protein in the TLR-mediated signaling pathway. Recent evidence demonstrated that Mal connects TLR2 to PI3K activation (160). Similarly, stimulation of TLR3 (152), TLR4 (97), TLR9 (52, 53, 60), and IL-1R (132) results in activation of PI3K/Akt signaling through an adaptor protein called RIP. However, there is compelling evidence that TLR and the PI3K/Akt signaling pathways counter-regulate each other (52, 53, 60, 97, 149, 180). For example, mice deficient in the p85 subunit of PI3K show enhanced TLR responses to ligand stimulation (53), whereas activation of the PI3K/Akt signaling pathway limits the proinflammatory effects of the TLR4 agonist, LPS, in cultured monocytes (60). These data strongly suggest that PI3K/Akt may be a negative feedback mechanism that prevents excessive innate immune and/or inflammatory responses (52, 53, 180). However, the precise mechanisms by which PI3K/Akt signaling negatively regulates TLR pathways are unknown. It is also unclear whether TLR-mediated pathways will negatively regulate PI3K/Akt signaling. Recent studies have shown that blunting TLR4-mediated signaling with an immune modulator significantly increased PI3K/Akt activity in ischemic hearts (105, 180). Further, genetic depletion of TLR4 or blunting of MyD88 signaling resulted in increased basal levels of phospho-Akt (63, 70, 71). In addition, TRAF6 deficiency results in hyperactivation of the PI3K/Akt signaling pathway in T cells (91). Collectively, these data suggest that there is crosstalk between the TLR/NF-κB and PI3K/Akt signaling pathways and modulation of the crosstalk could protect the myocardium from I/R injury (Fig. 3).

TLRs Cooperate with Other Coreceptors

Recent evidence suggests that TLRs act together with the other coreceptors on the cell surface that assist PAMP recognition. For example, TLR2 associates with Dectin-1, a C-type lectin that binds fungal β-glucan and induces its internalization (56). The class B SR CD36 acts together with TLR2-TLR6 heterodimer to sense some TLR2 ligands (173). In addition, CD36 ligands promote sterile inflammation through assembly of a TLR4 and TLR6 heterotrimer (161). CD36 recognizes both oxidized low-density lipoprotein and amyloid-β peptide to stimulate an inflammatory cascade, which signals through Src kinases to induce a TLR heterodimer of TLR4 and TLR6 (161).

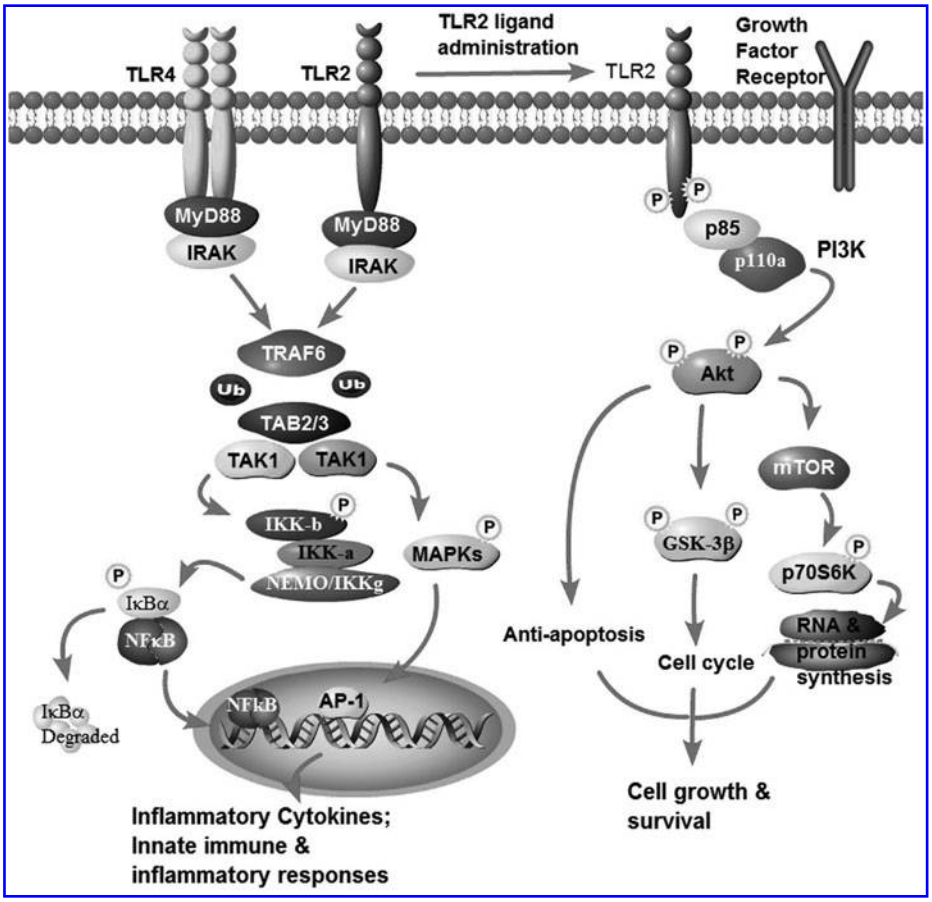

FIG. 3. TLRs crosstalk with the phosphoinositide 3-kinase (PI3K) signaling pathway. Stimulation of TLR4 or TLR2 leads to tyrosine phosphorylation of TLRs. Subsequently, the phosphorylated TLRs associate with the p85 subunit of PI3K, resulting in PI3K activation. Activated PI3K phosphorylates Akt at serine 473 and threonine 308 residues. Activated Akt phosphorylates glycogen synthase kinase 3-beta (GSK-3β) and leads to p70S6K phosphorylation through mammalian target of rapamycin (mTOR)-dependent mechanism.

Recently published data also suggest that macrophage SR class A (SR-A) is a coreceptor for TLRs to facilitate innate immune recognition and response, resulting in an over exuberant response (155). SR-A (aka CD204) was initially discovered due to its ability to bind and internalize modified low-density lipoprotein (55). Subsequently, SR-A has been demonstrated to recognize and clear modified host components, apoptotic cells, and pathogens (57, 143). SR-A has recently been demonstrated to act as a pattern recognition receptor (PRR) and can recognize several PAMPs, such as LPS, LTA, bacterial CpG DNA, dsRNA, and yeast zymosan/β-glucans (12, 69, 109, 128, 194). A growing body of evidence suggests that SR-A could be a coreceptor for TLRs to facilitate innate immune recognition and response (155). As an example, TLR ligands synergize with SR-A to mediate bacterial phagocytosis (6), induce SR-A expression (184), and promote SR-A binding to the TLR4 ligand, LPS (184). SR-A interacts with TLR4 to promote a proinflammatory, proapoptotic phenotype in LPS-exposed macrophages (155). SR-A may also suppress IRF3-mediated IFN-β production (155). On the other hand, SR-A ligands trigger apoptosis in endoplasmic reticulum-stressed macrophages by cooperating with TLR4 (155) and serve as a negative regulator of TLR4 in mediating immune responses (190). Collectively, these data suggest that SR-A promotes activation of innate immune and inflammatory responses (69, 109, 194), by acting as a coreceptor to TLR4 (155), and by suppressing the prosurvival signaling pathway (155). Further studies are needed to determine whether TLRs will interact with coreceptors, such as SRs during myocardial I/R, which would provide new mechanisms of TLRs in myocardial I/R injury.

TLRs and Reactive Oxygen Species Signaling Pathways

It is well documented that reactive oxygen species (ROS) play an important role in myocardial I/R injury. Recent evidence suggests that ROS activates NF-κB through a TLR4-dependent mechanism. For example, hydrogen peroxide can trigger nuclear translocation of NF-κB, whereas many of the agents that stimulate NF-κB activation increase the generation of intracellular ROS (45, 76). A broad range of antioxidants prevents NF-κB nuclear translocation and activation, suggesting that NF-κB activation is induced when intracellular ROS generation is increased. However, it is still unclear which molecules are involved in ROS generation leading to NF-κB activation.

A recent study by Asehnoune *et al.* (15) examined the mechanisms by which ROS modulate the transcriptional activity of NF-κB in response to TLR4-dependent signaling. The authors observed that antioxidant treatment inhibited LPS-stimulated production of inflammatory cytokines, and activation of the kinases (IKK-α and IKK-β) and IRAKs in nitrophiles. The results demonstrated that TLR4-mediated signaling is oxidant dependent and that ROS can modulate NF-κB-dependent transcription through their involvement in early TLR4-mediated cellular responses (15). Matsuzawa *et al.* (119) have shown that apoptosis signal-regulating kinase 1 (ASK1) is required for LPS-induced septic shock (119). These authors demonstrated that LPS-induced ASK1 activation was dependent on the generation of ROS. Pretreatment of cells with ROS inhibitors abolished LPS-induced ASK1 and p38 activation. Further, LPS-induced ASK1 and TRAF6 interaction was also dependent on ROS production because ROS inhibitors abolished the interaction of ASK1 and TRAF6. However, it is still unclear the mechanism by which LPS induces ROS generation.

Park *et al.* (138) have demonstrated that LPS-induced ROS generation and NF-κB activation are mediated by a direct interaction of TLR4 with NADPH oxidase 4 (Nox4) in HEK293T cells. HEK293T cells, which do not express TLR4, were transfected with TLR4 and accessory proteins MD2 and CD14 (TLR4/MD2/CD14). LPS stimulation resulted in ROS generation leading to NF-κB activation in the HEK293T cells expressing TLR4/MD2/CD14. Pretreatment of the HEK293T cells with an NADPH oxidase inhibitor (DPI) abolished LPS-mediated ROS generation and NF-κB activation, suggesting that NADPH oxidase plays a role in LPS-mediated ROS generation and NF-κB activation. The authors further demonstrated that the COOH-terminal region of Nox4 directly interacts with TIR of TLR4. Specific silencing of *Nox4* gene expression in HEK293T cells expressing TLR4/MD2/CD14 decreased NF-κB activation by 90% in response to LPS stimulation. Collectively, these data suggest that TLR4-mediated NF-κB activation is required for ROS-activated intracellular pathways and that targeting of the TLR4-mediated NF-κB pathway could limit ROS-induced cellular damage.

TLRs and miRNA in Cardiovascular Disease

Recently discovered miRNA has been identified as a novel regulator in gene expression at the post-transcriptional level by binding to target messenger RNAs (mRNAs), thus affecting many biological systems, including the mammalian immune system (157, 163) and the cardiovascular system (154, 175). miRNAs are 21–23 nucleotide non-protein-coding RNA molecules that negatively regulate gene expression and modulate the translational efficiency and/or the stability of target mRNAs. At present, several hundred miRNAs have been identified in the human genome and they regulate >30% of all protein encoding genes.

There are a number of excellent reviews describing the role miRNAs in cardiovascular disease (154, 175) and in the innate immune and inflammatory responses (157, 163), respectively. Here, we briefly summarize recent reports and connect the possible role of miRNAs in the regulation of innate immune and inflammatory responses mediated by TLRs and in myocardial I/R injury. We hope that the summarized information will provide basic knowledge of how modulation of innate immune responses mediated by TLRs will induce protection against myocardial I/R injury.

miRNA and the Innate Immune Response

Innate immune and inflammatory responses involve activation of the TLR-mediated signaling pathways that stimulate the expression of several hundred genes. This process must be tightly regulated so that pathogens and/or danger molecules are cleared, meanwhile avoiding the consequences of uncontrolled over-response by the innate immune system and inflammation. Growing evidence has demonstrated that miRNAs play a critical role in the negative regulation of innate immune and inflammatory responses (157, 163) (Fig. 4). Taganov *et al.* reported that the TLR4 ligand, LPS, increased the expression of miR-146a, miR-155, and miR-132 in monocytes (164). Ligands for TLR2, TLR4, and TLR5 also induce miR-146a/b expression. Importantly, TRAF6 and IRAK-1,

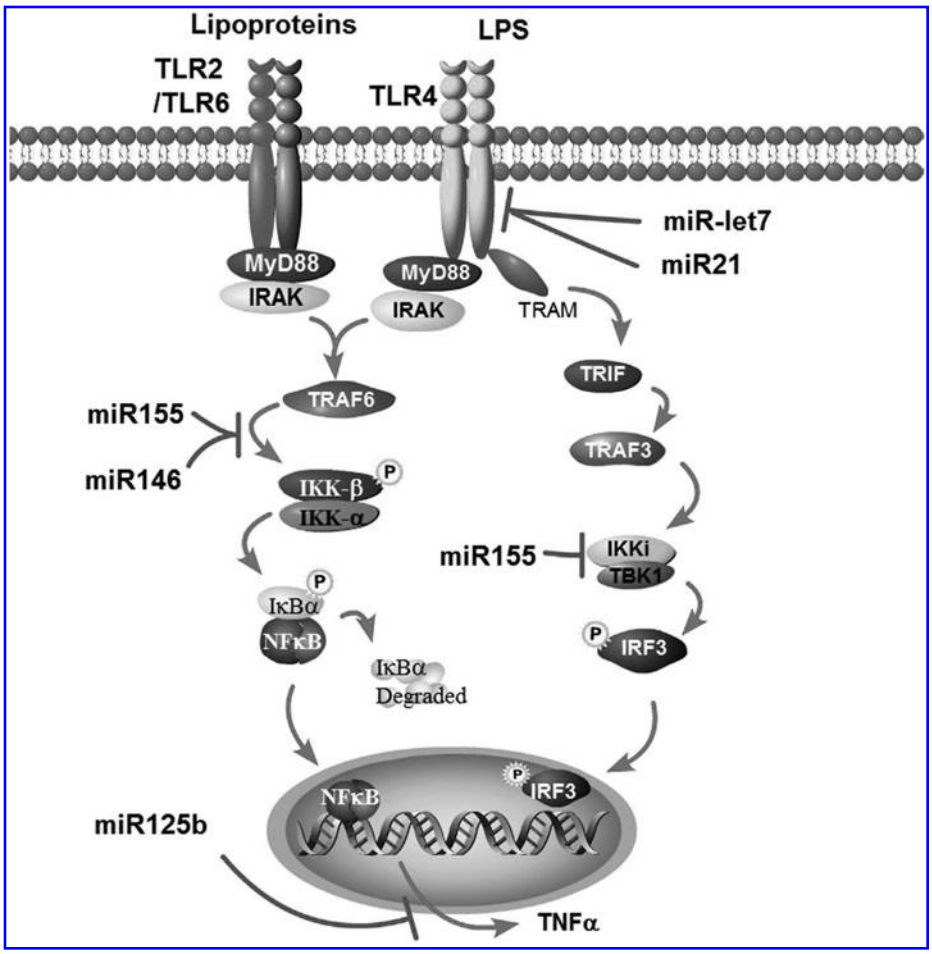

FIG. 4. microRNAs (miRNA) regulate innate immune and inflammatory responses activated by TLR-mediated NF-κB activation pathways. TLRs recognize bacterial compounds and activate intracellular signaling pathways, resulting in inflammatory responses. Recent evidence suggests that miR-146 and miR-155 serve as negative feedback regulators in TLR-mediated signaling. miR-125b inhibits TNF-α production and miR-let7 and miR-21 target TLR4 mRNA at the post-transcriptional level.

which are the key molecules in TLR-mediated NF-κB activation, are miR-146 targets (164). These data suggest that miR-146 may function as a negatively feedback regulator of TLR-mediated signaling in innate immune and inflammatory responses.

Ligands for TLR3 and TLR4, and cytokines such as IFN-β and IFN-γ, stimulate miR-155 expression through TNF-α autocrine/paracrine signaling (131). miR-155 targets FADD, IKK-ε, and Ripk1 (receptor-interacting serine/threonine-protein kinase-1) mRNA (168), suggesting that miR-155 is a component of the innate immune response to inflammatory stimulators that enhances TNF-α translation. The TLR4 ligand, LPS, suppresses macrophage miR-125b expression, which targets TNF-α (168). Downregulation of miR-125b by LPS may be needed for the macrophage inflammatory response through enhancing TNF-α production.

Several miRNAs that are involved in the response to ischemic challenge and/or innate immune response have been reported to directly target TLR4. For example, *Cryptosporidium parvum* stimulation decreased expression of miRNA-*let-7* in a MyD88/NF-κB-dependent manner, while increasing expression of TLR4. Forced overexpression of miR-*let-7i* significantly decreased TLR4 protein expression (30).

miRNA and myocardial I/R injury

Published data indicated that miRNAs, such as miR-1/106, miR-133, miR-21, miR-320, miR-199a, and miR-92a, are implicated in ischemic heart disease (44, 154, 175). miR-1 and miR-133 expression was involved in cardiac myocyte apoptosis in ischemic myocardium (186). However, miR-1 is proapoptotic by targeting Hsp60 and Hsp70, whereas miR-133 is anti-apoptotic through targeting caspase-9 (183).

miR-21 protects cells from oxidative stress-induced damage (31) and myocardium from ischemic injury (31). Cheng and colleagues reported that overexpression of miR-21 reduced infarct size, which was associated with the inhibition of proapoptotic genes and an increase in anti-apoptotic genes, whereas a miR-21 inhibitor abolished this effect (37). Ren *et al.* demonstrated that miR-320 is involved in I/R-induced cardiac injury and dysfunction *via* regulation of Hsp20 (144). Transgenic overexpression of miR-320 increased apoptosis and infarction size in I/R hearts, whereas *in vivo* treatment with antagomir-320 reduced infarct size (144).

It would be interesting to determine the role of miRNAs that regulate innate immune response by TLR-mediated signaling pathways, in myocardial I/R injury. This information could enhance our understanding of the mechanisms by which modulation of innate immune responses, mediated by TLRs, will induce cardioprotection against ischemic insult.

TLRs and Stem Cells in Treatment of Ischemic Hearts

Ventricular muscle cells of adult mammals are considered to be terminally differentiated cells that have lost their ability

to replicate for the repair or reconstitution of damaged myocardium (81, 141). Cellular cardiomyoplasty has gained significant interest in experimental studies and clinical treatment of patients with heart attack or heart failure. However, conditions in the infarcted myocardium, such as hypoxia and/or ischemia and inflammatory responses that cause oxidative stress and apoptosis of transplanted stem cells, limit the effectiveness of cellular cardiomyoplasty. Therefore, tolerance (preconditioning) of stem cells before implantation, improvement of survival of implanted cells and enhancement of immunosuppressive capacity of stem cells, are important factors for cellular cardiomyoplasty to be clinically effective. There are reviews on the topic of cellular cardiomyoplasty (50, 81, 141, 181). In this section, we briefly summarize current published data on the role of modulation of TLRs that are expressed on stem cells in improvement of survival and enhancement of immunosuppressive capabilities of implanted stem cells.

Human mesenchymal stem cells (MSCs) express TLRs (TLR1–6, and TLR9) and respond to TLR ligand stimulation by activation of TLR-mediated signaling (170). When stem cells are transplanted for cellular cardiomyoplasty, the transplanted cells are often exposed to unfavorable conditions, such as hypoxia and inflammation. In addition, TLRs expressed on transplanted MSCs sense endogenous ligands or danger signals released by injured tissues, which, in turn, affect the tissue repair processes by transplanted stem cells. Therefore, TLRs on the stem cells may determine the fate of transplanted stem cells. Interestingly, stimulation of human adipose-derived MSCs with TLR ligands induced the expression of manganese superoxide dismutase, which is a key protective molecule against oxidative stress in the mitochondria (111). Thus, TLR ligand stimulation may improve engraftment or survival of implanted stem cells at the injured sites, resulting in enhanced therapeutic effects. Indeed, activation of TLR4 by its ligand, LPS, protected MSCs from oxidative stress-induced apoptosis (178). More significantly, LPS-induced preconditioning of bone marrow (BM) MSCs significantly improved survival, engraftment of implanted MSCs, and increased release of vascular endothelial growth factor (VEGF), in a model of rat acute myocardial infarction (187). These data suggested that modulation of TLRs on stem cells could enhance the therapeutic effect of stem cell transplantation.

The immunosuppressive capability of MSCs could be another key factor for successful cellular cardiomyoplasty. Opitz *et al.* reported that TLRs expressed on human MSCs enhanced the immunosuppressive phenotype of MSCs (134). Tomic *et al.* reported that the capability of human MSCs to migrate, invade, and secrete immune modulating factors was drastically affected by specific TLR-agonist engagement (171). For example, TLR3 ligand stimulation leads to the secretion of factors with mostly immune suppressive properties, whereas stimulation of TLR4 with LPS resulted in the secretion of proinflammatory factors (179).

Given the facts that MSCs express TLRs and have the capacity for modulation of immune responses, it would be interesting to determine whether modulation of TLRs on MSCs will interfere with or enhance homing, bio-distribution, and immunosuppressive capacity of MSCs in the injured heart. In addition, it would be important to define whether recognition of endogenous ligands by TLRs on MSCs would be a new therapeutic strategy for ischemic injury and heart failure.

TLRs and Gene Therapy in Ischemic Hearts

The strategies for gene therapy are mainly dependent on the specific cardiac disease. Many cardiovascular diseases are caused by ischemic injury; therefore, angiogenic gene therapy is getting much attention as a therapy to improve ischemic heart failure. In this brief review, we are focusing on TLRs and angiogenic gene therapy in ischemic hearts. VEGF is the most thoroughly investigated growth factor that induces angiogenesis in the ischemic heart (99, 146, 151). Administration of plasmid VEGF increased collateral blood flow and improved cardiac function in an animal model with chronic ischemic heart (66). Angiogenic gene therapy in a clinical trial suggested that $VEGF_{165}$ delivered by myocardial injection and intramyocardial transfection to patients showed significant improvement of ischemic myocardium area with increased perfusion and reduced angina, respectively (83). The levels of VEGF in the plasma were also significantly increased after administration of VEGF plasmid.

The major problem of naked DNA in gene therapy is very low transfection efficiency and expression levels. Therefore, viral vectors are commonly employed for gene therapy (151). A phase I clinical trial showed that transfection of adenovirus expressing $VEGF_{121}$ into the myocardium resulted in an improvement of angina and exercise time with increased levels of VEGF in plasma (162). Similarly, injection of adenovirus expressing fibroblast growth factor into epicardial fat also showed similar results as those observed for $VEGF_{121}$ (58).

Interestingly, growing evidence suggests that innate immunity mediated by TLRs is involved in angiogenesis. For example, Cho *et al.* reported that a TLR ligand mediates upregulation of the angiogenic factor, VEGF, in human rheumatoid synovial fibroblasts (32). Varoga *et al.* have shown that TLR2 mediates induction of VEGF in cartilage in septic joint disease (176). In addition to TLR2, TLR4 on endothelial cells regulates fibrosis-associated angiogenesis in the liver (75). TLR3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor-α (137). Pinhal-Enfield *et al.* have shown that ligands for TLR2, TLR4, TLR7, and TLR9, but not TLR3 and TLR5, synergize with adenosine A(2a) receptor (A(2A)R) agonists and adenosine to upregulate VEGF, while simultaneously strongly downregulating TNF-α expression (142). In the absence of adenosine or A(2A)R agonists, these TLR ligands strongly upregulate TNF-α expression, with no effect on VEGF. The authors proposed a novel signaling pathway in murine macrophages involving synergy between TLR2, 4, 7, and 9 and A(2A)Rs that upregulates VEGF and downregulates TNF-α, thus acting as an angiogenic switch. This angiogenic switch may play an important role in ischemia when TLR agonists are present. Recently, Grote *et al.* reported that the TLR2 ligand, macrophage-activating lipopeptide 2 (MALP-2), induces angiogenesis *via* granulocyte-macrophage colony-stimulating factor that was released by endothelial cells and monocytes in a TLR2/6-dependent manner (59).

When considered as a whole, recent data suggest that modulation of TLRs could induce angiogenesis *in vitro* and that gene therapy has a great potential to treat ischemic heart disease. However, the transfection efficiency, stability, and

long-term expression of the therapeutic genes should be further evaluated for the treatment of patients with ischemic heart disease. Modulation of TLRs to promote angiogenesis would be a potential use of TLR ligands for an angiogenic therapy after ischemic insult.

TLR-Mediated NF-κB Signaling Plays a Central Role in Myocardial I/R Injury

Numerous studies have demonstrated that innate immune and inflammatory pathways play a role in myocardial I/R injury and CHF (17, 22, 25, 82, 88, 105, 135, 172). However, the mechanisms by which innate immune and inflammatory responses are involved in myocardial I/R injury have not been elucidated entirely. Innate immune recognition is mediated by germ-line-encoded receptors/signal transduction molecules that respond to highly conserved macromolecular structures in pathogens called PAMPs (122, 123). Recent studies have highlighted the role of TLRs in the induction of innate immune and inflammatory responses (122, 123). TLR-mediated signaling predominantly activates NF-κB (104, 106, 108). NF-κB is a ubiquitous inducible transcription factor that stimulates gene expression, in particular those genes that promote immune and inflammatory responses (16). The importance of TLR-mediated NF-κB activation and inflammation in myocardial I/R injury has been well documented (34, 48, 104, 105, 106, 108, 126, 136, 153). Li and colleagues reported that I/R rapidly increased myocardial IKK-β activity, IκBα phosphorylation/degradation, and NF-κB activation (104, 106, 108). Blunting NF-κB activation significantly reduced myocardial injury after I/R, improved cardiac functional recovery (105, 126, 153), and downregulated inflammatory cytokine and adhesion molecule gene expression (78). These data suggested that the TLR-mediated NF-κB signaling pathway contributed to cardiac ischemic injury. Among TLRs, the role of TLR4 and TLR2 in cardiac ischemic injury has been extensively studied. There are consistent reports regarding the role of TLR4 in myocardial I/R injury; that is, TLR4 deficiency protected the myocardium from ischemic injury. Similarly, protection was also observed in the other organs such as brain, lung, liver, kidney, and intestines that were subjected to I/R. There are controversial reports on the role of TLR2 in myocardial ischemic injury and other important organs. The differences may be due to the various models and experimental designs, in particular, the times of ischemia and reperfusion that were examined.

TLR4 Deficiency Reduced Myocardial Infarction After Myocardial I/R

In 1999, Frantz *et al.* reported an increased TLR4 expression in human heart failure and in ischemic rat hearts (48). Subsequently, numerous studies have demonstrated that the TLR4-mediated signaling pathway contributes to myocardial I/R injury. TLR4-deficient mice exhibited decreased myocardial infarction after I/R compared with wild-type (WT) I/R mice (10, 34, 41, 71, 136, 145). Interestingly, administration of a specific TLR4 antagonist, Eritoran, to mice significantly reduced cardiac infarct size compared with untreated I/R mice (158). Either TLR4 deficiency or TLR4 antagonist administration blunted I/R-increased NF-κB binding activity in the myocardium and decreased inflammatory cytokine production after myocardial I/R (10, 28, 158). MyD88 is an important adaptor protein in the TLR4-mediated signaling pathway. Blocking the MyD88-dependent signaling pathway significantly reduced myocardial I/R injury. Hua *et al.* reported that transfection of adenovirus expressing dominant negative MyD88 (Ad5-dnMyD88) into the myocardium significantly reduced myocardial infarction after myocardial I/R injury compared with control hearts that were transfected with adenovirus expressing green fluorescent protein (70). Transfection of Ad5-dnMyD88 also significantly blunted I/R increased NF-κB binding activity (70). This observation was supported by a study using transgenic mice with MyD88 deficiency (43). Feng *et al*. (43) showed that infarct size in MyD88-deficient mice was significantly reduced after myocardial I/R compared with WT mice. Cardiac function after myocardial I/R in MyD88-deficient mice was significantly improved compared with WT mice. In addition, there was less neutrophil infiltration and inflammatory cytokine expression in the myocardium of MyD88-deficient mice compared with WT mice (43). IRAK-4 is an important kinase downstream in the Toll-mediated MyD88-dependent signaling pathway. IRAK-4-deficient mice had an improved survival rate 4 weeks after myocardial infarction and showed attenuated cardiac dilation and decreased inflammation in the infracted myocardium (113). Collectively, these data demonstrated that the TLR4-mediated MyD88-dependent signaling pathway plays a deleterious role in myocardial I/R injury. The TLR4-mediated MyD88-dependent signaling pathway could be a target for treatment of cardiovascular disease related to an ischemic insult.

TLR4-Mediated Signaling Contributes to Cardiac Dysfunction

Cardiac dysfunction is an important event that occurs after myocardial I/R. As mentioned above, the TLR4-mediated MyD88-dependent signaling pathway contributes to cardiac remodeling and cardiac dysfunction, whereas TLR4 deficiency or modulation of the TLR4-mediated MyD88-dependent signaling pathway resulted in significant improvement of cardiac function in both early and late stages after myocardial I/R. Since TLR4 is expressed on both cardiac myocytes and circulating cells, it is not clear which TLR4-expressing cells contribute to cardiac dysfunction after myocardial I/R. Using isolated hearts from TLR4-deficient mice, Cha *et al*. (28) have shown that cardiac function was significantly improved after global myocardial I/R compared with the hearts isolated from WT mice. Since there were no circulating cells in this isolated heart model during global I/R, the results suggested that TLR4 expressed by cardiac myocytes may contribute to cardiac dysfunction during myocardial I/R. Consistent with this observation, Fallach *et al*. (41) recently reported that cardiac myocytes expressing TLR4 play a critical role in cardiac dysfunction in both myocardial I/R and sepsis models. These authors observed that cardiac function in TLR4-deficient mice was significantly improved after myocardial I/R or endotoxin challenge compared with WT I/R mice (41). To determine the role of cardiac TLR4 *versus* TLR4 in circulating cells in cardiac dysfunction after I/R or endotoxin challenge, these investigators prepared chimeras of BM transplantation to substitute TLR4 expression in the immunohematopoietic system. They observed that the chimeric mice that express TLR4 only in the cardiac myocytes showed

cardiac dysfunction 4 h after myocardial I/R or endotoxin challenge. Since the authors did not observe acute inflammatory infiltration into the myocardium at 4 h, they concluded that TLR4 expressed by cardiac myocytes plays a major role in mediating cardiac dysfunction. Tavener *et al.* reported that myocardial TLR4 is the primary mediator of cardiac suppression after endotoxin administration (4 h) (167). However, Binck *et al.* reported that using chimeric mice expressing TLR4 either in the hematopoietic compartment or the heart, TLR4 expression in both compartments contributes to cardiac dysfunction 18 h after endotoxin challenge (19). The difference between the studies could be due to differences in the observation time. In the Binck's study, cardiac function was examined 18 h after LPS administration, which would give sufficient time for infiltration of inflammatory cells into the hearts. The infiltrated inflammatory cells release important mediators that suppress cardiac contractility (19).

There are also inconsistent reports regarding the role of TLR4 in cardiac function after myocardial I/R. Kim *et al.* (90) reported that TLR4 deficiency reduced infarct size but did not gain in function. However, Fallach *et al.* (41) reported that TLR4 deficiency significantly improved cardiac function after myocardial I/R. Similarly, cardiac function in TLR4-deficient mice was significantly improved 6 days after I/R compared with WT mice. Infarct size was smaller and left ventricle (LV) remodeling was reduced as evidenced by decreases in the levels of atrial natriuretic peptide, total collagen, and decreased ratio of heart weight/body weight compared with WT mice (41). These data suggest that in the early stage, TLR4 expressed by cardiac myocytes plays a critical role in the induction of cardiac dysfunction; however, in the late stage, TLR4 in both cardiac myocytes and inflammatory cells contribute to cardiac dysfunction.

TLR4 Ligand Induces Cardioprotection Through Preconditioning Mechanisms

It is well known that administration of a high dose of LPS increases the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the host through the TLR4-mediated NF-κB activation pathway. These inflammatory cytokines are responsible, in part, for high-dose LPS-induced cardiac dysfunction and injury (65, 129), Interestingly, numerous studies have shown that pretreatment of animals with a small dose of LPS 24 h before injury induces protection against subsequent myocardial I/R injury and improves recovery of cardiac function after I/R (24, 120, 148, 188, 189, 193). This effect is not unique to LPS, because LTA, a cell wall component of gram-positive bacteria, also reduces infarct size when administered to rats 8–24 h before ischemia (191). Interestingly, TLR4 recognizes both LPS and LTA (124), suggesting that activation of TLR4 by its agonists at a small dosage will induce cardioprotection. This phenomenon is called preconditioning. However, the TLR4 agonists must be administered at least 8–24 h before induction of I/R to be effective. This suggests that the mechanisms involve *de novo* synthesis of cardioprotective proteins, such as heat shock proteins and inducible nitric oxide synthase (177). A recent study by Ha *et al.* (62) has shown that pretreatment of mice with a small dose of LPS significantly increased the levels of myocardial phospho-Akt, suggesting that LPS administration will activate the PI3K/Akt signaling pathway, which negatively regulates TLR/NF-κB-mediated inflammatory responses (52, 53, 180). It has been demonstrated that there is crosstalk between the TLR and PI3K/Akt signaling pathways (11, 132). For example, stimulation of TLR4 can result in activation of PI3K/Akt-dependent signaling (11, 105). Activation of PI3K/Akt-dependent signaling protects cardiac myocytes from I/R injury and inhibits I/R-induced cardiac myocyte apoptosis (51, 117). These authors speculated that high levels of myocardial PI3K/Akt activity in LPS pretreated mice may be responsible for the cardioprotection against I/R injury. To test their hypothesis, Ha *et al.* administered the PI3K inhibitor, LY294002, to LPS pretreated mice before myocardial I/R. Pharmacologic inhibition of PI3K with LY294002 abrogated cardioprotection in LPS-pretreated mice after I/R injury. LPS-induced cardioprotection was also abolished in kinase defective Akt (kdAkt) transgenic mice treated with LPS 24 h before the hearts subjected to I/R. Thus, using both pharmacologic and genetic approaches that inhibit PI3K and Akt, the authors demonstrated that LPS-induced cardioprotection is mediated through a PI3K/Akt-dependent mechanism.

Mechanisms of TLR4 Deficiency-Induced Protection Against Ischemic Injury: Role of the PI3K/Akt Signaling Pathway

It is well known that TLR4 deficiency or modulation of the TLR4-mediated MyD88-dependent signaling pathway will induce cardioprotection against myocardial I/R injury. Most published reports suggest that the mechanisms involve decreased infiltration of inflammatory cells, blunting the production of inflammatory cytokines, and reduction of cardiac myocyte apoptosis. However, it is still unclear why TLR4 deficiency limited I/R-increased infiltration of circulating cells, blunted I/R-increased inflammatory cytokine production, and reduced I/R-induced cardiac myocyte apoptosis. The mechanisms by which TLR4 deficiency protected the myocardium against I/R have not been elucidated entirely.

Interestingly, Hua *et al.* recently reported that NF-κB binding activity is significantly reduced in TLR4-deficient mice after myocardial I/R (71). In addition, the levels of phosphorylated Akt in the myocardium of the TLR4-deficient mice subjected to I/R were significantly increased compared with WT mice (63, 71). This observation suggests that a deficiency of TLR4-mediated signaling will increase activation of the PI3K/Akt signaling pathway. To determine the role of activation of the PI3K/Akt signaling pathway in TLR4 deficiency-induced cardioprotection, Hua *et al.* administered PI3K pharmacological inhibitors LY294002 or Wortmannin to TLR4-deficient mice before myocardial I/R. Importantly, PI3K inhibition completely abolished the cardioprotection in TLR4-deficient mice after myocardial I/R injury (71). This suggests that there was crosstalk between the TLR4 and PI3K/Akt signaling pathways during myocardial I/R. In addition, these data suggest that there is reciprocal regulation of TLR- and PI3K-mediated responses. Activation of the PI3K/Akt signaling pathway is responsible for TLR4 deficiency-induced cardioprotection. These data may also point to a possible strategy for reducing heart injury in acute myocardial infarction. Specifically, downregulation of TLR4/NF-κB-dependent signaling while simultaneously stimulating PI3K/Akt-dependent signaling may decrease the morbidity and mortality associated with myocardial infarction.

The Role of TLR2 in Myocardial I/R Injury

Several studies have shown a role for TLR2 in mediating myocardial I/R injury. Sakata *et al.* reported that after *in vitro* I/R using isolated hearts, the recovery of LV developed pressure in WT mice was relatively lower than that observed in TLR2-deficient mice (150). However, the creatinine kinase levels, which indicates damage of cardiac myocytes, were similar in both WT and TLR2-deficient mice, suggesting that zero-flow ischemia resulted in similar myocyte damage in both groups (150). Favre *et al.* reported that mice with TLR2 deficiency exhibited a smaller infarct size after 30 min of ischemia followed by 60 min of reperfusion (42). Arslan *et al.* (13) recently reported that circulating cells expressing TLR2-mediated myocardial I/R injury. TLR2-deficient mice showed a smaller infarct size after 30 min of ischemia followed by reperfusion for 24 h compared with WT mice. Transplantation of TLR2-deficient mice with BM cells that were derived from WT mice abolished protection against myocardial I/R injury in TLR2-deficient mice, suggesting that circulating cells that express TLR2-mediated myocardial I/R injury. Importantly, administration of anti-TLR2 antibody 5 min before reperfusion significantly improved cardiac performance 28 days after reperfusion. Administration of anti-TLR2 antibody also significantly reduced infarct size, leukocyte influx, cytokine production, and proapoptotic signaling activation (13). MSC transplantation has also been shown to significantly improve cardiac function in heart failure patients and experimental animals. To determine the role of TLR2 and MSCs in recovery of cardiac function, Abarbanell *et al*. (1) isolated MSCs from WT and TLR2-deficient mice, respectively, and added them to the isolated rat hearts 5 min before the hearts were subjected to I/R. They observed that the isolated hearts that received MSCs from WT mice showed significant improvement of LV function recovery, whereas the hearts treated with MSCs from TLR2-deficient mice did not. The authors suggested that TLR2 may be essential for MSC-mediated recovery of myocardial function (1).

TLR2 Ligand Induced Protection Against Myocardial I/R Injury

While the TLR2-mediated signaling pathway contributes to myocardial I/R injury, recent studies have shown that TLR2 is needed for ischemic preconditioning-induced LV functional recovery after myocardial I/R in isolated hearts (36). Treatment of the hearts from WT mice with Pam3CSK4, a specific synthetic ligand for TLR2, significantly improved LV functional recovery after I/R. However, Pam3CSK4 did not induce cardiac function improvement in TLR2-deficient hearts, suggesting that Pam3CSK4 mimicked the effects of ischemic preconditioning through a TLR2-dependent mechanism (64). Mersmann *et al.* (125) have shown that *in vivo* administration of Pam3CSK4 to mice 24 h before myocardial I/R significantly reduced infarct size, improved cardiac function, and decreased chemokine (CXCL1) with subsequent reduction of leukocyte infiltration to ischemic tissues.

However, preconditioning-induced protection requires pretreatment for at least 8–24 h. Interestingly, a recent study by Ha *et al.* (64) has shown that administration of TLR2 ligands, either Pam3CSK4 or PGN, 1 hr before myocardial I/R significantly reduced infarct size and improved cardiac function after myocardial I/R. Treatment of mice with Pam3CSK4 also induced protection against cerebral ischemic injury (72) and attenuated cardiac dysfunction in septic mice (64). The protective effect induced by TLR2 ligands was abolished in TLR2-deficient mice, suggesting that TLR2 ligand-induced cardioprotection is mediated through a TLR2-dependent mechanism (61). This study showed a rapid induction of cardioprotection by TLR2 ligands. To determine why modulation of TLR2 by its ligands rapidly induces cardioprotection, Ha *et al.* examined whether there is an interaction between TLR2 and activation of the PI3K pathway. The authors observed that TLR2 ligand administration significantly increased the levels of phosphorylated Akt in the myocardium, suggesting that the PI3K/Akt signaling pathway was activated. More significantly, PI3K inhibition or defective Akt abolished TLR2 ligand-induced cardioprotection, demonstrating that TLR2 ligand-induced cardioprotection is mediated through a TLR2/PI3K/Akt-dependent mechanism. It is well known that the cytosolic domain of TLR2 contains a PI3K binding motif (YXXM), which binds the phosphorylated form of the p85 subunit of PI3K (11). TLR2 ligand administration significantly induced TLR2 tyrosine phosphorylation and increased the association of the p85 subunit of PI3K with TLR2 (61). Previous studies have shown that stimulation of TLR2 resulted in the recruitment of active Rac1 and PI3K to the TLR2 cytosolic domain, resulting in activation of the PI3K/Akt pathway (11). Mal, an adaptor in TLR-mediated signaling, has been shown to connect TLR2 to PI3K activation (160). When considered together these data indicated that TLR2 ligand administration increased phosphorylation of TLR2 with subsequent recruitment of the p85 subunit of PI3K, which resulted in activation of PI3K/Akt-dependent signaling.

Concluding Remarks

Current published reports suggest that both TLR2 and TLR4 ligands can induce cardioprotection. However, the cardioprotective mechanisms of modulation of TLR2- *versus* TLR4-mediated signaling are quite different. Specifically, signaling mediated by modulation of TLR2 induces cardioprotection rapidly, whereas signaling through modulation of TLR4 requires prolonged pretreatment to be effective. Interestingly, the cardioprotection induced by modulation of either TLR2- or TLR4-mediated pathways was abolished by inhibition of PI3K/Akt activation (61, 71, 105, 195). These data suggest crosstalk or counter regulatory mechanisms between TLRs and PI3K/Akt. Future studies are needed to elucidate the mechanisms by which modulation of TLRs will activate the PI3K/Akt pathway, leading to protection of the myocardium from I/R injury. In addition, it would be interesting to investigate whether intracellular TLRs, such as TLR3 and TLR9, will be involved in the pathophysiology of myocardial I/R injury.

It is unclear whether TLR2 or TLR4 will cooperate with other coreceptors such as SRs CD36 or SR-A during myocardial I/R. As mentioned above, a growing body of evidence suggests that both TLR2 and TLR4 cooperate with SRs CD36 and SR-A. Interestingly, either deficiency of TLR4 (27, 73, 74) or TLR2 (101) protected the brain from cerebral I/R injury. Similar observations have been found in either CD36 (33, 89, 96) or SR-A knockout mice (112). Deficiency of either CD36 (33, 89, 96) or SR-A (112) protects the brain from cerebral I/R

injury. A recent study has shown that SR-A plays a critical role in mediating the pathophysiology of myocardial I/R injury (107). After myocardial I/R injury, infarct size in SR-A-deficient mice was smaller compared with WT mice. I/R increased the IRAK-mediated NF-κB signaling pathway in WT mice but was significantly reduced in SR-A-deficient mice. I/R activated the FasL/Fas-mediated apoptotic signaling pathway and induced cardiac myocyte apoptosis in WT mice but did not in SR-$A^{-/-}$ mice. The data suggest that SR-A-mediated activation of NF-κB and apoptotic signaling contribute to the pathophysiology of myocardial I/R. Although the authors did not examine the association between TLRs and SR-A during myocardial I/R, it would be interesting to know if there is an interaction between SR-A and TLRs during myocardial I/R since knockout of either TLR4 or SR-A induces cardioprotection against I/R injury. Elucidation of the association between macrophage SRs and TLRs during myocardial I/R would highlight the role of pattern recognition receptors in myocardial I/R injury and provide basic scientific knowledge for development of therapeutic approaches for myocardial I/R injury.

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Abbreviations Used

Ad5-dnMyD88 = adenovirus expressing dominant negative MyD88
AP-1 = activation of the transcription factor complex-1
ASK1 = apoptosis signal-regulating kinase 1
BLP = bacterial lipoprotein
BM = bone marrow
CHF = congestive heart failure
CpG = cytidine phosphate guanosine
dsRNA = double-stranded RNA
FADD = Fas-associated death domain
FasL = Fas ligand
GSK-3β = glycogen synthase kinase-3β
IκBα = inhibitor of kappa B alpha
IFN-β = interferon-beta
IFN-γ = interferon gamma
IKK-α = IKαPPaB kinase alpha
IKK-β = IKαPPaB kinase beta
IL-1R = interleukin-1 receptor
IL-1β = interleukin-1 beta
I/R = ischemia/reperfusion
IRAK = interleukin-1 receptor-associated kinase
IRF3 = interferon regulatory factor 3
LAM = lipoarabinomannan
LBP = LPS binding protein
LPS = lipopolysaccharide
LRR = leucine-rich repeats
LTA = lipoteichoic acid
LV = left ventricle
MALP-2 = macrophage-activating lipopeptide 2
MAPK = mitogen-activated protein kinase
MD2 = myeloid differentiation factor 2
miR- = microRNA subtypes
miRNA = microRNA
MSC = mesenchymal stem cell
mTOR = mammalian target of rapamycin
MyD88 = myeloid differentiation primary response gene 88
NADPH = reduced form of nicotinamide adenine dinucleotide phosphate
NEMO = NF-κB essential modulator
NF-κB = nuclear factor-kappaB
NLRs = NOD-like receptors
NOD = nucleotide binding oligomerization domain
Nox4 = NADPH oxidase 4
PAMP = pathogen-associated molecular pattern
PDK1, 2 = phosphoinositide-dependent kinase 1, 2
PGN = peptidoglycan
PI3K = phosphoinositide 3 kinase
PIP2 = phosphatidylinositol 4,5-bisphosphate
PIP3 = phosphatidylinositol (3,4,5)-trisphosphate
PKB = protein kinase B
Poly I:C = polyinosinic-polycytidylic acid
PRR = pattern recognition receptor
Rac1 = Ras-related C3 botulinium toxin substrate 1
RIG 1 = retinoic acid inducible-gene-1
RIP1 = receptor-interacting protein 1
RLR = RIG-like receptor

Abbreviations Used (Cont.)

ROS = reactive oxygen species
SARM = sterile alpha and Armadillo motif-containing protein
SR-A = scavenger receptor class A
ssRNA = single-stranded RNA
TAB 1,2,3 = TAK1-binding protein 1,2,3
TAK1 = TGF-β- activated kinase
TANK = TRAF family member-associated NF-κB activator
TBK1 = TANK binding kinase 1
TGF-β = transforming growth factor-beta
TIR = toll interleukin-1 receptor
TIRAP = TIR domain containing adaptor protein
TLR = toll-like receptor
TNF-α = tumor necrosis factor alpha
TNFR = tumor necrosis factor receptor
TRAF6 = TNFR-associated factor 6
TRAFDD = TNFR-associated death domain
TRAM = TRIF-related adaptor molecule
TRIF = TIR domain containing adaptor inducing IFN-β
VEGF = vascular endothelial cell growth factor
WT = wild type

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