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Toll-like receptor and its roles in myocardial ischemic/reperfusion injury

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Summary

The innate immune system, mediated via toll-like receptors (TLRs), represents the first line of defensive mechanisms that protects hosts from invading microbial pathogens. TLRs are a family of pattern recognition receptors (PRRs), and are pathologically activated by a set of pathogen-associated microbial patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs deliver signals via a specific intracellular signaling pathway involving distinctive adaptor proteins and protein kinases, and ultimately initiate transcriptional factors resulting in inflammatory responses. TLR4 is a paramount type of TLRs, located in the heart, and plays an important role in mediating myocardial ischemic reperfusion (I/R) injury. Loss-of-function experiments and animal models using genetic techniques have found that the MyD88-independent and the MyD88-dependent pathways together participate in the pathological process of myocardial I/R injury. Some other distinctive signaling pathways, such as the PI3K/AKt and AMPK/ERK pathways, interacting with the TLR4 signaling pathway, were also found to be causes of myocardial I/R injury. These different pathways activate a series of downstream transcriptional factors, produced a great quantity of inflammatory cytokines, such as IL, TNF, and initiate inflammatory response. This results in cardiac injury and dysfunction, such as myocardial stunning, no reflow phenomenon, reperfusion arrhythmias and lethal reperfusion injury, and other related complication such as ventricular remodeling. In the future, blockades aimed at blocking the signaling pathway could benefit developments in pharmacology.

key words: **toll-like receptors (TLRs) • ischemic/reperfusion (I/R) • innate immune • cardiomyocytes • inflammatory cytokines**

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Background

The innate immune system recognizes and eliminates specific invaders, maintaining tolerance to self-tissues and modulates adaptive immune responses [1,2]. It can sense pathologic organisms via pattern recognition receptors (PRRs), recognizing diverse exogenous or endogenous molecules [3,4].Toll-like receptors (TLRs), belonging to the PRRs family and located on cell surfaces or intracellular compartments, can recognize a number of molecules and promote initiation of immune responses [5]. TLRs are crucial for immune defense and host survival in chronic inflammation-mediated tissue damage [6–8]. In addition, TLRs are also capable of responding to stress and modulating tissue damage under "sterile inflammation" such as hypoxia or ischemia in the lungs, liver, brain and heart [9–12].

Ischemic heart disease is the primary cause of mortality and morbidity in most developed and in some developing countries [13–15]. Rapid reperfusion is critical in the treatment of unexpected myocardial ischemic incidents. Despite the use of effective reperfusion strategies, severe post-perfusion lesions still occur [16]. Therefore, it is necessary and urgent to understand the mechanisms of myocardial I/R injury and explore effective therapies to minimize the adverse effects. Recently published studies have documented that TLRs play an important role in myocardial I/R injury, but have only partially explained the injury mechanisms involved. We review the related literature to summarize the basic understanding of TLRs and their roles in myocardial ischemic/reperfusion injury.

TLRs

The structure and location of TLRs

TLRs are type-I transmembrane proteins composed of 3 structural domains: a C-terminal leucine-rich repeat (LRR) as ligand recognition domain, a central transmembrane domain, and an N-terminal cytoplasmic toll/interleukin-1 receptor (TIR) homologous signaling domain as an effector domain that mediates homotypic interactions facilitating downstream signaling or substrate processing [17,18].

The ectodomians of TLRs are mainly composed of LRRs of differing sizes and abundances and are considered to play a prominent role in the promotion of ligand-receptor interaction in a variety of biological settings. The LRR family, which are subdivided into 7 groups using LRR hybrid technology [19], share a highly conserved 11-residue tandem repeat modular sequence (L××L×L××N×L) and horseshoe-like 3D structures (Figure 1). The "×" residue in this sequence is exposed toward the concave surfaces of the horseshoe-like structure mediating interaction with ligands [19–32]. The "asparagines ladder" is another key characteristic of the LRR family [31–33]. The TLRs ectodomians form a relatively rigidly curved solenoid or spring shape, and gain flexibility to some degree by virtue of irregular loops (hinges) of the structure. The first several residues of each LRR form a short beta-strand, which is aligned in the overall structure to form a beta-sheet. The entire structure is bent, with the beta-sheet forming a concave surface, and stabilized by both an asparagines ladder and a hydrophobic interaction. The solenoid architecture that extends

Figure 1. Crystal structure of the TV8 hybrid of human TLR4 and hagfish VLRB.61. (The picture is supplied by NCBI protein structure database, PDB 2Z63).

from the cell surface forms an arc in which the N-terminus bends back toward the membrane. The ends of the solenoid are capped with disulfide bond motifs that isolate the hydrophobic residues from the surrounding aqueous environment [20,21,24].

On the cytoplasmic side, TLRs have a specific domain called the TIR signaling domain, which contains 2 distinct sites: an oligomerization site for maintaining dimeric interactions between the TLRs subunits, and a site for recruiting cytoplasmic adapter proteins. The adaptor proteins also contain oligomerization sites and form extended chains in the cytoplasm of the cell following activation by "face-to-face" and "back-to-back" interactions [25].

In mammals, the TLR family, to our knowledge, is comprised of 11 members (TLRs 1-11), and most of the members are widely expressed in different cell types, either from the immune system, including dendritic cells (DCs), macrophages (Ms), natural killer cells (NKs), mast cells, neutrophils (N), B and T cells, or from the non-immune system, such as fibroblasts, epithelial cells (ECs) and myocardia. TLR1, 2, 4, 5, 6 and 10 are primarily located on the cell surface, and recognize bacterial-related ligands; whereas TLR3, 7, 8 and 9 are located in the endocytic compartments and mainly recognize viral products. Recently, TLR11, as well as 2 new members –TLR12 and TLR13 – have been discovered in mice, and are believed to be plasma membrane localized [26–31].

Ligands recognized by TLRs

TLRs enable the innate immune system to discriminate different pathogen-associated molecule patterns (PAMPs), which are unique to distinctive microbial classes. These PAMPs, of varying sizes, may originate from bacterial cell wall components, hydrophilic nucleic acids, small-molecule anti-viral molecules and immunomodulatory compounds, and consequently launch the specific immune defense response [32,33].

TLRs can identify various components of the bacteria [34,35]. Lipopolysaccharides (LPS) and lipotechoic acid (LTA) from Gram-negative bacteria can induce TLR4 activation [17,36,37]; peptidoglycans (PGNs) from Gram-positive bacteria, bacterial lipoproteins (BLP) and lipoarabinomannan (LAM) from *mycobacteria* are recognized by TLR2 [3,4,38–40]; diacyl and triacyl lipopeptides from bacteria, mycobacteria and mycoplasm can be sensed by TLR1/2 or TLR2/6 complex [41]; TLR5 is best known as a receptor for flagellin, a monomeric subunit of bacterial flagella [42]; and genomic DNA rich in unmethylated CpG from bacteria can be discriminated by TLR9. Bacterial RNA products in the lysosomal compartment can be discerned by TLR7 [43–64]. Spirochetes of *Borrelia burgdorferi* can be sensed by TLR1 and TLR2.

Viral products, composed of envelope proteins and nucleic acids, also activate TLRs. The envelope proteins from *respiratory syncytial virus* (*RSV*) and *mouse mammary tumor virus* (*MMTV*) are recognized by TLR2, 4 and 6 [50,51,54]. TLR2/6 complex can bind diacylated mycoplasmal macrophage-activating lipopeptide (MALP2). Several DNA viruses such as *herpes simplex virus* (HSV) and *murine cytomegalovirus* (MCMV) encompassed by unmethylated CpG DNA motifs are activated by TLR9 [27,54,55,63]. The single strand (ss)/double strand (ds) RNA-containing viruses, which are rich in uridine or uridine/guanosine, can be recognized by TLR3 and TLR7/8 complex (in humans only) [17,34,35,44,45].

Some components of fungi and protozoa are also recognized by TLRs. Phospholipomannans and beta-glucans from fungi are recognized by TLR2, while glucuronoxylomannans are recognized by TLR4 [54,55]. TLR2 is also responsible for the recognition of the yeast cell-wall particle zyosam; glycoinlsitolphospholipids (GIPLs) and glycosylphosphatidylinositol anchors (GPI-anchors) from *Trypanosoma* species, *P. falciparum,* and *T. gondii* are recognized by TLR2 and TLR4. Unsaturated alkylacylglycerol and lipophosphoglycan (LPG) from *Trypanosoma* species and *Leishmania* species are recognized by TLR9. Beta-hematin crystal made from hemin from *P. falciparum* is also identified by TLR9. Profilin-like protein from *T. gondii* is reported to be discerned by mouse TLR 11 [34,35,46,47].

TLRs can sense synthetic antiviral compounds, as well as endogenous tissue fragments. R848, imiquimod and some gyanine nucleotide analogs, such as loxoribine, are recognized by TLR7/8, and the synthetic analog of dsRNA, poly IC, is also recognized by TLR3 [17,34,35,43–45]. Some endogenous tissue fragments from injured and inflamed tissues, such as HMGB1, hyaluronan, S 100 proteins, heat shock protein (HSP), and the spliced extra domain A of fibronectin, can activate the TLR4 signaling pathway [33] (Table 1).

TLRs signaling pathway

TLRs are capable of recognizing distinguishable ligands such as PAMPs and damage-associated molecular patterns (DAMPs), which initiate TLRs activation and mediate intracellular signaling. TLRs activation entails the recruitment of various TIR domain-containing adaptors [17,34,35,48]. There are 5 TIR domain-containing adaptors: Myeloid Differentiation Primary-Response Protein 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR-domain-containing adaptor protein inducing TNF-beta-mediated transcription factor (TRIF), TRIF-related adaptor molecule (TRAM), and a sterile-alpha and armadillo-motif-containing protein (SARM) [49]. TLRs1, 2, 4, 5, 6, 7, 8, 9 and 11 bind to their specific ligands produced downstream of TLRs signaling via MyD88, while TLRs1, 2, 4, 6, TLR1/2 and TLR2/6 recruit TIRAP (also known as MAL). Similarly, TLR3 and 4 uniquely and separately recruit TRIF and TRAM. TIRAP and TRAM serve as a "bridge" linking the TIR domain of TLRs to MyD88 and TRIF, respectively [17,34,35,48,50]. Docking studies can predict the mechanisms of interaction between MyD88 and TIR domains of TLRs. The first hypothesis, called "face-to-face" interaction, mediates receptor: adaptor binding and involves the BB loop and Poc site. The second hypothesis, named "back-to-back" interaction, mediates binding between 2 receptors or 2 adaptors, and is characterized by homotypic interactions between alphaE helices [51,52]. Dimerization of TLRs is mediated in "back- to-back" fashion, followed by recruitment of adaptor to receptor by "face to face" interaction. Signal amplification may be achieved by the subsequent recruitment of additional adaptors, which bind to each other with alternating "face to face" and "back to back" modes.

The TLRs signaling pathway is generally classified into MyD88-dependent pathway and TRIF-dependent pathway (MyD88-independent pathway) [49]. The former starts with activation of all the TLRs except TLR3. Upon recruitment of MyD88, IL-1 receptor-associated kinase (IRAK) family is activated, followed by production of TNF receptor-associated factor 6 (TRAF6). TRAF6 induces activation of TNF receptor-associated factor (TRAF) family member-associated NF-κB activator binding kinase (TAK1), which consequently results in activation of the transcription factor NF-κB and the Mitogen-Activated Protein Kinases (MAPKs) p38 and c-jun kinase (JNK) [49,53–55]. The latter finally produces activation of NF-κB initiated by TLR3 and TLR4 recognition via 2 distinctive processes: N-terminal domain of TRIF interacts with TRAF6 and activates NF-κB directly; while the C-terminal domain of TRIF interacts with receptor-interacting protein (RIP1) and activates NF-κB indirectly. These 2 pathways both induce activation of NF-κB, which activates a cascade of inflammatory responses. Otherwise, the TRIF-dependent pathway also induces type 1 interferon (IFN-1) through Interferon Regulatory Factor-3 (IRF3), which is phosphorylated and activated by IKK-related kinase, Tank-Binding kinase 1 (TBK1) and Inhibitor of κ Light Polypeptide Gene Enhancer in B-Cell Kinase of ε (IKKε) [36,49,56].

TLR4, which is recognized by the bacterial LPS, LTA and endogenous molecules, signals via both MyD88-dependent and -independent pathways (Figure 2). TLR4 could directly bind to LTA through cluster of differentiation 14 (CD14) and LPS through CD14 and LPS-Binding Protein (LBP). Upon formation and activation of TLR4/MD2/CD14 complex following TLR4-ligand interaction, the cytoplasm TIR domain recruits distinct adaptor proteins, activates the signaling cascade, and consequently produces diverse inflammatory mediators. In MyD88-dependent pathway, TLR4 recruits downstream IRAKs through adaptors Mal and MyD88, which both contain an N-terminal death domain (DD) and a C-terminal TIR domain. MyD88 binds to TLR4 through TIR-TIR interaction

Table 1. TLRs, their ligands, cofactors, signaling pathway, adaptor proteins, transpritional factors and cytokines.

TLRs	Ligands for TLRs	Cofacors	TLRs Signaling Pathways	Adaptor Proteins	Transcriptional Factors	Cytokine
TLR1/2	Tri-acylated lipopeptide(Pam_3CSK_4) Glycolipids	CD14 CD36 Dectin 1	MyD88-Dependent Pathway	MyD88 TRIAP	NF-κB	TNF IL, et al.
TLR2/6	Diacylated MALP2 (Mycoplasmal macrophage-Activating Lipopeptide) LTA (*Streptococcus*) Zymosan (*Saccharomyces*)	CD14 Dectin 1	MyD88-Dependent Pathway	MyD88 TRIAP	NF-κB	TNF IL, et al.
TLR2	LAM (Lipoarabinomannan) BLP (bacterial Lipoprotein) PGN (Peptideglycans) Zymosan Hemagglutinin (*Measles virus*) Phospholipomannan (*Candida*) Gkycosylphosphophatidyl inositol mucin (*Trypanosoma*) HSP (heat shock protein) hyaluronan	CD14 CD36 RP105	MyD88-Dependent Pathway	MyD88 TRIAP	NF-κB	TNF IL, et al.
TLR3	Double-standed Viral RNA (dsRNA) Single-stranded viral RNA(ssRNA) polyI: C		Trif-Dependent Pathway (MyD88-independent Pathway)	TRIF	IRF3,7 NF-κB	TNF IL and IFN, et al.
TLR4	LPS (Lipopolysaccharide) LTA (Lipotechoic Acid) Mannan (*Candida*) Glycoinositolpholipids (*Trypanosoma*) Envelope proteins Heparan Hyaluronate HSP(heat shock protein) Fibronectin Fibrinogen Hyaluronic acid Heparan sulfate Hyaluronan Lung surfactant protein A	CD14 MD2, LBP RP105	MyD88-Dependent Pathway	MyD88 TRIAP	NF-κB	TNF IL, et al.
			Trif-Dependent Pathway (MyD88-independent Pathway)	TRIF TRAM	IRF3,7	IFN
TLR5	Bacterial Flagellin		MyD88-Dependent Pathway	MyD88	NF-κB	TNF IL,et al
TLR7	Small anti-viral components Single-stranded RNA (ss-RNA) Imiquimod loxorbine		MyD88-Dependent Pathway	MyD88	IRF3,7 NF-κB	TNF IL and IFN, et al.
TLR9	CpG islands of bacterial DNA dsDNA virus Hemozoin (*Plasmodium*)		MyD88-Dependent Pathway	MyD88	IRF7,8 NF-κB	TNF IL and IFN, et al.
TLR11(mouse)	Uropathgenic bacteria Profillin-like molecule		MyD88-Dependent Pathway	MyD88	NF-κB	TNF IL, et al.
TLR12	Unknown		MyD88-Dependent Pathway	MyD88	NF-κB	TNF IL, et al.
TLR13						

and binds to IRAKs via DD-DD interaction. MyD88 first recruits IRAK4, which is a multidomain protein consisting of a conserved N-terminal DD and a central kinase domain, permitting engagement of IRAK1 [57]. IRAK1 is phosphorylated by

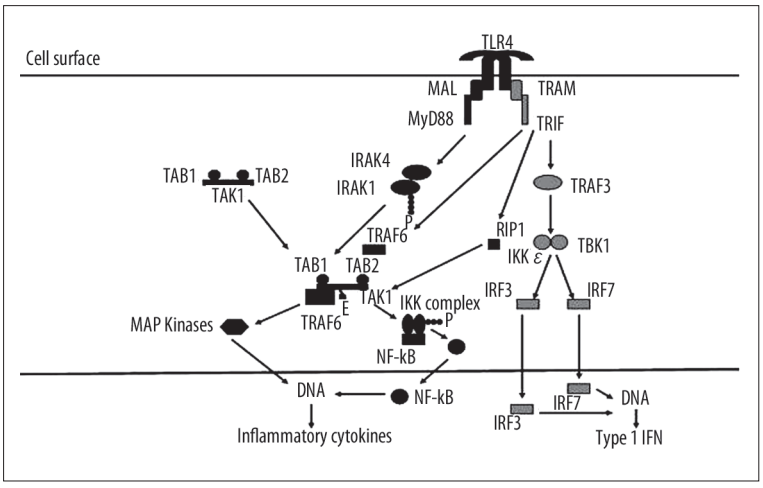

Figure 2. TLR4 signaling pathway: TLR4 is localized on cell surface for ligand recognition and activates both MyD88-dependent pathway and TRIF- dependent pathway. In MyD88-dependent pathway, TLR4 recruits MAL to link TIR domain of TLR4 with MyD88. MyD88 recruits the IRAK family of proteins and TRAF6. TRAF6 activates TAK1. The activated TAK1 not only activates the IKK complex activating NF-κB, also activates the MAPKs. In TRIF-dependent pathway, TLR4 recruits.TRAM to link TIR domain of TLR4 with TRIF. TRIF interacts with RIP1 and TRAF6, which could activate NF-κB and MAPKs; TRIF interacts with TRAF3 and activates TRAF3/IKKε which activates IRF3 and IRF7.

IRAK4 and further recruits Tumor Necrosis Factor-Receptor-Associated Factor-6 (TRAF6) [58]. Phosphorylated IRAK1 and TRAF6 together dissociate from cytoplasmic domain protein complex, and constitute a new complex with GTF-beta-Activated kinase 1 (TAK1), TAK1-Binding Protein-1 (TAB1) and TAK1-Binding Protein-2 (TAB2). TRAF6, TAK1, TAB1 and TAB2, binding to ubiquitin-conjugating enzyme, lead to ubiquitylation of TRAF6 and activation of TAK1. Activated TAK1 phophorylates MAPKs, p38 and JNKs via Mitogen-Activated Protein Kinase Kinase-3, 6, and 7 (MMKs3, 6 and 7). P38 and JNKs enter the cell nucleus and promote target genes expression. TAK1 also phophorylates the inhibitor of κ Light Polypeptide Gene Enhancer in B-Cell Kinase (IKK complex). The IKK complex degrades I-κB, resulting in NF-κB activation, transferring NF-κB into the nucleus and inducing expression of target genes [59,60]. In TRIF-dependent pathway, TLR4 signals via TRAM and TRIF, and activates NF-κB via direct and indirect mechanisms via interacting domains of TRIF with RIP1 and TRAF, respectively. This pathway also induces phophorylation of IRF3 and IFN-regulatory factor 7 (IRF7), which play key roles in initiating expression of type 1 IFN while translocation into nucleus occurs [61]. All the TLRs are listed in Table 1.

TLRs and Myocardial I/R Injury

The evidence of myocardial I/R leading to cardiac injury and dysfunction

Myocardial ischemia and reperfusion (I/R) injury was first discovered in 1960 by Jennings, who described the histological features of reperfused ischemic canine myocardium [62]. The process of restoring blood flow to the ischemic myocardium can induce myocardial injury and produce a spectrum of reperfusion-associated pathologies. This process is defined as myocardial I/R injury, which is characterized by an acute inflammatory process in which activated leukocytes and endothelial cells (EC) are primarily involved [63]. Myocardial I/R injury occurs in some conditions of cardiovascular diseases (e.g., arteriosclerosis, coronary artery spasm and thrombosis [64,65]) and therapeutic strategies (e.g., percutaneous coronary intervention (PCI), coronary artery bypass grafting (CABG), cardiopulmonary bypass (CPB) in cardiac operation and cardiac transplantation [66,67]). CPB and cardiomyocytes reperfusion are the leading causes of postoperative morbidity and mortality. A number of cardiac surgery-associated complications are often attributed to myocardial I/R injury [68]. A series of postoperative complications occur within patients who underwent CABG, including prolonged contractile dysfunction, low-output syndrome, perioperative myocardial infarction and heart failure. Restoration of blood flow achieved with PCI can result in an acute cardiac local inflammatory response and myocardial and endothelial cell damage [69–71].

I/R injury causes severe clinical symptoms, including cardiac contractile dysfunction, arrhythmias, cell death mediating heart failure, and sudden death. Myocardial I/R leads to 4 types of myocardial damage, with different magnitudes and patterns:

Myocardial stunning

After short episodes of myocardial I/R, prolonged mechanical dysfunction persists, although no prominent histological features of irreversible myocardial injury exist. The phenomenon was called "myocardial stunning" [72–74]. Myocardial stunning is characterized by reversible myocardial contractile dysfunction, within a prolonged period after reperfusion of ischemic myocardial tissue. The myocardiocytes are capable of recovering from this reversible form of injury after several days or weeks. The clinical symptoms of stunned myocardium range from cardiac arrest in open cardiac surgery and PCI, to thrombolysis, and unstable and stable angina [66,74,75].

No-reflow phenomenon

Originally defined as "inability to reperfuse a previously ischemic region" [76]. It refers to myocardial I/R injury that obviously impairs EC function, which disturbs restoration of blood in microvasculature. This phenomenon is secondary to vasoconstriction, platelet and leukocyte activation, increased oxidant production, and increased fluid and protein extravasation [77–79]. No-reflow phenomenon usually occurs after more prolonged episodes of myocardial ischemia [66].

Reperfusion arrhythmias

This phenomenon is mediated by myocardial I/R injury, and is potentially harmful, but effective treatments are efficient [80].

Lethal reperfusion injury

Lethal reperfusion injury is defined as death (necrosis or apoptosis) of cardiomyocytes, which are viable at the end of ischemia. The process primarily occurs in severely ischemic myocardium and is regarded as severe, deadly and irreversible.

The evidence of inflammation linking TLRs to myocardial I/R injury

It has been well established that the innate immune system plays a significant role in the pathogenesis of myocardial I/R injury. TLR is an important constitutive component of the innate immune system, and mediates a series of innate immune responses. Specifically, the best known TLR4 is central to early activation of the innate immune response in myocardial I/R injury. It was demonstrated that a number of endogenous molecules are released from ischemic, damaged, or dying cells and tissues, and can serve as triggers for activation of inflammatory response, and exacerbate tissue injury in myocardial I/R injury [81]. Kaozoroswki [82] and Aol [83] determined that high mobility group box 1 (HMGB1) and 70-kD heat shock cognate protein (HSC70), which were considered to be endogenous molecules released from I/R cardiac myocytes, mediate the expression of cardiac depressants via TLR4-dependent inflammatory response. Zou [84] also reported that TLR4 were activated by HSC70, which were constitutively expressed in the myocardium and released from I/R myocardium, to mediate TLR4-dependent inflammatory response. These endogenous molecules produced in myocardial I/R are regarded as activators and triggers for cardiac and myocardial I/R injury.

TLR4 mediate myocardial I/R injury via multiple hierarchies of inflammatory response. Activated proinflammatory cytokines and chemoattractants are important factors for TLR4-mediated myocardial I/R injury. Yang and Cha [85–87] both reported that activated and up-regulated TLR4 play a role in myocardial I/R injury by increasing TNF-alpha, IL-6 and IL-1beta expression. Additionally, it was also reported that the TLR4 signaling pathway can mediate production of TNF-alpha and IL-1beta, contributing to cardiac dysfunction in global myocardial I/R. Kaczorowski et al. [88] employed syngeneic heart transplant models to test the hypothesis that TLR4 mediated early inflammatory response in cold I/R in murine hearts. They demonstrated that serum TNF, IL-6, JE/monocyte chemotractant protein (MCP)-1, IL-1beta, as well as intragraft TNF, IL-1beta, IL-6, early growth response (EGR)-1, intercellular adhesion molecule (ICAM)-1, and inducible nitric oxide synthase (iNOS) mRNA levels were significantly lower in the C3H/HeJ (deficient in TLR4 signaling, donors)→C3H/HeJ mice (recipients) compared to the C3H/HeOuJ (wild type, donors)→C3H/HeOuJ mice (recipients). Treatment with anti-HMGB1 neutralizing antibody or anti-HSC70-specific antibody during the period of mouse myocardial I/R resulted in suppression of cytokine expression (such as IL-6, TNF-alpha and intracellular adhesion molecule-1 [ICAM-1] messenger RNA [mRNA]), either from the layer of protein or mRNA, and improved cardiac functional recovery [82,89]. Activated inflammatory cells also act as a significant factor for TLR4-mediated I/R injury. It was shown that hearts isolated from TLR4-defective mice undergoing global myocardial I/R reduced neutrophil infiltration and expressed lower levels of keratinocytic-derived chemokine (KC) and monocyte chemoattractant protein-1 (MCP-1). In the syngeneic heart transplant model described above, less infiltration along with less expression of chemoattractants occurred in the C3H/HeJ mice→C3H/HeJ mice compared to C3H/HeOuJ mice→C3H/HeOuJ mice [88]. Neutrophil activation and infiltration may be associated with chemoattractants in myocardial I/R injury [83]. Abundant evidence substantiates the role of neutrophils in myocardium undergoing I/R – neutrophils are activated and recruited to the I/R area within the period during which endothelial and myocyte injury occurs [90–94]. Activated neutrophils can aggravate such injury via interaction with other cells and production of oxidants and proteases.

Effect of TLR4-mediated cytokines in I/R injury on cardiac function

The cytokines mediated by the TLR4-mediating signaling pathway in the myocardial I/R primarily consist of the TNF family, IL family, chemoattractant and adhesion molecules family, which all can act to induce I/R cardiac dysfunction.

TNF-alpha is the principal cytokine induced by TLR4 in myocardial I/R. From 1986 to 1998, many animal experiments were performed, and it was concluded that TNF-alpha appears to be an important cardiodepressant for myocardium and depressed myocardial contractility [95–97]. Clinical data also indicated that TNF-alpha contributes to myocardial dysfunction and hemodynamic instability following CPB [98–102]. TNF-alpha produces its acute negative inotropic effects by interfering with Ca^+ homeostasis, inducing direct cytotoxicity, disrupting excitation-contraction coupling, desensitizing the beta- and alpha- catechol receptor, and feeding back to induce other myocardial depressants such as IL-1beta [103]. TNF-alpha induces these negative inotropic effects by 2 different mechanisms in early and delayed phases [97,104]. In the early phase of inflammatory response, TNF-alpha may depress alpha- and beta- adrenergic responsiveness of myocytes via an NO-independent manner that is probably mediated by sphingolipid metabolites and caused by TNF-alpha effecting on intracellular calcium transient [105–107]. In the delayed phase of inflammatory response, TNF-alpha induces cardiac dysfunction via an NO-dependent pathway, which is mediated by production of nitric oxide (NO) and by depressed sensitivity of the myofilament to Ca^+ [107,108]. Myocardial apoptosis (programmed cell death) is another mechanism leading to TNF-alpha-mediated cardiac depression. TNF-alpha antagonists can reduce cardiac infarct size after myocardial I/R injury and improve cardiac function, which is regulated by the magnitude of myocardial apoptosis. Myocardial apoptosis is characterized by programmed cell death with the maintenance of cell membrane integrity and without releasing creatine kinase. Apoptotic myocardial cells can still retain their ability to contract in response to calcium ionophores [109]. Myocardial apoptosis is mainly induced via a TNF-alpha receptor-1 and TNF-alpha receptor-2 with a death domain (TRADD). Specific signal transduction molecules initiated by exogenous or endogenous stimuli deliver apoptotic signals between TRADD and a kinase domain to activate caspases that facilitate DNA fragmentation and eventual cell apoptosis. This process is thought to be mediated

by sphingosine and NO. Cardiac myocyte apoptosis-associated clinical syndromes include chronic heart failure, ischemia arrhythmogenic right ventricular dysplasia, viral myocarditis and dilated cardiomyopathy, as well as cardiogenic arrest [103,110].

IL is another important cytokine inducing TLR4-mediated myocardial I/R injury. IL-1 induces the negative inotropic effect of myocardium and depresses myocardial contractility via expression of iNOS, p38 and p42/p44 MAPK signaling pathways [111–113]. It can result in arrhythmogenesis after myocardial I/R via decreases in Ca^+ regulatory gene expression [114–116], and mediates neonatal myocardium apoptosis, identical to TNF-alpha [117]. Clinical research data establishes that increased levels of IL-6 are associated with cardiac dysfunction after CPB [118]. IL-6 is a multifunction cytokine produced by different cell types, and is reported to serve as a direct cardiodepressant, inhibiting contractility in the hamster myocardium [119]. The primary mechanism of IL-6-mediated myocardial contractility inhibition is shown by reduced peak systolic intracellular Ca^+ transient levels, increased iNOS-induced NO production, and, subsequently, decreased levels of cGMP-mediated L-type Ca^+-channel [119–121]. In addition, IL-6 induces activation and production of diverse chemoattractants, intercellular adhesion molecules (ICAM) and complements, as well as C-reactive protein (CRP), which is an acute phase protein produced by the liver. These cytokines have a damaging effect on myocardium and lead to cardiodepression [122–125].

ICAM-1, a member of the adhesion molecule family, is a type of transmembrane protein, with a molecular weight of 80–114 kDa. This cytokine is capable of being expressed by immune cells and non-immune cells, including leukocytes and endothelial cells. ICAM-1 plays an important role in the pathogenesis of I/R injury and seems to be of particular importance for attachment and transendothelial migration of leukocytes [126]. Entman et al. demonstrated that ICAM-1 induced leukocyte-mediated cardiomyocte damage after CPB [127]. Appleyard and Cohn, deploying a sheep model, showed that myocardial stunning induced by cardiac I/R can be attenuated by blocking neutrophil-endothelial cell interaction with ICAM-1 receptors [128]. Siminiak reported that administration of antibodies that block the function of adhesion molecules attenuated PMN-mediated tissue destruction during on-pump CABG surgery [129]. In summary, ICAM-1 actually mediates cardiomyocyte damage via neutrophil-endothelial cell interaction after cardiac I/R.

TLR4 signaling pathway and myocardial I/R injury

TLR signals via 2 different pathways, known as MyD88-dependent pathway and MyD88-independent pathway (TRIF-dependent pathway). Both of these distinct signaling pathways may act together to mediate myocardial I/R injury. Kaczorowski [82] found that the hearts of MyD88 knock-out (KO) mice and TRIF KO mice undergoing cold I/R exhibited lower levels of TNF-alpha, IL-6, IL-1beta, ICAM-1 and MCP-1 located both in serum and intragrafts through syngenieic mouse heart transplant models, concluding that MyD88 and TRIF both contributed to the inflammation response occurring after cold I/R. NF-κB, which is a downstream transcription factor in the TLR4-mediated signaling pathway, can be activated, thereby entering the cell nucleus to promote a series of proinflammatory cytokine genes expression-mediating inflammatory responses. Myocardial I/R injury is closely related to TLR4/NF-κB signaling pathways and also involves the iNOS-dependent mechanism. Yang and Jiang [130] applied Valsartan, which is a kind of angiotensin II type I receptor blocker, to preconditioning in rat hearts before I/R, and demonstrated that Valsartan preconditioning inhibited TLR4 and NF-κB expression concomitant with an improvement in myocardial injury. HSC70 [84], which is produced during myocardial I/R as a kind of endogenous ligand binding to TLR4, induced NF-κB activation and depressed myocardial contractility in a TLR4-dependent manner. LPS, which is a kind of exogenous ligand binding to TLR4, can induce cardiac myocyte damage via a TLR4-dependent signaling pathway that is also induced by cardiomyocyte I/R injury. Baumgarten [111] applied LPS in both wild-type and C3H/HeJ mice. He found that the levels of NF-κB and iNOS were upregulated in control mice *vs.* mutant mice. Inhibition of iNOS could prevent the influence of LPS on contractile activity in control myocytes. A syngenieic heart transplant model performed by Kaczorowski showed less myocardial NF-κB translocation at the mutant→mutant group compared to the wild-type→wild-type [88].

Myocardial I/R injury occurs not only through the TLR4 signaling pathway itself, but also through other related signaling pathways inter-regulating with the TLR4 signaling pathway, such as PI3K/AKt signaling and AMPK and ERK signaling. Zhao [132] proposed that cardioprotective effects against ischemic myocardial injury without TLR4 signaling may be mediated through regulating AMP-activated protein kinase (AMPK) and ERK signaling pathway, and demonstrated that ERK and AMPK signaling was augmented during ischemia in C3H/HeJ (TLR4-deficient) mouse hearts *vs.* C3H/HeN (wild type) mouse hearts. Isolated cardiomyocytes from C3H/HeJ hearts showed resistance to contractile dysfunction compared to those from C3H/HeN hearts, which were associated with greater hypoxic activation of AMPK and ERK signaling in C3H/HeJ hearts *vs.* C3H/HeN hearts. It appears that the TLR4 signaling pathway inhibits AMPK and ERK signaling pathways to cause myocardial I/R injury. Another signaling pathway, TLR4/PI3K/AKt-dependent signaling pathway, may be the mechanism of cardiomyocyte I/R injury. It was reported that TLR4 (–/–) mice experienced decreased myocardial injury following I/R. Pharmacologic inhibitors of PI3K (wortmannin or LY294002) were administered before myocardial I/R. The blockade can abrogate myocardial protection in TLR4(–/–) following I/R. Protection against myocardial I/R injury in TLR4 (–/–) mice is mediated through a PI3K/AKt-dependent mechanism. The mechanisms by which PI3k/Akt are augmented in the TLR4(–/–) myocardium may involve increased phosphorylation/inactivation of myocardial phosphatase, tension homology deleted on chromosome 10, and increased phosphorylation/inactivation of myocardial glycogen synthase kinase-3beta. Myocardial I/R injury may be mediated by suppressing the PI3k/Akt signaling pathway, which is induced by TLR4 (+/+) [133]. We conclude that multiple signaling pathways interacting with each other lead to myocardial I/R injury.

Left ventricular remodeling is a complication secondary to myocardial I/R injury and myocardial infarction. Left

ventricular (LV) remodeling is strongly associated with inflammatory responses and extracellular matrix degradation. Therefore, we proposed that MI and myocardial I/R injury mediates LV remodeling via the same mechanism. Riad [134] demonstrated that TLR4 KO mice undergoing MI showed improved LV function and reduced LV remodeling (reduced levels of atrial natriuretic factor and total collagen, as well as a reduced heart weigh-to-body weight ratio) *vs.* wild type-MI mice. This was associated with a reduction of protein levels of the intracellular TLR4 adaptor protein MyD88 and enhanced protein expression of the anti-hypertrophic JNK in KO-MI mice *vs.* wild type-MI mice. Thus, LV remodeling could be the combined effects of TLR4-dependent pathway and JNK.

A number of endogenous molecules are released by different immune cells and non-immune cells during myocardial I/R. These endogenous ligands bind to TLR4 and activate diverse innate immune responses. The immune response processes are mediated by the TLR4 signaling pathway (MyD88-dependent pathway and TRIF-dependent pathway) interacting with other signaling pathways (PI3K/Akt and AMPK, ERK signaling pathway). Finally, transcriptional factor NF-κB is activated and enters the nucleus to promote the inflammatory response. This is the basic mechanism of TLR4 signaling pathway-mediated myocardial I/R injury.

Conclusions

TLRs-mediated innate immune response and adaptive immune response represent the first line of defense against invading pathogens. In addition to the central role they play in host immune defense against micro-organisms, TLRs are capable of responding to stress and modulating inflammation and tissue damage following noninfectious impairments such as cardiac ischemia and reperfusion injury. Ischemia and perfusion injury is defined as the process of restoring blood flow to the ischemic myocardium, inducing myocardial injury and producing a spectrum of reperfusion-associated pathologies. CPB during cardiac surgery, CABG, and PCI all may experience myocardial I/R injury. Thus, research on TLRs-mediated myocardial I/R is of great clinical significance in the search for measures to diminish or eliminate the adverse effects of myocardial I/R injury. We could solve these problems through neutralizing TLRs activator, disrupting TLRs signaling pathway or obstructing distal effectors by specific blockades that thwart development of effective pharmacologic therapies. Some laboratories paradoxically have discovered that TLR4 has a beneficial effect on myocardium, as demonstrated by the fact that LPS, at sublethal doses, has a preconditioning-like effect, protecting the heart against subsequent I/R injury. The mechanism involved in the LPS-inducing beneficial impact on myocardial I/R injury requires further investigation.

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