

Role of Toll-like receptor mediated signaling pathway in ischemic heart

Yasuchika Takeishi¹, Isao Kubota²

¹First Department of Internal Medicine, Fukushima Medical University, Fukushima, and ²Department of Cardiology, Pulmonology, and Nephrology, Yamagata University School of Medicine, Yamagata, Japan

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1. ABSTRACT

Stimulation of TLRs by exogenous and endogenous ligands triggers expression of several genes that are involved in innate immune responses. Recently, a role of TLR4 in the myocardial response to injury separate from microbial pathogens has been examined in experimental studies. TLR4 deficient mice sustain significantly smaller infarctions compared with wild-type control mice given similar areas at risk. Levels of serum cytokines such as IL-1 β , IL-6, and TNF α are increased after ischemia/reperfusion, but these responses are attenuated in TLR4 deficient mice compared to control mice. TLR2 signaling also importantly contributes to cardiac dysfunction following ischemia/reperfusion. MyD88, a key adaptor protein for TLR signaling, is responsible for the protective effects of TLR signaling inhibition in ischemia/reperfusion injury. TLR4 gene polymorphism (Asp299Gly) attenuates innate immune responsiveness, reduces the risk for coronary artery disease, and increases a chance of longevity. The innate immune system is clearly involved in the pathogenesis of cardiovascular diseases and could be a new therapeutic target.

2. INTRODUCTION

Host defense against invading microbial pathogens is elicited by the immune system, which consists of two approaches: innate immunity and acquired immunity (1). The innate immune system is limited to the recognition of evolutionary highly conserved pathogen motifs and is considered as a first line of defense (2, 3). The induction time of innate immune responses is fast. The innate immune response appears in primitive organisms in evolution. The Toll gene was first discovered as encoding for a receptor responsible for the dorsal ventral polarity in *Drosophila* (4). Toll was shown to be an essential receptor for host defense against fungal infection in *Drosophila*, which has only innate immunity. Subsequently, a mammalian homolog of the Toll receptor, Toll-like receptor (TLR), was identified to induce expression of genes involved in inflammatory responses (5). Recently, accumulating interest emerges from the cardiovascular research field in this TLR family (6). This review will focus on TLR signaling pathway in ischemic heart and ischemia/reperfusion injury.

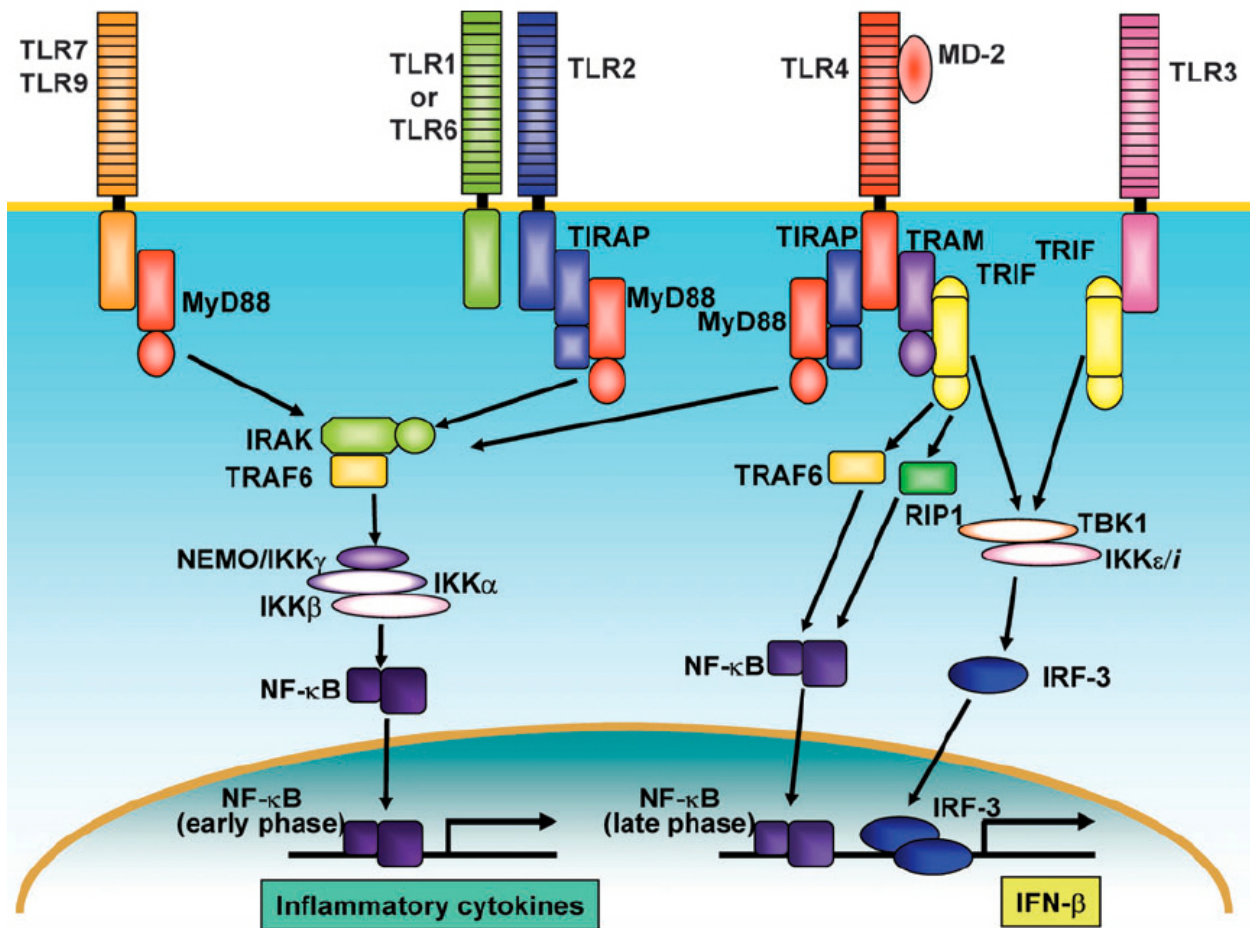


Figure 1. TLR signaling pathway. Reproduced with permission from (1).

3. EXPRESSIONS OF TLRs IN THE HEART AND VASCULATURE

Expression of the TLRs is very ubiquitous throughout species like mammals, fruit flies, chicken, and plants (7). Macrophages and T-lymphocytes express TLRs that recognize molecular patterns that are foreign to mammalian organism but commonly found on pathogens (8). The principal effector cells of innate immunity are macrophages, natural killer cells, and mast cells, and those cells play important roles in the development and progression of cardiovascular diseases. The macrophage secretes cytokines that guide and regulate function of other cells that are associated with atherogenesis and progression of atherosclerotic disease.

Expression of TLRs has been found in most cardiovascular cells such as cardiomyocytes (9), endothelial cells (10), adventitial fibroblasts (11), and dendritic cells (12). In neonatal rat cardiomyocytes, TLR2, TLR3, TLR4, and TLR6 are expressed (9). In murine myocardium, TLR4 expression is increased after myocardial infarction. Focal areas of intense TLR4 staining are observed in human heart tissue from patients

with dilated cardiomyopathy (9, 13). Compared to cardiomyocytes, endothelial cells have about 5-fold higher TLR4 expression (9). Human endothelial cells express predominantly TLR4 and very low level of TLR2 (10). Lipopolysaccharide (LPS) and interferon (IFN)- γ induce a 2-fold increase in TLR2 and TLR4 expression in endothelial cells (14). TLR2 and TLR4 are primarily expressed in endothelial cells and macrophages in the atherosclerotic lesions (15, 16).

4. TLR SIGNALING PATHWAY

Stimulation of TLRs by exogenous and endogenous ligands triggers expression of several genes that are involved in immune response. Ligand recognition by TLRs facilitates dimerization of TLRs (1, 2). Dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic Toll/interleukin-1 receptor (TIR) domain (Figure 1). In the signaling pathways downstream of the TIR domain, a TIR domain-containing adaptor, myeloid differentiation factor 88 (MyD88), is essential for induction of inflammatory cytokines such as tumor necrosis factor (TNF)- α ,

interleukin (IL)-4, IL-12, and IFN- γ (17, 18). MyD88, harboring a C-terminal TIR domain and an N-terminal death domain, associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor associated kinase (IRAK)-4 to TLRs through interaction of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TNF receptor associated factor 6 (TRAF6), leading to the activation of two distinct signaling pathways. One is activation of mitogen-activated protein (MAP) kinases, leading to AP-1 transcription factor. Another is the TAK1/TAB complex, which enhances activity of the I κ B kinase (IKK) complex. The IKK complex induces phosphorylation and subsequent degradation of I κ B, which then leads to nuclear translocation of NF- κ B transcription factor.

In MyD88-deficient macrophages, TLR4 ligand-induced production of inflammatory cytokines is not observed, but activation of NF- κ B is observed in the delayed phase (19). These data suggest that a MyD88 independent component exists in TLR4 signaling. TLR4 stimulation leads to activation of interferon regulatory factor 3 (IRF3) as well as the late phase activation of NF- κ B in a MyD88-independent manner (20). Activation of IRF3 produces IFN- γ .

5. ROLE OF TLR4 SIGNALING IN MYOCARDIAL ISCHEMIA/REPERFUSION

Currently, more than 10 mammalian TLRs have been discovered, and TLR4 is most extensively investigated among them. A point mutation in the TLR4 gene has been identified in a mouse strain (C3H/HeJ), which is unresponsive to LPS (21). The TLR4 mutant mouse enables us to investigate functional roles of TLR4 *in vivo*. In addition, immunologist Shizuo Akira of Osaka University in Japan and his colleagues deleted systemically the mouse genes for TLRs to reveal their specific functions (22). TLR4 is an essential receptor for recognition of LPS from Gram-negative bacteria, and also has been shown to recognize endogenous ligands such as heat shock proteins, fibronectins, and hyaluronic acid (6).

Recently, a role of TLR4 in the myocardial response to injury separate from microbial pathogens has been examined in experimental studies. Myocardial ischemia/reperfusion was performed on TLR4 mutant mice (23), which do not express functional TLR4 because of naturally occurring mutations in the TLR4 gene (21). Mice were subjected to 1 hour of coronary ligation followed by 24 hours of reperfusion. TLR4 mutant mice sustained significantly smaller infarctions compared with wild-type control mice given similar areas at risk. In TLR4 mutant mice, fewer neutrophils infiltrated into the myocardium, and fewer lipid peroxides and less complement deposition were contained compared with control mice after ischemia/reperfusion (23). These data suggest that TLR4 serves a proinflammatory role in murine myocardial ischemia/reperfusion injury. Levels of serum cytokines such as IL-1 β , IL-6, and TNF α were increased after ischemia/reperfusion, but these responses were attenuated

in TLR4 mutant mice compared to control mice (24). Although infarct size was smaller in TLR4 mutant mice following ischemia/reperfusion than in wild-type mice, left ventricular developed pressure measured with a left ventricular catheter was lower in TLR4 mutant mice. Thus, although the blockade of TLR4 signaling reduces myocardial infarction size and cytokine production, it does not preserve myocardial function (24).

A signaling cascade downstream of TLR4 was examined after ischemia/reperfusion using TLR4 mutant mice. After 1 hour of regional ischemia and 2 hours of reperfusion, significant activation of extra-cellular signal regulated kinase (ERK), p38 MAP kinase and c-Jun N-terminal kinase (JNK) was observed in wild-type mouse hearts (25). However in TLR4 mutant mice, JNK activity, but not ERK or p38 MAP kinase, was significantly reduced compared to wild-type mice. Nuclear translocation of NF- κ B and AP-1 was significantly reduced in TLR4 mutant mice. IL-1 β , monocyte chemotactic factor-1 (MCP-1), and IL-6 were detectable in reperfusion ischemic myocardium, but the expression of these mediators was significantly decreased in the myocardial tissue of TLR4 mutant mice (25). A role of TLR4 in ischemia/reperfusion injury was also examined by pharmacological blockade of TLR4. Eritoran, a specific TLR4 antagonist, developed significantly smaller infarct size, reduction in JNK phosphorylation, less NF- κ B translocation, and a decrease in cytokine expression after 30 minutes of ischemia and 120 minutes of reperfusion in mice (26).

Hua *et al.* examined the role of phosphoinositide 3-kinase (PI3K) and Akt signaling in TLR4-mediated cardioprotection following ischemia/reperfusion injury (27). TLR4 mutant mice and wild-type control mice were subjected to 45 minutes of ischemia followed by reperfusion for 4 hours. Pharmacological inhibition of PI3K with wortmannin or LY294002 abrogated myocardial protection in TLR4 mutant mice, suggesting that protection against myocardial ischemia/reperfusion injury in TLR4 mutant mice is mediated through a PI3K/Akt-dependent mechanism (27).

In a clinical study, expression of TLR4 on circulating monocytes from normal controls, patients with stable angina, unstable angina, and acute myocardial infarction was examined with the use of flow-cytometry (28). Circulating TLR4-positive monocytes were 2.5-fold increased above controls and patients with stable angina in patients with unstable angina and acute myocardial infarction. This was paralleled by enhanced transcript levels of TLR4 and MyD88 in patients with unstable angina and acute myocardial infarction. Acute coronary syndrome is associated with enhanced expression and signaling downstream of TLR4 in circulating monocytes.

6. FUNCTIONAL ROLE OF TLR2 IN ISCHEMIC HEARTS

TLR2 recognizes a variety of microbial components. These include lipoproteins from various pathogens, peptidoglycan and lipoteichoic acid from Gram-

positive bacterias, soluble modulin, and yeast (6). The broad ligand specificity of TLR2 may be accounted by the fact that TLR2 dimerizes with other TLRs to detect optimally these ligands. In addition, it has been demonstrated that oxidative stress, an important factor for ischemia and ischemia/reperfusion injury, mediates nuclear translocation of NF- κ B and AP-1 by a TLR2-dependent mechanism (29).

Recently, a functional role of TLR2 in response to ischemia- and ischemia/reperfusion-induced myocardial injury separate from microbial pathogens has been demonstrated in experimental studies using TLR2 knockout (KO) mice. Left anterior descending coronary artery was ligated in TLR2-KO mice and wild-type mice (30). Survival rate was significantly higher in TLR2-KO mice than in wild-type control mice 4 weeks after myocardial infarction. Although infarct size and degree of inflammatory cell infiltration in infarct area were similar, myocardial fibrosis in non-infarct area of TLR2-KO mice was much less compared to WT mice accompanied with reduced transforming growth factor- β 1 and collagen type 1 mRNA expressions. One and four weeks after surgery, left ventricular dimensions at end-diastole and end-systole were smaller and fractional shortening was higher in TLR2-KO mice compared with control mice (30). These data suggest that TLR-2 plays an important role in ventricular remodeling after myocardial infarction.

TLR2 also mediates inflammation in response to non-infectious injury. Isolated cardiac function was examined in Langendorff-perfused hearts from TLR2 deficient mice and wild-type mice (31). Contractile performance after ischemia/reperfusion was significantly impaired in hearts from wild-type mice as demonstrated by a lower recovery of left ventricular developed pressure relative to TLR2 deficient hearts. Contractile dysfunction in wild-type hearts was associated with elevated cardiac levels of TNF α and IL-1 β . Ischemia/reperfusion-induced cardiac dysfunction was reversed by treatment with the recombinant TNF blocking protein etanercept (31). TLR2 signaling importantly contributes to cardiac dysfunction following ischemia/reperfusion.

In TLR2 deficient mice, ischemia/reperfusion-induced endothelial dysfunction was improved compared to wild-type mice (32). TLR2 deficiency was also associated with a smaller infarct size and reduced reperfusion-induced production of reactive oxygen species and leukocyte infiltration. TLR2 contributes to coronary endothelial dysfunction after ischemia/reperfusion, possibly through stimulation of neutrophil- and free radical-mediated endothelial injury.

7. MyD88 IS A KEY ADAPTOR PROTEIN FOR TLR SIGNALING

MyD88 is a key adaptor protein that plays a major role in the innate immune pathway (Figure 1). MyD88 contributes to cardiac inflammation and cytokine production after Cocksackie virus exposure (33). Hua *et al.* examined whether blocking the MyD88 could protect

myocardium from ischemia/reperfusion injury by adenovirus-mediated transfection of dominant negative MyD88 into the myocardium of rats (34). The hearts were subjected to 45 minutes of ischemia and 4 hours of reperfusion at 3 days after adenovirus transfection. Dominant negative MyD88 significantly reduced infarct size compared with rat hearts transfected with GFP. Transfection of dominant negative MyD88 inhibited ischemia/reperfusion-induced NF- κ B activation and increased the levels of Akt phosphorylation. The MyD88 blockade also attenuated cardiomyocyte apoptosis after ischemia/reperfusion (34). These data suggest that MyD88 is responsible for the protective effects of TLR signaling inhibition in ischemia/reperfusion injury.

8. CLINICAL SIGNIFICANCE OF TLR GENE POLYMORPHISM

TLR, the activity of which may be modulated by genetic polymorphisms, is a signaling receptor in innate immunity, and the inflammatory mediators produced by its activation exert various atherogenic effects. Kolek *et al.* examined whether the Asp299Gly polymorphism in the TLR4 gene, which impairs inflammatory response, is associated with reduced vascular inflammation and a decreased risk for coronary artery disease in 1894 patients (35). The level of C-reactive protein was lower in G-allele carriers than wild-type (AA) subjects. G-allele carriers also had a lower prevalence of angiographic coronary artery disease. Another group studied the Asp299Gly polymorphism in 105 young Sicilian men admitted for acute myocardial infarction (mean age 41 years), 127 Sicilian men matched by age strata (mean age 38 years), and 55 Sicilian oldest old men (mean age 100 years) (36). The prevalence of G-allele carriers was lower in patients with acute myocardial infarction and higher in the oldest old men compared to healthy young controls. These findings suggest that attenuation of innate immune responsiveness beneficially modifies the risk for coronary artery disease and increases a chance of longevity.

9. SUMMARY AND PERSPECTIVE

The innate immune system is clearly involved in the pathogenesis of cardiovascular diseases. New treatments could include drugs that block TLR4, TLR2 or MyD88 in a modern environment with reduced pathogen load and improved control of severe infections by antibiotics. Such drugs might cripple a person's infection-fighting ability if delivered systemically, but administering them locally to the coronary arteries reduces that risk.

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Abbreviations: TLR: Toll-like receptor; LPS: lipopolysaccharide; IFN: interferon; MyD88: myeloid differentiation factor 88; TNF: tumor necrosis factor; IL: interleukin; IRAK: IL-1 receptor associated kinase; TRAF6: TNF receptor associated factor 6; MAP: mitogen-activated protein; IKK: I κ B kinase; IRF3: interferon regulatory factor 3; ERK: extra-cellular signal regulated kinase; JNK: c-Jun N-terminal kinase; MCP-1: monocyte chemotactic factor-1; PI3K: phosphoinositide 3-kinase; KO: knockout

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Send correspondence to: Yasuchika Takeishi, First Department of Internal Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima, Japan 960-1295, Tel: 81-24-547-1188, Fax: 81-24-548-1821, E-mail: takeishi@fmu.ac.jp

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