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# **Toll-like receptors signaling pathways as a potential therapeutic target in cardiovascular disease**

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## **Running title:** TLR in CAD

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## **Abstract**

Cardiovascular disease (CVD) is one of the most important causes of morbidity and mortality, and associated with an important economic burden globally. Over the last decade, the prevalence of CVD has been rising globally, and is now associated with millions of death annually in both developed and developing countries. There is good evidence that the immune system is involved in the pathophysiology of CVD. Toll-like receptors (TLRs) and their down-stream signaling pathways play an important role in the immune system. Recent studies have suggested that the TLRs are involved in atherogenesis, including stroke, myocardial infarction, ischemia-reperfusion injury, cardiac remodeling and development of heart failure (HF). In this review we have summarized the recent studies investigating the role of TLRs in CVD and the potential for using TLRs signaling pathways as a therapeutic target in CVD.

**Key words:** cardiovascular disease, immune system, Toll-like receptors, signaling pathway

## **Introduction**

Cardiovascular disease (CVD) is a major health problem with a high prevalence of morbidity and mortality worldwide, and particularly in developing countries. Over the last decade, the prevalence of CVD has risen alarmingly and it is now estimated that about 17 million deaths, and a very high rate of complications result from CVD and this imposes huge economic burden on healthcare system (1, 2). Ischemic heart disease and cerebrovascular disease are responsible for a large percentage of patients with CVD (3). Although a number of studies have been performed to clarify the mechanisms involved in the pathogenesis of CVD, it remains a complex disease that is incompletely understood. Recently molecular investigations of CVD pathophysiology have revealed some interesting new insights, that may have a bearing on its treatment and prevention. Toll-like receptors (TLRs) and their signaling pathways have emerged as being potentially important in the pathophysiology of CVD.

The immune system has two main arms; the innate immune system (also called non-specific) and adaptive immune system (also called acquired or specific). The innate immune system plays a fundamental role as the first line of defensive mechanisms for sensing and eliminating invading pathogens (4). The Toll-like receptors (TLRs) are a group of receptors which related to a family of pattern recognition receptors (PRRs) that distinguish different molecular pattern of invading microbial pathogens called pathogen associated molecular patterns (PAMPs) (5). TLRs are expressed in various cell types, including immune cells and several non-immune cell types such as cardiovascular system cells (6, 7). After recognition of PAMPs the stimulation of TLRs results in multi-steps events which finally activates expression of a variety of genes related to

inflammation and immune response (8-10). There is increasing evidence that shows that there are many interactions between the immune system and various human disease (7, 11). There are associations between polymorphisms of the TLRs and signaling pathways which are related with different diseases such as autoimmune diseases, diabetes, cancers and also cardiovascular disease (7). The TLRs are involved in many pathophysiological processes suggests that with pharmacological manipulation of TLRs pathway as one of these signaling pathways can be a potential target for therapeutic management of cardiovascular disease. The aim of this review is to summarize recent studies in exciting field of role of TLRs in CVD for improvement of understandings pathophysiology and using TLRs signaling pathways as a therapeutic target in CVD (Table 1-2).

### **Characteristics of Toll-like receptors**

The Toll-like receptors (TLRs) are type I transmembrane glycoproteins, that play a role as a part of immune system which related to a family of pattern recognition receptors (PRRs) that distinguish different molecular pattern of invading microbial pathogens called pathogen associated molecular patterns (PAMPs) such as lipid, protein, lipoprotein and nucleic acid (5). TLRs have two major domains: an intracellular portion that called the Toll/IL-1 receptor (TIR) domain and extracellular portion which contains leucine-rich repeats (LRRs) (5). Eleven types of TLRs (TLR1-TLR11) have been identified in human and for recognition of cytoplasmic and extracellular PAMPs, some of them are localized on the cell surface and others exist in intracellular compartments, localized in endosome, lysosome, endolysosome and endoplasmic reticulum (6, 12). Cell surface TLRs include type 1, 2, 4, 5, 6, 10 and 11 and in contrast

to these TLRs, intracellular TLRs that known as antiviral TLRs include type 3, 7, 8 and 9 (6). Stimulation of the TLR leads to initiating activation several signaling pathways which some of them are particular to specific type of TLR and some of them are common to all TLRs (6). TLRs are being expressed in various cell types, including immune cells in innate and adaptive system and several non-immune cell types such as fibroblast cells, endothelial cells and epithelial cells (13). The stimulation of TLRs results in a multi-step process which finally lads to the activation of regulatory pathways of the innate and adaptive immune system by producing inflammatory cytokines and other mediators (12).

### **Toll-like receptors activation in immune and non-immune related conditions**

As mentioned above, TLRs can recognize conserved structural motifs of microbial pathogens known as PAMPs such as lipid, protein, lipoprotein and nucleic acid (5). For example, TLR2 recognizes specific components of cell wall in gram-negative or positive bacteria, viruses, mycobacteria, parasites and fungi, TLR4 recognizes lipopolysaccharide (LPS) of gram-negative bacteria and TLRs 3, 7, 8 and 9 (14) bind with viral PAMPs. Other TLRs bind to protein ligands (14). It was demonstrated that dendritic cells as a one type of immune system cells can be activated without existence of microbial pathogen and host tissue injury (15). There has been increasing evidence that in certain conditions TLRs can be also be activated by non-pathogen molecules such as host-derived molecule that could be release from tissues underwent physiological stress or any injury which is known as danger signal. These molecules are referred to as a danger-associated molecular pattern (DAMPs) include heat shock proteins (HSPs), fibronectin, fibrinogen, low-molecular-weight hyaluronic

acid, surfactant A protein, heparin sulfate, single- or double-strand RNA and high mobility group box-1 (HMGB-1) (16, 17). Alterations in the extracellular matrix could be a trigger for TLRs stimulation in the any presence of pathogens (6). Stimulation and activation of TLRs with host-derived molecules has risen attention to possible association between role of TLRs and CVD especially in development of atherosclerosis and heart failure.

### **Toll-like receptors signaling and its association with microRNA**

TIR domain-containing adaptor molecules are different in TLRs signaling (18). At present, four major TIR domain-containing adaptors were identified: 1) myeloid differentiation factor 88 (MyD88), 2) TIR domain-containing adaptor protein (TIRAP), 3) TIR domain-containing adaptor inducing interferon  $\beta$  (TRIF), 4) TRIF-related adaptor molecule (TRAM) (6, 18). Following the recruitment of distinct adaptor molecules, downstream TLRs signaling can be separated into two different pathways: MyD88 dependent and MyD88-independent signaling pathways, or the so-called TRIF dependent pathway. MyD88 is bound by all TLRs except TLR3 (19). Each signaling pathway results in activation of inflammatory gene transcription factors (including nuclear factor- $\kappa$ B (NF- $\kappa$ B), interferon-regulatory factors (IRFs), activator protein 1 (AP1) (19). On the other hand, it has been observed that there is crosstalk between TLRs signaling pathways and the phosphoinositide 3 kinase (PI3K)/ Akt signaling pathway, so that activation of each one could be able to activate the other (18).

TIRAP is an essential molecule for connecting of TLR2 and TLR4 to MyD88 (18). After connection of MyD88 and activated TLR, MyD88 communicates with IL-1 receptor-

associated kinases 4 (IRAK-4) and after that IRAK-4 stimulate IRAK-1 and IRAK-2. Following this, the IRAKs is separated from MyD88 and associate with tumor necrosis factor receptor-associated factor 6 (TRAF6). The complex of IRAKs and TRAF6 associate with another membrane complex, and activates the complex consisting TAK1 transforming growth factor- $\beta$ -activated kinase 1 which also called mitogen-activated protein kinase kinase kinase 7 (MAP3K7), TAK1-binding protein 1 (TAB1), TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3) which phosphorylates the inhibitor of nuclear factor kappa B (NF- $\kappa$ B) kinase (IKK) and mitogen-activated protein kinase kinase 6 (MAP2K6) and other downstream molecules such as JUN N-terminal kinase (JNK), P38 and ERK activate NF- $\kappa$ B and AP-1. Subsequently NF- $\kappa$ B and AP-1 translocate into the cellular nucleus and induce a number of gene transcription of proinflammatory cytokines and chemokines (16, 20, 21).

In addition to MyD88-dependent signaling, there is another signaling pathway in which NF- $\kappa$ B activates in the absence of MyD88 association and known as MyD88-independent signaling that in this signaling pathways the main adaptor protein are TRIF and TRAM. The TLR3 transduces its signal mainly via MyD88-independent signaling pathways (22). In addition to activation of the MyD88-dependent pathways, TRIF-dependent pathways is another way that TLR7 and TLR9 could be able to transduce their signals and produce IFNs (10). TLR4 is able to transduce its signals via TRIF-dependent pathways or MyD88 signaling pathways. In this pathway TRIF associates with TRAF family member-associated NF- $\kappa$ B activator (TANK)-binding kinase 1 (TBK1) and IKK-related kinases and IKK which make interferon regulatory factor 3 (IRF3) become active. IRF3 then transfer to the nucleus which leads to interferon (IFN- $\alpha$  and



IFN- $\beta$ ) production. The TRIF-dependent pathway may also lead to the activation of NF- $\kappa$ B (18).

There is evidence of an association between the expression of microRNA (miRNA or miR) and regulation of TLRs-mediated signaling (18). miRNAs are short, non-coding RNA comprised of 19–22 nucleotides which play key roles in post-transcriptionally regulation of many biological activities in the cells such as gene expression in cardiovascular and immune system cells (1). In this regard, Taganov et al. observed that TLR4 ligand could be able to increase miR-132, miR-146a and miR-155. As well, elevation in miR-146a expression also observed by TLR2 and TLR5 ligands (23). They also reported that TRAF6 and IRAK-1 are two targets of miR-146 (23). Similarly, O’Connell et al. showed that TLR3 and TLR4 ligands and IFN, enhance miR-155 expression (24). Tili et al. have suggested that the expression of miR-155 increases the expression of TNF- $\alpha$  (25). They also reported that TLR4 ligand down-regulates miR-125b expression.

### **Role of TLRs signaling in atherosclerosis and angiogenesis**

Although the inflammatory nature of atherosclerosis process is well established, the potential factors driving the pro-inflammatory process are not fully established (Table 1-2) (6). In this regard, infectious factors such as chlamydia pneumonia and cytomegalovirus have previously been suggested as being involved in the development of plaque formation and act as TLRs ligand (26). Several studies have evaluated polymorphisms of the genes encoding TLRs in CVD. The most evaluated polymorphisms are Asp299Gly and Thr399Ile (6). Kiechl et al. in a cohort study evaluated the effect of Asp299Gly and Thr399Ile polymorphisms of the TLR4 gene in

carotid artery atherosclerosis and found that subjects with specific alleles of these polymorphisms were associated with reduced risk of atherosclerosis and reduced intima-media thickness (27). However, later studies performed on larger sample size did not support this association (28-30). The relationship of these two polymorphisms and coronary artery disease (CAD) progression and risk of myocardial infarction (MI) have also been assessed. Several of these studies reported that the TLR4 Asp299Gly and Thr399Ile polymorphisms are associated with a reduced risk of MI (31-33). In contrast, Edfeldt et al. in an investigation of 2774 subjects reported that these polymorphisms were associated with an increased risk of MI in men, but not women (34). However Zee et al. and Koch et al. found no association between MI and these TLR4 polymorphisms (35, 36). Satoh and colleagues in 2005 evaluated serum proinflammatory cytokines and TLR4 expression on monocyte isolated from peripheral blood in patients with MI on admission and 14 days after MI and observed that baseline and after 14 days post MI onset; the expression of TLR4 and pro-inflammatory cytokines was higher in patients than healthy subjects. Also these levels were higher in patients with heart failure (HF) following a MI, than patients without HF (37). Methe et al. found that in patients with unstable angina (UA) and acute MI, levels of circulating TLR4-positive monocyte was significantly higher in UA than healthy subject and patients with stable angina (SA). In this study the increased level of TLR4 was associated with increased levels of IL-12 and B7-1 (38). In addition to atherosclerosis progression, there is evidence that TLRs might also have role in angiogenesis so that, activation of TLRs particularly TLR2, TLR4, TLR7 and TLR9 with activating ligands such as LPS could activate endothelial cell even without presence of further cytokines with the mechanisms of increased adenosine and

adenine nucleotide concentration in involved tissue which makes releasing endothelial growth factors and induct angiogenesis (39, 40). Recently, Carnevale et al. has proposed that TLR4 as well as gut derived LPS can play an important role in atherosclerosis process in human. They have found that *Escherichia coli* LPS can be localized in carotid plaque and facilitate formation of atherosclerotic lesion (41). LPS can amplify platelet responses to common agonists upon binding to its receptor, TLR4. It has been proven that platelet activation and aggregation requires an active TLR4 pathway (42). Carnevale et al. study highlighted the possible role of bacterial LPS in atherosclerotic plaque formation via triggering the inflammation response through TLR4 (41). According to this study, dietary modifications can play an important role in reducing the risk of cardiovascular disease. Although still controversial, drugs and diets which are helpful in lowering the circulating LPS and bacteria can possibly modify the atherosclerosis development in susceptible patients. Buerger's disease is characterized by peripheral arterial occlusive disease in young male smoker and is mostly presented with limb ischemia and pain, intermittent claudication and severe limb ulcers. The molecular pathogenesis of this vascular disease is not fully understood. Recently, the role of TLRs in Buerger's disease is being better understood. A single nucleotide polymorphisms in the MyD88 gene has been reported to be associated with Buerger's disease (43). Both myeloid MyD88-dependent and independent TLR signaling pathways can result in monocyte adhesion and therefore, inflammatory response in vessel walls. TLR activators are newly developed immunomodulators that are proposed as possible therapeutic approaches for Buerger's disease and other vascular disease with similar mechanisms

## **Role of TLRs signaling in myocardial ischemia-reperfusion injury**

Myocardial ischemia-reperfusion injury (IRI) is a condition that occurs after blood flow returns to myocardial tissue in an ischemic area after coronary artery occlusion (44). In this condition, neutrophil accumulation and excessive generation of oxygen radicals by ischemic myocardial and endothelial cells after restoration of blood supply result in further cellular damage and deleterious consequences (44). According to previous studies, TLRs are expressed in the myocardium are TLR2, TLR3, TLR4 and TLR6 and could be able to impact on myocardial disease (20, 45). Animal studies had shown that activation or conversely inhibition of activation of some of TLRs can cause adverse or favorable clinical effects, for example it was seen that stimulation of TLR4 leads to reduction in cardiac myocyte apoptosis (46). The immune system plays an important role in IRI and therefore contributes to cardiac remodeling and incidence of heart failure (HF) but the exact involved mechanisms have not been clarified (47, 48). Recently a numbers of studies have indicated that the TLRs signaling pathway could be a treatment target in patients with MI. in this regard, activation of NF- $\kappa$ B after stimulation of TLRs after myocardial reperfusion has shown that related to increase myocardial damage and inhibition this activation could be beneficial in reducing myocardial injury and improve cardiac function (49-51). In an animal study, Eritoran a TLR4 blocker was reported to reduce infarct size after myocardial infarction (MI) (52). Other animal studies conducted on TLR4-deficient mice represent similar results by reducing inflammatory cell infiltration and cytokine expression (53-55). Consistent with these observations, in another study evaluated transgenic mice results showed that infarct size in MyD88-deficient mice was significantly smaller. Furthermore, there was significantly better

cardiac function and less inflammatory cells infiltration to ischemic area after MI in these mice (56). Similarly, a study in IRAK4-deficient mice showed they were partially cardioprotected effects against IRI (57). Interestingly despite these results, in several animal studies it was reported that activation of TLR4 with administration of low dose of TLR4 agonists such as LPS 8-24 h before induction myocardial ischemia-reperfusion could be protective and reduced infarct size (58). This suggests that due to the existence of crosstalk between TLRs and PI3K/Akt signaling pathways activation of these signaling pathways modulated inflammatory responses and protect myocardial cells against apoptosis (59). It has been shown that PI3K $\gamma$  blockade is advantageous in cancer therapy. The blockade will result in both reduction of tumor growth and may also protect against anthracyclines cardiotoxicity (60). Moreover, TLR2 and TLR4 has been reported as an early markers of other drugs induced cardiomyopathy including doxorubicin. Patients who develop diastolic dysfunction will express higher rate of TLR2 and TLR4 (61).

Ha et al. observed that administration of a PI3K inhibitor eliminated the cardioprotection of pretreating with low dose of LPS (62). Furthermore, TLR2 plays a role in IRI so that although in several studies it was observed that TLR2 deficiency or the administration of anti-TLR2 antibody, before myocardial reperfusion results in decreased infarct size and cytokine secretion and improved cardiac function (63, 64), but in a study of Ha et al. administration of TLR2 agonist before reperfusion resulted in beneficial cardiac effects (18). Overall, cardiac protection induced by both TLR4 and TLR2 appear to be related to PI3K/Akt signaling and studies have revealed that inhibition of this pathway abolished cardiac protection of TLR2 and TLR4 (18, 65).

## **Role of TLRs signaling in post-MI cardiac remodeling and development of heart failure**

Due to negligible endogenous self-regenerative capacity of cardiomyocytes of human heart, elimination of necrotic cardiac tissue after MI and infiltration of immune cells ultimately leads to replacement with scar tissue (66). This is dependent of activation of immune system and production of cytokines which is a hallmark of MI (66). Although activation of TLRs-mediated signaling pathways as one of immune pathways in the early phase after MI, this may be beneficial but continuing this activation can accentuate detrimental remodeling in cardiac tissue injury and occurring heart failure (HF) which is defined as inability of pumping sufficient blood to different organs and tissues (67, 68). The two important factors in patient outcome following an MI are: infarct size and left ventricular (LV) remodeling. Although the exact role of activation of TLRs in cardiac pathology is not fully-understood, but there are evidences that TLR4 is highly expressed in heart and has a major role in response of immune system after incidence of MI (69). Oyama et al. observed smaller infarct size and suppressed inflammation in mice with TLR4 deficiency (70). Shishido et al. found that TLR4 deficiency was a protective condition in mice against adverse remodeling after MI. However, the infarct size was the same in mice with and without TLR2 deficiency, although TLR2-deficient mice had less TGF- $\beta$ 1 expression and type 1 collagen deposition, and also less fibrosis in histological examinations, ventricular remodeling and mortality (71). Furthermore, decreased LV remodeling and preserved systolic function in following MI was observed by Timmers et al. in TLR4-deficient mice. They also reported that although the collagen density was higher in the infarct area,

inflammatory cytokine expression was lower in TLR-4 deficient mice (72). On the other hand, Birks et al. showed that patients requiring left ventricular assist devices had higher levels of TLR4 and IL-1 receptor expression in their myocardium. They also observed significant association between expression of TLR4 and IL-1 receptor (49). Riad et al. reported that in mice with an induced MI, 6 days after MI those with TLR4 deficiency had improved LV function, lower LV remodeling and level of atrial natriuretic peptide and collagen density (73).

### **Role of TLRs signaling in septic cardiomyopathy and myocarditis**

Septic cardiomyopathy is systolic and diastolic dysfunction of both sides of the heart following infection (74). As mentioned above, bacterial components such as LPS are recognized by surface TLRs and bacterial or viral nucleic acids are recognized by intracellular TLRs (19). Consequently, TLRs especially TLR4 may be involved in septic cardiomyopathy and cardiac dysfunction. In animal models it was shown that TLR4 or IRAK1 deficiency are protective against septic cardiomyopathy (6). Results of the study conducted by Tavener et al. showed that administration of LPS to mice with TLR4-deficient in myocardial cells but TLR4-positive in circulating leukocytes leads to impaired myocardial function. In contrast, giving LPS to TLR4-deficient in circulating leukocytes but positive in myocardial cells led to no change in cardiac function (75). However transplantation of TLR4-deficient bone marrow with the aim of cardioprotection did not lead to a positive effect (76). This could be because of roles of other TLRs such as TLR2 in myocardial cells or other tissues. In the studies investigated the role of TLR2 in bacteria-induced cardiomyopathy, results revealed the protective effect of TLR2-deficient condition during sepsis (77). Although the effect of Eritoran administration, a

synthetic TLR4 antagonist, in prevention of cardiomyopathy in severe septic patients are not fully demonstrated, Tidswell et al. in their clinical trial reported no significant reduction in all-causes mortality in patients with severe sepsis by administration of Eritoran (78).

The most common cause of acute myocarditis is viral infection (19). The immune system and its responses are active in the subclinical phase of myocarditis (79). The definite diagnosis of myocarditis is made by endomyocardial biopsy and its histological evaluation (80). Activation of host immune response and TLRs signaling following viral infection and replication produce a variety of inflammatory cytokines that make infiltration of inflammatory cells found to be important factors with protective or deleterious effects on incidence of myocarditis and cardiomyopathy (81, 82). TLRs can be activated by double or single stranded RNA such as TLR3 have more correlation with viral myocarditis (19). Despite many animal studies on the roles of TLRs in pathogenesis of myocarditis, there is little clinical data available. In an animal model study, Negishi et al. evaluated the role of TLR3 signaling pathway in infection with coxsackie virus, TLR3 deficient mice was more susceptible to be infected by some viral infection such as Coxsackie virus, whilst expression of TLR3 makes resistant against viruses (83). It was seen that TLR3 is important in controlling infection caused by Coxsackie virus due to subsequent activation of anti-viral infection (19). In viral myocarditis in mice deficient in TLR3 due to more viral replication in the primary phase, results in greater myocardial injury and increases risk of adverse clinical outcome (84). Also it was observed that in these mice infection with encephalomyocarditis virus resulted in more severe cardiac damage and earlier death (19). Gorbea et al. showed a



polymorphism of TLR3 gene in patients diagnosed with enteroviral myocarditis, reduce the ability to inhibit viral replication because of decrease in type I interferon (85). As mentioned previously, TLR3 transduces its signal through TRIF-dependent pathways. Although the presence of TLR3 inhibits viral replication, activation of TRIF which possesses pro-apoptotic activity, could give viruses, which are the obligatory intracellular pathogens, to replicate in infected cells. Therefore more studies should be undertaken to clarify its role in viral infection. On the other hand, it has been suggested that after viral infection, TLR4 deficient mice showed significantly lower viral replication in myocytes and reduced production of IL-1 $\beta$  and IL-18 and therefore less severe myocarditis (86). In this regard, Satoh et al. investigated 44 patients with myocarditis and found higher levels of TLR4 in these patients compared with control group. In addition, they found a positive association between the level of enteroviral RNA and TLR4 level (87). Pauschinger et al. observed that a greater enteroviral RNA level correlated with left ventricular dysfunction (88). Furthermore, Fuse et al. reported that Absence of MyD88 is associated with higher survival rate and lower viral load and reduction of production of interferon- $\gamma$  (IFN- $\gamma$ ), IL-18, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and cytokines of T-helper 1. However levels of IFN- $\beta$  were significantly increased (89).

### **Role of TLRs signaling in infective endocarditis**

Infective endocarditis (IE) is an important cardiac disease with a high mortality rate and in spite of available treatments its prognosis is poor (90). Several studies have reported the relationship between TLRs and pathogenesis of IE. Bustamante et al. investigated R753Q and R677W polymorphisms of TLR2 and D299G and T399I polymorphisms of TLR4 in 65 patients with IE and 66 healthy participants, and report that the TLR2

R735Q polymorphism was significantly associated with endocarditis and presence of this polymorphism makes individuals more susceptible to development of infective endocarditis (91). Golovkin et al. also investigated several polymorphisms of TLRs: rs5743551 and rs5743611 of TLR1, rs3804099 and rs5743708 of TLR2, rs4986790 and rs4986791 of TLR4 and rs3775073 and rs5743810 of TLR6. One hundred ten patients with infective endocarditis and 300 healthy matched participants were evaluated. They concluded that among mentioned polymorphisms, only TLR6 rs3775073 polymorphism was significantly correlated with decreased risk of IE (92). Single nucleotide variants of TLR4 were investigated by Weinstock et al. using blood that was obtained from 148 patients with IE and 185 controls. No significant difference in genetic variants of TLR4 was found between IE and control groups (93). Tsaganos et al. in their animal study induced bacterial endocarditis by two types of staphylococcus aureus, methicillin-susceptible (MSSA) and methicillin-resistant (MRSA), in rabbits. Results showed that after addition of TLR4 antagonist, infection with MSSA resulted in higher stimulation of TNF- $\alpha$ . This study revealed different pattern of TNF- $\alpha$  stimulation, independent to TLR4 mechanisms in MSSA and MRSA endocarditis (94). Banks et al. investigated Streptococcus sanguis as one of main causes of IE. They observed that the secreted components of this bacteria which was able to inhibit activation of monocytes by binding to a complex of TLR4 and CD14 (Table 1-2)(95).

### **Role of TLRs signaling in atrial fibrillation**

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia (96) and MI is an independent risk factor of new-onset AF (97). It has been reported that TLRs and its associated inflammation may be correlated with AF. Therefore they can be used as

potential predictor of new-onset AF after MI. In this regard, Zhang et al. investigated TLRs in peripheral blood mononuclear cell in 84 patients with MI and 85 patients with new-onset AF after MI. Eighty-two healthy individuals were selected as control group. Results showed significantly higher expression of TLR2 and TLR4 and their signaling proteins in group of patients with AF compared with MI patients without AF and also control group (97). Similarly, eighty six patients with AF and 86 participants with sinus rhythm were enrolled in a study conducted by Gurses and colleagues. The expression of TLR2 and TLR4 in peripheral blood monocyte were evaluated. At the end of the study, they found that AF patients had significantly higher TLR2 and TLR4. As well, after 17 months follow they found that the expression level of monocyte TLR4 was independent predictor of AF recurrence. These results suggest that TLR4 may be a potential therapeutic target in AF patients (96). Consistent with this result, Gurses et al. evaluation platelet TLR2, TLR4 and HMGB-1 in 53 AF patients and 22 controls. Left atrial and peripheral blood were obtained. This study showed significantly higher level of peripheral blood and left atrial TLR2, TLR4 and HMGB-1 in AF patients. Patients with persistent AF had higher serum HMGB-1 and also left atrial platelet TLR2 and TLR4 than patients with paroxysmal AF (98). Wang et al. found that patients with AF had higher level of TLR2 compared to controls and in contrast to a previous study comparison two group of AF patients revealed that TLR2 was significantly higher in paroxysmal AF than persistent AF patients (99). The most important complication of AF is atrial thrombi formation which can be resulted in ischemic accidents such as ischemic stroke (98). Association between TLRs signaling and thrombosis in patients with AF was investigated by Xu et al. with evaluation three groups: 15 AF patients without

thrombus, 15 AF patients with thrombus and 15 participants with sinus rhythm. Ultimately, they observed that in comparison to other groups, MyD88 and HMGB1 were significantly higher in AF patients with thrombus. However expression level of TLR4 was not different between three groups (100).

### **Role of TLRs signaling in cerebrovascular disease**

Cerebrovascular diseases refer to conditions in which the blood vessels supplying the brain are primarily involved (101). The most common manifestation of this group of disease is stroke that can be occurred as an ischemic or hemorrhagic which can cause major disability and mortality (101). Different endogenous ligands such as high-mobility group box 1 (HMGB1) protein, heat shock proteins (HSPs), hyaluronic acid and mRNA are released from damaged cells following to CNS injuries could be able to activate TLRs signaling pathways and result in triggers immune responses and infiltration of inflammatory elements and therefore further neuronal damage (102). Although the exact roles of TLRs in cerebrovascular disease is not fully-understood, two aspect of their effects have been suggested in stroke: on one hand the neuroprotective effects and neurodegenerative effects on the other hand. In this regard one of the important factors in outcomes resulted from activation of TLRs in nervous system depends on type of involved cells express TLR and another one is time course. It has been observed that TLRs activation could lead to the expression of anti-inflammatory cytokines such as IL-10 production that result in barricade neurodegenerative of several cytokines such as IFN- $\gamma$  and TNF- $\alpha$  (103, 104). Samarasinghe et al. have reported that 12-24 h after activation of TLRs, the levels of serum IL-10 was raised (105). MyD88 deficient hematopoietic cells were investigated by Downes et al. who observed that

MyD88 deficient mice had a larger cerebral infarct size (106). Microglial cells have key role in stroke pathogenesis and study conducted by Jung et al. showed that the inhibition of activation of TLR4 leads to the release of IFN- $\beta$  which contributes to induction of apoptosis in these cells (107). Aravalli et al. observed that blocking of TLR2 signaling pathway prevent progression of microglial cells towards apoptosis (108). Stevens et al. found that blockage of TLR9 also leads to neuroprotection (109). Tang et al. found that within 1 h after incidence of ischemic stroke the expression of TLR2 and TLR4 by neurons was elevated (110) and within 24 h microglial cells in ischemic area express high TLR2. This means activation of neuronal response prior to activation of microglial cells. Cao et al. showed that a reduced infarct size and lower levels of proinflammatory cytokines and overall better outcome in TLR2 and also TLR4 deficient mice with brain ischemia (111). Hyakkoku et al. confirmed these findings (112). Tang et al. concluded that activation of TLR 2 and TLR4 expressed by neurons with activation of caspase 3 can result in apoptosis (110). However, no significant difference was reported by Brea et al. in expression of TLR3 and TLR9 between good outcome and poor outcome in evaluation of 110 patients with ischemic stroke (113). Yang et al. found a correlation between higher TLR4 level in monocytes in peripheral blood and adverse neurological outcome (114). A positive association was also found by Ferronato et al. between TLR4 expression and expression of cyclooxygenase-2 (COX-2), an enzyme important in inflammation, after ischemic stroke which can cause more CNS damage (115). miRNAs act as key regulators of the expression of different genes post-transcriptionally (1). Xu et al. found high levels of miR-1906 in the ischemic zone and also in a peri-ischemic zone. They also found that after administration of exogenous

miR-1906, the expression of TLR4 and infarct zone was reduced and neurological functional outcomes were improved. However this protective effect was not seen in TLR4 knockout mice (116). Lin et al. investigated TLR4 gene polymorphisms among ethnic Chinese people and observed that Asp119Cys polymorphism significantly correlated with increased risk of ischemic stroke (117).

### **Role of TLRs in therapeutic effect of cardiac stem cell therapy and bacteria based approaches**

Rejection of cardiac stem cells is a major limitation of stem cell therapy that is mostly due to low tolerance of transplanted cells against hypoxia and inflammation which leads to apoptosis (Table 1-2). Disrupting the immune response is considered as a possible solution for improving the survival of transplanted stem cells. Modulation of toll like receptors is an interesting issue which under active research. TLR1-TLR6 and TLR9 which are expressed on outer surface or within intracellular organelles of mesenchymal stem cells (10, 118). TLRs signaling pathways can be activated by endogenous molecule secreted from damaged tissue. TLR4 is a well-known example of this issue. LPS which is a ligand for TLR4 will promote releasing of proinflammatory factors and prevent stem cell apoptosis (119, 120). Preconditioning of mesenchymal stem cells with LPS will results in better engraftment and greater survival rate of stem cells. Also it has been reported that angiogenesis will be enhanced by releasing of vascular endothelial growth factor (VEGF) (121). As well, activation of TLR3 will increase immunosuppressive capacity of transplanted stem cell by releasing immunosuppressive factors (120).

As mentioned earlier, TLRs play an important role in inflammation and inflammatory pathways. According to the complexity of inflammatory pathways, there is still controversy about the exact therapeutic effect of TLR targeting. Activation of TLR will result in inflammatory response and this phenomenon has been reported with *Escherichia coli* and chlamydia infection (41, 122). Despite the established effect of gut microbiota on various metabolic and cardiovascular conditions, the role of antibiotic therapy on gut microbiota is not widely studied. Antibiotics can alter microbiota and induce or prevent some disease (123). By selective disruption of gut microbiota a certain bacteria can be targeted. Using highly specific antibiotics will eradicate a specific bacteria and therefore may interfere with specific pathways such as inflammation or atherogenesis. Another emerging issue about the therapeutic approaches for cardiovascular disease are probiotics. It has been suggested that use of different strains may interact with TLR and suppress inflammatory cytokine production (124). However, despite of recent trends toward new therapeutic approaches for cardiovascular disease, use of probiotics and selective antibiotics is not widely studied and the available data is controversial.

### **Conclusion:**

Cardiovascular disease is among the leading cause death. Toll-like receptors and their down-stream signaling pathways play a crucial role in the immune system and its potential link with myocardial infarction, and heart failure, which is activated via endogenous molecule secreted from damaged tissue, indicating its value as a therapeutic target. Atherosclerosis is associated with inflammation and involvement of immune responses. TLR play an important role in macrophage activation within atherosclerotic lesions. There is growing body of data on TLR inhibitors and agonists in

clinical trials for inflammatory conditions such as asthma, cancer, and autoimmune diseases, although studies in the context of CVD is in its infancy and many questions are still remained unanswer, therefore a deeper understanding of the role of TLRs in cardiovascular diseases via further experimental studies including randomized controlled trials, are warranted.



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**Table1.** Summary of the most relevant studies investigating TLRs signaling pathways in animal model of CVD

Author (Reference)	Population (number)	Investigated TLR or other component	Chief findings
Shimamoto et al. (52)	Mouse	TLR4	Decrease infarct size after MI with blocking of TLR4
Ao et al. (53)	Mouse	TLR4	Decreased neutrophil infiltration and chemokines expression in TLR4-deficient hearts
Chong et al. (54)	Mouse	TLR4	Decreased inflammatory cytokines expression in myocardial tissue in TLR4-deficient mice
Fallach et al. (55)	Mouse	TLR4	Cardioprotective effect of myocardial TLR4 deficiency during MI
Feng et al. (56)	Mouse	MyD88	Significantly smaller infarct size and better cardiac function after MI in MyD88-deficient
Maekawa et al. (57)	Mouse	IRAK4	Cardioprotective effect of deficiency of IRAK4 against IRI
Zacharowski et al. (58)	Rat	TLR4	Decrease infarct size with administration of TLR4 agonist before myocardial reperfusion
Ha et al. (62)	Mouse	PI3K	Cardioprotective effect with administration of PI3K inhibitor
Ha et al. (18)	Mouse	TLR2	Cardioprotective effect with administration of TLR2 agonist
Favre et al. (64)	Mouse	TLR2	Smaller infarct size after cardiac ischemia in deficiency of TLR2
Arslan et al. (63)	Mouse	TLR2	Significantly improved cardiac function and smaller infarct size with administration of anti-TLR2 antibody
Oyama et al. (70)	Mouse	TLR4	Decrease infarct size in deficiency of TLR4
Shishido et al. (71)	Mouse	TLR4, TLR2	Decreased ventricular remodeling after MI in deficiency of TLR4 and TLR2 No association between infarct size and deficiency of TLR2
Timmers et al. (72)	Mouse	TLR4	Decrease ventricular remodeling and better systolic function after MI and higher collagen density in infarct area in TLR4-deficient
Riad et al. (73)	Mouse	TLR4	Decrease ventricular remodeling and better cardiac function after MI in TLR4 deficient
Tavener et al. (75)	Mouse	TLR4	Administration of LPS to TLR4-deficient in myocardium but TLR4-positive in circulating leukocytes led to myocardial dysfunction
Binck et al. (76)	Mouse	TLR4	No cardioprotective effect was observed with transplantation of TLR4-deficient bone marrow
Zou et al. (77)	Mouse	TLR2	Cardioprotective effect in deficiency of TLR2 during sepsis
Negishi et al. (83)	Mouse	TLR3	More susceptibility to viral infection in TLR3-deficient
Richer et al. (84)	Mouse	TLR3	Important role of TLR3 in controlling coxsackie virus infection Increase myocardial injury and risk of adverse clinical outcome in TLR3-deficient mice
Gorbea et al. (85)	Mouse	TLR3	Poor controlling of viral replication in TLR3-deficient mice with enteroviral myocarditis

Fairweather et al. (86)	Mouse	TLR3	Significantly lower viral replication and severity of myocarditis in TLR4 deficiency in myocytes
Fuse et al. (89)	Mouse	MyD88	Association between absence of MyD88 and higher survival in viral myocarditis
Downes et al. (106)	Mouse	MyD88	Larger infarct size after ischemic stroke in MyD88 deficient mice
Jung et al. (107)	Mouse	TLR4	Blocking of TLR4 led to releasing IFN- $\beta$ and cells apoptosis
Aravalli et al. (108)	Mouse cell line	TLR2	Blocking of TLR2 prevented progression of microglial cells toward apoptosis
Stevens et al. (109)	Mouse	TLR9	Blocking of TLR9 made neuroprotective
Tang et al. (110)	Mouse	TLR2, TLR4	Elevation in expression of TLR2 and TLR4 within 1 h after ischemic stroke
Cao et al. (111)	Mouse	TLR2, TLR4	Decrease in infarct size after stroke in TLR2 and TLR4-deficient mice
Hyakkoku et al. (112)	Mouse	TLR3, TLR4, TLR9	Neuroprotective effect of TLR4 deficiency in brain ischemia but not with TLR3 and TLR9
Xu et al. (116)	Mouse	TLR4	High level of miR-1906 in ischemic and peri-ischemic area Association between miR-1906 administration and decrease TLR4 level and size of infarct area and improvement in neurological function
Tsaganos et al. (94)	Rabbit	TLR4	Different pattern of TNF- $\alpha$ stimulation, independent to TLR4 mechanisms in methicillin-susceptible and methicillin-resistant staphylococcus aureus endocarditis
Banks et al. (95)	Streptococcus sanguis	TLR4	Inhibition activation of monocytes by binding to a complex of TLR4 and CD14 by secreted components of Streptococcus sanguis

**Table2.** Summary of the most relevant studies investigating TLRs signaling pathways in CVD patients

Author (Reference)	Population (number)	Investigated TLR or other component	Chief findings
Kiechl et al. (27)	Human (n=810)	TLR4	Decreased risk of atherosclerosis in existence of Asp299Gly and Thr399Ile polymorphisms
Labrum et al. (28)	Human (n=3000)	TLR4	No association between Asp299Gly and Thr399Ile polymorphisms and intima-media thickness
Netea et al. (29)	Human (n=493)	TLR4	No association between Asp299Gly polymorphism and atherosclerosis
Norata et al. (30)	Human (n=1256)	TLR4	No association between Asp299Gly and Thr399Ile polymorphisms and intima-media thickness
Ameziane et al. (31)	Human (n=399)	TLR4	Decreased risk of MI in Asp299Gly polymorphism
Boekholdt et al. (32)	Human (n=885)	TLR4	Association between Asp299Gly polymorphism and decreased risk of cardiovascular event in patients receiving statin treatment
Holloway et al. (33)	Human (n=166)	TLR4	Association between Asp299Gly polymorphism and decreased risk of MI in patients receiving statin treatment
Edfeldt et al. (34)	Human (n=2774)	TLR4	Increased risk of MI in men in existence of Asp299Gly and Thr399Ile polymorphisms
Zee et al. (36)	Human (n=1390)	TLR4	No association between risk of MI and Asp299Gly polymorphism
Koch et al. (35)	Human (n=5264)	TLR4	No association between Asp299Gly and Thr399Ile polymorphisms and risk of MI
Satoh et al. (37)	Human (n=85)	TLR4	Increase level of TLR4 after MI and also higher TLR4 level in MI patients with HF compared with MI patients without HF
Methe et al. (38)	Human (n=118)	TLR4	Higher TLR4 level in patients with UA
Birks et al. (49)	Human (n=36)	TLR4	Higher TLR4 in patients with requiring ventricular assist devices
Tidswell et al. (78)	Human (n=300)	TLR4	No significant reduction in all-cause mortality in severe septic patients received Eritoran
Satoh et al. (87)	Human (n=49)	TLR4	Higher TLR4 level in patients with myocarditis Positive association between level of TLR4 and enteroviral RNA
Brea et al. (113)	Human (n=110)	TLR3, TLR9	No difference in expression of TLR3 and TLR9 between patients with good and poor outcome after ischemic stroke
Yang et al. (114)	Human (n=65)	TLR4	Association between higher level of TLR4 and adverse neurological outcome
Ferronato et al. (115)	Human (n=60)	TLR4	Positive association between TLR4 and expression of COX-2 enzyme which can cause more CNS damage after ischemic stroke
Lin et al. (117)	Human (n=457)	TLR4	Increased risk of ischemic stroke in existence of Asp119Cys polymorphism
Bustamante et al. (91)	Human (n=131)	TLR2, TLR4	Increased risk of infective endocarditis with presence of TLR2 R735Q polymorphism
Golovkin et al.	Human (n=410)	TLR2, TLR4,	Decreased risk of infective endocarditis with

(92)		TLR6	presence of TLR6 rs3775073 polymorphism
Weinstock et al. (93)	Human (n=333)	TLR4	No significant difference in genetic variants of TLR4 between patients with infective endocarditis and controls
Zhang et al. (97)	Human (n=251)	TLR2, TLR4	Significantly higher expression of TLR2 and TLR4 and their signaling proteins in patients with AF compared with MI patients without AF
Gurses et al. (96)	Human (n=172)	TLR2, TLR4	Significantly higher TLR2 and TLR4 in AF patients and expression level of monocyte TLR4 was associated with AF recurrence
Gurses et al. (98)	Human (n=75)	TLR2, TLR4	Significantly higher level of peripheral blood and left atrial TLR2 and TLR4 in AF patients and also higher left atrial platelet TLR2 and TLR4 level in persistent AF than patients with paroxysmal AF
Wang et al. (99)	Human (n=48)	TLR2	Higher level of TLR2 in AF patients and significantly higher of TLR2 level in paroxysmal AF compared to persistent AF patients
Xu et al. (100)	Human (n=45)	TLR4, MyD88	Significantly higher MyD88 and HMGB1 in AF patients with thrombus and no difference in level of TLR4 between AF patients with and without thrombus
Xu et al. (125)	Human (n=163)	TLR2	Significantly higher TLR2 level in patients with persistent AF compared to paroxysmal AF

**Figure1.** Schematic view of TLRs signaling pathways. Downstream TLRs signaling is divided into two different pathways: MyD88-dependent and -independent signaling pathways, which after several steps ultimately resulted in proinflammatory gene expression and production of various cytokines and chemokines