

Targeting the Toll-System in Cardiovascular Sciences

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Abstract: Toll-like receptors (TLRs) are a family of pattern recognition receptors that serve as a key part of the innate immune system. TLRs play a role in coordinating the organism's first line of defence against invading microbes or tissue injury. TLR-mediated inflammation is an important pathogenic link between innate immunity and a diverse panel of clinical disorders. Among these processes are cardiovascular disorders such as atherosclerosis, heart failure, viral myocarditis or diabetic angiopathies. In the new area of TLRs, this has generated a lot of interest from pharmaceutical companies as well as the investment communities. The improved understanding of TLRs, their key ligands and signaling cascades brought a number of diagnostic methods and compounds into clinical development. The first potential applications for TLR compounds include therapies for cardiovascular disease. The idea of this article is to describe the molecular basis of TLR signaling and review corresponding new inventions relating to TLR system and drug targets in cardiovascular disease.

Keywords: Toll-like receptors, cardiovascular disease, atherosclerosis, innate immune system, lipopolysaccharides, developmental drugs, patent.

INTRODUCTION

Toll-like receptors (TLRs) form a family of pattern recognition receptors that have emerged as important mediators of innate immunity. The members of the TLR family are believed to have an ancient evolutionary origin, and may be the oldest components of the immune system [1]. Eleven TLRs (named TLR1 to TLR11) have been identified in humans so far, and it has been estimated that most mammalian species express between ten and fifteen types of TLRs [2-4]. Additionally, there are proteins that share similarities with certain regions of TLRs, such as RP105 [5,6]. The discovery of TLRs has been a milestone in the understanding of the molecular link between innate immunity, inflammation, and a wide variety of diseases and provided a foundation for the development of innovative therapeutic targets. There has been a major effort in recent years, with significant success, to discover new drug compounds that act by stimulating or suppressing certain key aspects of the TLR system. The first potential applications for TLR compounds include therapies for cardiovascular disease. This paper reviews the molecular basis of TLR signaling and corresponding recent drug investigational findings.

LIGAND RECOGNITION OF TLRs

Recognition of pathogen-associated molecular signatures is critically important in proper activation of the immune system. The expression of individual TLRs is triggered by a

distinct repertoire of conserved microbial particles as well as host-derived factors, thereby sensing both the presence of invading pathogens and tissue damage [3,7,8]. Whilst most TLRs are expressed on the cell surface, TLR3, TLR7, TLR8, and TLR9 are expressed intracellularly. In fact, the last three are part of the endosomal compartment [9,10]. TLR forms either a homodimer or heterodimer, each dimer having different ligand specificity, in the recognition of specific molecular determinants present on the microorganisms (Fig. 1). Activating ligands can be divided into several groups: naturally occurring molecules that are derived from a variety of microorganisms, synthetic structures based on microbial products, fully synthetic small molecules and endogenous ligands. TLRs 1, 2, 4, 5 and 6 specialize mainly in recognizing bacterial products that are unique to bacteria and not made by the host. These include bacterial outer cell membrane lipopolysaccharides (LPS) (detected by TLR4), peptidoglycan, lipoproteins and lipoteichoic acid (by TLR2) and proteins such as flagellin from bacterial flagella (by TLR5) [11-13]. TLRs 3, 7, 8 and 9, in contrast, specialize in viral detection and recognize nucleic acids, which are not unique to the microbial world. These ligands comprise unmethylated cytosine/guanine (CpG) dinucleotides (by TLR9) [14,15], double-stranded RNAs such as polyI.polyC (by TLR3) [15], analogues of adenosine and guanosine, and single-stranded viral RNAs (by TLR7) [10]; and certain other RNA and DNA. TLR8 binds imidazole quinolines while the natural ligands are still unknown [16]. TLRs - particularly TLR4 - are known to recognize endogenous molecules, including cellular fibronectin [17], fibrinogen [18], oxidized lipids [16], surfactant protein-A [17] and heat shock proteins [19]. More recently, it has emerged that a class of ligands derived from endogenous molecules is capable of initiating innate immunity by stimulating TLR

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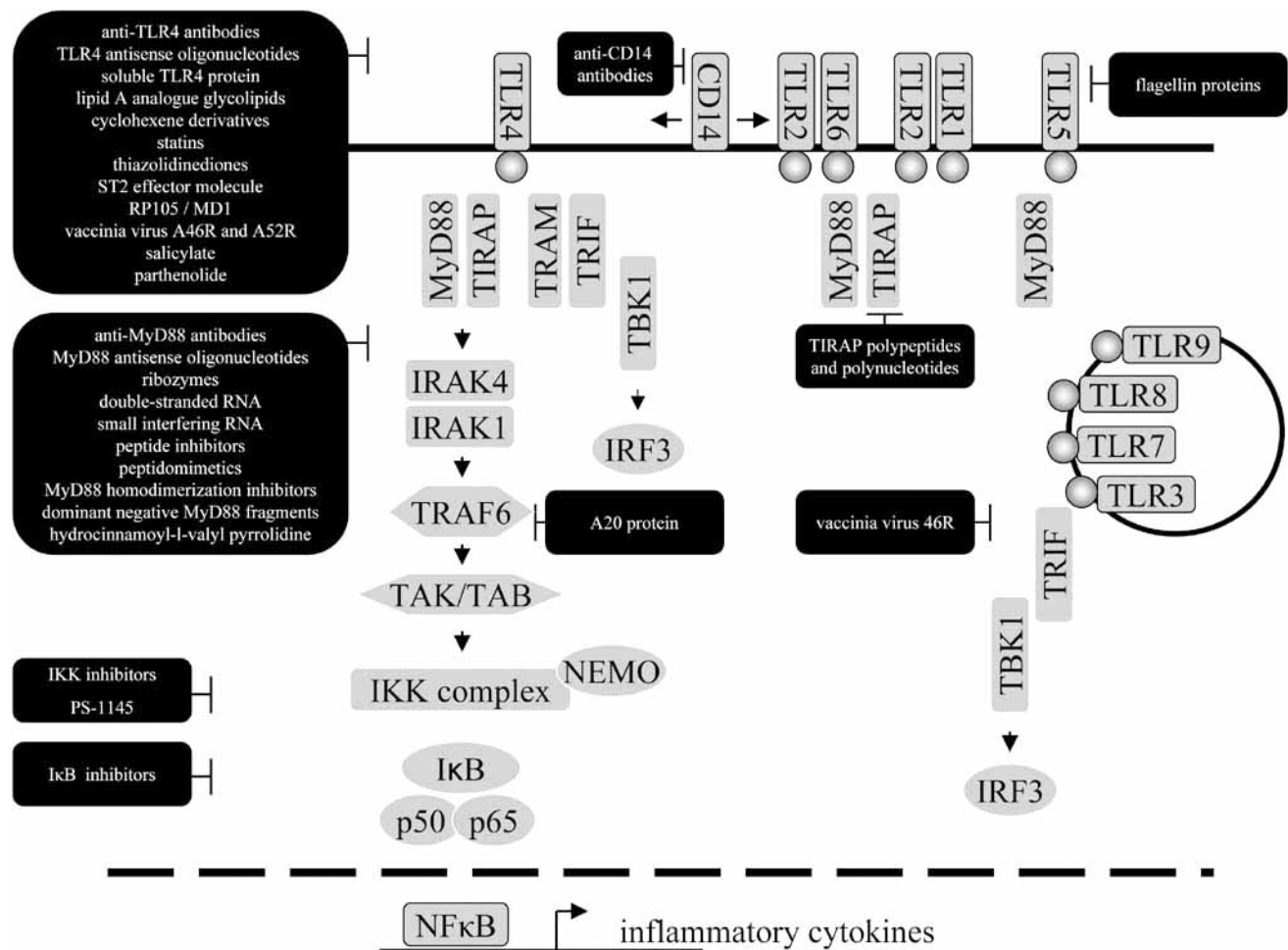


Fig (1). Potential inhibition of TLR-mediated signaling in cardiovascular system.

The TIR (Toll/IL-1 receptor homologous region) adaptor proteins (MyD88, TIRAP, TRAM, TRIF and TRAF) associate with one another and are drawn as dimers. Recruitment of adaptor molecules into receptor complexes leads to the initiation of kinase cascades and the induction of gene expression of inflammatory cytokines after the activation and nuclear translocation of NF- κ B. TLR-based cardiovascular drug development efforts are focused primarily on inhibitory compounds targeting cell surface receptors TLR4, TLR5 and CD14 and intracellular (endosomal) TLR7, TLR8, TLR9 and TLR3. Potential inhibitors of downstream signaling molecules are also available. MyD88, myeloid differentiation factor 88; TIRAP, TIR-related adaptor protein; TRAM, Toll-associated molecule; TRIF, Toll-associated activator of interferon; TRAF, IL-1 receptor associated factor, NEMO, transcription factor NF- κ B essential modulator; TAK, transforming growth factor- β -activated kinase; TBK, TAK1-binding proteins; IRF, interferon regulatory factor; IKK, I- κ B kinase kinase.

signaling [20]. These ligands are generally generated via the degradation of macromolecules arising as a result of inflammation, cellular rupture, and activation of proteolytic cascades during times of tissue injury, stress or remodeling or oxidative stress [21] and may act as auto-antigens early in the disease (Fig. 2) [22,23]. For CD14, signaling co-receptor for TLR4 and TLR2, LPS is the major ligand. Recent data indicate that CD14 can interact with other molecules such as lipoteichoic acid, soluble peptidoglycan, muramyl dipeptide, and lipoarabinomannan. Endogenous molecules such as heat shock proteins and ceramide can also interact with CD14 [24].

TLR SIGNALING

TLRs do more than merely recognize pathogens via their molecular patterns. Upon binding, TLRs tend to cluster,

recruit other extracellular and intracellular accessory proteins to the complex, and trigger signaling cascades that ultimately impact transcription of proinflammatory genes. To avoid pathological immune reactions, TLRs are coupled to signal transduction pathways, and the signaling network is regulated at multiple levels [7,25,26]. The understanding of the extensive crosstalk between the main bow-tie network and collateral subsystems as well as feedback and feed-forward controls may facilitate the design of novel approaches based on TLR signaling to treat or prevent human disease (Fig. 1). With the exception of TLR3, all TLRs are coupled to the cytoplasmic adaptor protein MyD88 (myeloid differentiation factor 88); TLR3 and TLR4 are also coupled to TRIF (Toll-associated activator of interferon) [27]. Other adaptor proteins such as TIRAP (Toll/interleukin (IL)-1 receptor-associated protein), and TRAM (Toll-associated

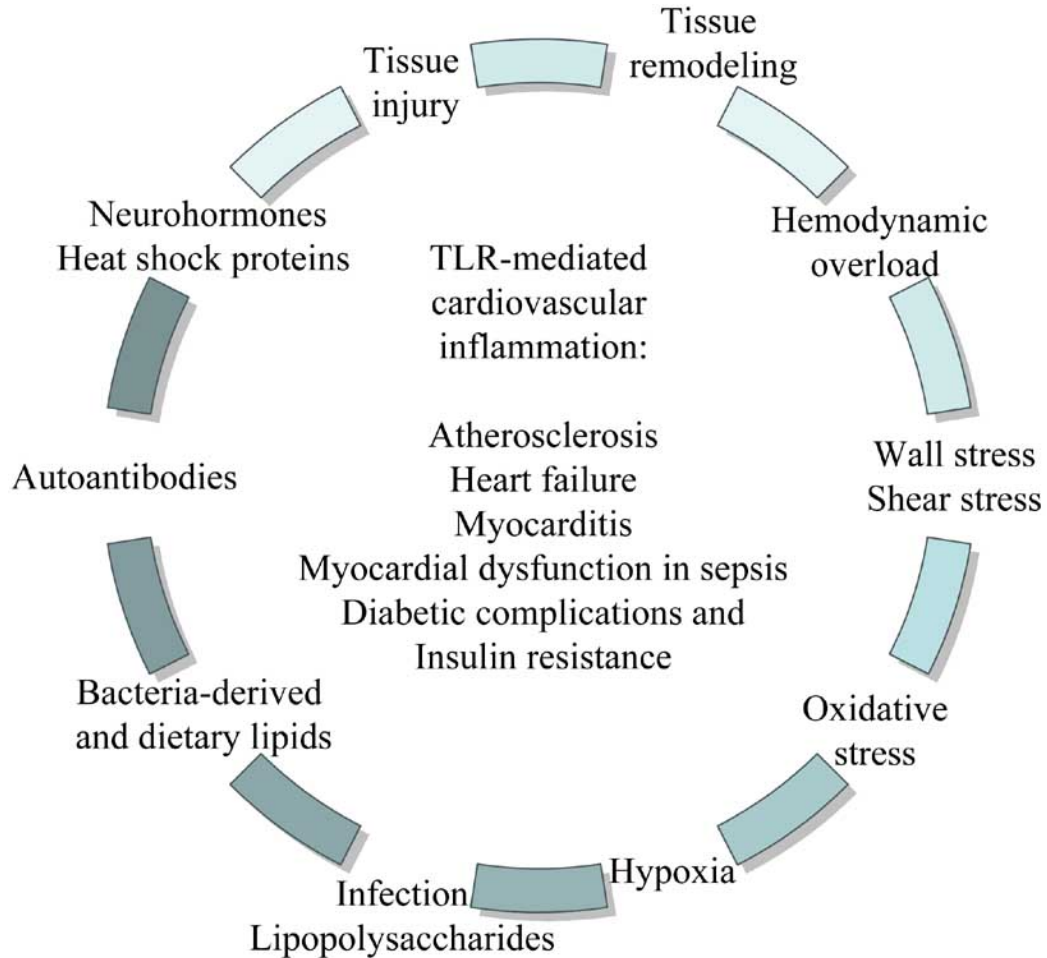


Fig. (2). Induction of inflammation in cardiovascular disease. Toll-like receptors are important for mediating innate immune response to various stimuli in the cardiovascular system. TLRs trigger inflammatory processes upon exposure to tissue damage, remodeling, stress, and certain external factors. On activation of TLRs, cells in the cardiovascular or immune systems are stimulated to produce pro-inflammatory cytokines, which, together with components of the activated complement and coagulation systems, may promote the development of myocardial dysfunction.

molecule) are differentially recruited by TLR2, TLR3 and TLR4, but their functions are less clear [28,29]. After MyD88 recruited, the receptor complex is joined by IRAK1 (IL-1 receptor-associated kinase 1), IRAK4 and TRAF6 (tumor necrosis factor receptor-associated factor 6). IRAK1 and TRAF6 then dissociate from the complex and associate with an other one composed of TAK1 (transforming growth factor-beta-activated kinase) and TAB 1 and 2 (TAK1-binding proteins). Active TAK1 triggers the activation of IKK (I B kinase kinase) complex. Activity of this complex is modulated by NEMO (transcription factor NF B essential modulator). IKK-mediated phosphorylation of I B leads to the translocation of NF B into the nucleus [7,30]. Transcription is followed by the production of proinflammatory cytokines such as IL-1, IL-6, and IL-8, chemokines, polyreactive antibodies and essential costimulatory molecules that permit an adaptive immune response, which, in turn, counteracts early spreading of infection, and promotes and guides specific adaptive immune responses [13,31]. TLRs are involved in activation of complement, coagulation, phagocytosis, and apoptosis functions in response to pattern detection [1,2,32].

ROLE OF TLR SIGNALING IN CARDIOVASCULAR DISEASES

Significant progress in understanding the etiology of cardiovascular disease has come from recent recognition that chronic inflammation plays a key role in its development. The underlying mechanisms of the inflammatory response, however, are not fully understood. The heart possesses a functionally intact intrinsic immune system and this can be activated non-specifically in response to various forms of tissue injury (Fig. 2) [33]. There is increasing evidence that short-term, self-limited expression of proinflammatory cytokines provides the heart with a rapid adaptive response to cardiac injury as part of an early warning system [34]. A sustained or excessive activation of the inflammatory signaling, as seen, for example in chronic heart failure [35], however may be deleterious and contravene these beneficial effects [36]. In addition to specialized tissues, such as spleen, lung, kidney and leukocytes, TLRs are highly expressed in endothelium and heart, suggesting a functional importance of TLRs in the cardiovascular system [37]. Two major approaches are available to understand the role of TLRs in

the progress of human cardiovascular disease. First, many details of TLR signaling have emerged from the studies of TLR2 and TLR4 gene-targeted mice. Studies indicate that the TLR4 gene maps to the critical region in LPS hyporesponsive mice [18]. Mutations in the TLR4 gene are found in mouse strains (C3H/HeJ and C57BL10/ScCr) that are defective in their response to LPS, and disruption of the TLR4 gene results in a LPS hyporesponsive phenotype [38,39]. Indeed, TLR4-deficient mice show attenuated myocardial dysfunction in response to LPS during inflammatory shock [33,40,41]. Similarly, downstream factor TRAM [42] and MyD88 knockout mice [43] are also shown to be non-responsive to LPS. These studies suggest that an impaired innate immunity mediated by TLRs is involved in cardiovascular disease in humans. TLR2-knockout mice demonstrate higher survival rate and less ventricular remodeling, more preserved cardiac function, associated with fewer pathological changes after myocardial infarction and in doxorubicin-induced oxidative stress [44,45]. Second, studies of specific polymorphisms in genes encoding TLRs and their downstream signaling molecules revealed links between TLR signaling and cardiac disease. The human TLR4 gene has been shown to be polymorphic [46]; so far, its best-studied functional polymorphism of the receptor is an amino acid substitution, from aspartic acid to glycine at position 299 (Asp299Gly). This variant was initially identified because of its association with hyporesponsive LPS phenotype in humans that interrupts LPS signaling [47,48]. Subsequently, other polymorphisms in TLR4, as well as in TLR2 and in genes such as those encoding IRAK4, NEMO, I B and caspase-12 have demonstrated that TLR signaling affects the development of several human diseases [30,49]. Based on papers published so far, it is believed that a number of diseases deriving from dysregulation of the signaling of the TLR system comprise to cardiac disorders such as atherosclerosis, heart failure, myocarditis, septic myocardial dysfunction, and diabetic angiopathies (Table 1). Furthermore, TLR polymorphisms play a role in asthma, air-way infections, autoimmune diseases and tumors [50]. Of note, new data demonstrate that TLR4 polymorphisms in humans are more frequent than previously estimated, and present in approximately 10 % of Caucasian individuals [51].

ATHEROSCLEROSIS AND RELATED DISEASES

Complications from atherosclerosis represent the leading cause of morbidity and mortality in developed countries [52]. The inflammatory nature of this disease process is widely accepted; however, the precise components of the atherogenic proinflammatory cascade remain controversial [53]. Beside hypercholesterolemia, experimental and epidemiologic studies suggest that microbial antigens are implicated in the pathogenesis of this disease [54-56]. The discovery of the TLRs has provided a molecular link between low-level chronic infection, inflammation, and atherosclerosis [51] and provided a foundation for the development of innovative diagnostic and therapeutic targets [57]. The association between TLR4 function and atherosclerosis is consistent with the data showing that TLR4 exhibits preferential expression in lipid-rich and macrophage-infiltrated coronary

Table 1. TLRs as Mediators of Cardiovascular Disease

Cardiovascular disease	Receptor	References
Atherosclerosis	TLR1	[67,68]
	TLR2	[66-68]
	TLR4	[48,51,62,63]
	TLR9	[69]
	MyD88	[54,57]
Coronary artery disease	TLR4	[64,70,71]
Myocardial infarction	TLR4	[65]
Ischemic stroke	TLR4	[150]
Cardiovascular complications of diabetes and hyperlipidemia	TLR4	[92,93]
	TLR3	[95]
	MyD88	[94]
Heart failure	TLR4	[65,134,136]
	TLR2	[21,44]
Ventricular remodeling	TLR2	[21,44]
Autoimmune myocarditis	TLR7/8	[139]
	MyD88	[141]
Viral myocarditis	TLR7/8	[140]
	TLR4	[139]
Sepsis-associated myocardial dysfunction	CD14	[113,130]
	TLR4	[33,40,41]
Doxorubicin-induced oxidative stress	TLR4	[45]

atherosclerotic plaques [58]. Adventitial TLR4 activation augments neointima formation in a mouse model, suggesting a link between TLR4 and intimal lesion formation [59,60]. Atherosclerosis prone hypercholesterolemic mice ApoE^{-/-} that also harbor a null mutation in either TLR4 or adaptor molecule MyD88 exhibit reduced aortic atherosclerosis, plaque lipid content, and plaque macrophage infiltration without altering circulating cholesterol levels or lipoprotein profiles [54,61]. TLR4 is involved not only in the initiation but also in expansive remodeling of atherothrombosis [62,63]. More recently, human studies have established that unstable angina and acute myocardial infarction are associated with enhanced expression and signaling events downstream of human TLR4 in circulating monocytes [64]. Monocyte TLR4 has been shown to be activated in acute heart failure after myocardial infarction [65]. TLR2 and TLR1 expressions are elevated in human atherosclerotic lesions [66] and injected exogenous TLR2/1 agonist exacerbated atherosclerosis [67,68]. Moreover, immunohistochemical staining revealed TLR9 expression in atheroma

tissue. Plaque plasmacytoid dendritic cells respond to TLR9 ligand CpG dinucleotides, with enhanced interferon-transcription and secretion [69].

Functional Asp299Gly polymorphism in TLR4 is associated with a reduced risk for carotid artery atherosclerosis [48,51], and acute coronary events [47,70,71]. The cumulative burden of cardiovascular disease is reduced by more than half in patients with both Thr399Ile and Asp299Gly TLR4 polymorphisms, as compared with the background population. People with the Asp299Gly variant have lower levels of circulating C reactive protein (CRP), adhesion molecules, and other acute phase molecules [51]. These molecules have well described functions in inflammation, which in turn is associated with atherosclerotic progression, plaque rupture and consequent vessel occlusion. Not surprisingly, patients with the Asp299Gly polymorphism are more susceptible to infections [48,72]. Based on these data, several inventions are provided that relate to TLR4 mutations and susceptibility to atherosclerosis as well as methods to treat an individual identified as being at increased risk [73].

In mice, pretreatment with TLR4 antagonist eritoran (E-5564) attenuates the inflammatory response to myocardial ischemia-reperfusion injury, as evidenced by a reduced infarct size, decreased nuclear NF B translocation and lower cytokine expressions [74]. Compounds that are known to inhibit TLR4 or MyD88 can also be used in the treatment of human disease and injury (Fig. 1). The therapeutic use of TLR4 inhibition is suggested in thrombosis, transplant atherosclerosis, restenosis after angioplasty and stenting, and vein-graft disease after bypass surgery [75]. Such patients may include those suffering from angina pectoris, stroke, myocardial infarct, congestive heart failure, and peripheral arterial disease. Inventions provide several means with which to inhibit TLR4 or MyD88 signaling pathways in treating atherosclerosis-based pathologies [61]. The vascular delivery of TLR4 and MyD88 inhibitors can be accomplished by a wide range of devices and methods. Among others, an intravascular device is developed which is coated with a compound that inhibits TLR4 [75] or MyD88 [76], thereby imparting an improved efficacy to the device. The coatings may eliminate or substantially reduce restenosis following stent placement, as well as late in-stent thrombosis following intracoronary brachytherapy [75]. TLR4 can also be inhibited by administering recombinant viral vectors that deliver genes expressing antisense TLR4 RNA [11,77], by high affinity soluble TLR4 protein that competes for TLR4 ligands, or by small-molecule antagonists [78,79]. Antisense therapy using oligodeoxynucleotides may inhibit TLR4 gene expression by specific base pairing of single stranded regions of the TLR4 mRNA [79]. Peptide mimetics refers to any structural analog of TLR4 or MyD88. As shown in Fig. 1, examples of suitable peptide mimetics include RP105 [6], ST2 effector molecule [80], vaccinia virus open reading frame A46R and A52R [81], and hydrocinnamoyl-L-valyl pyrrolidine [61,78]. Other signaling inhibitors include anti-apoptotic protein A20 [82], agonistic lipid A and its analogues [83,84], and inhibitors of I B2 [85], salicylate and parthenolide [86], IKK inhibitor PS-1145 [87], and inhibitors of p38 and JNK [88]. Anti-TLR4 and anti-MyD88 antibodies target the TIR domain of the TLR4 or the portion of MyD88

that binds to the TIR domain of the TLR4 [76]. MyD88 homodimerization inhibitors are peptidic substances that mimic a particular portion of MyD88, preventing its homodimerization and interfering with its interaction with the TIR domain. Inhibition of MyD88 homodimerization may result in the inhibition of numerous proinflammatory signals [89]. Additionally, a recent invention claims the use of TIRAP polypeptides and polynucleotides for antagonizing MyD88-independent signaling in response to TLR4 ligation [90].

CARDIOVASCULAR COMPLICATIONS ASSOCIATED WITH TYPE 2 DIABETES AND HYPERLIPIDEMIA

Accelerated atherosclerosis is a major complication of long-term diabetes mellitus, and this is partly due to associated abnormalities of lipoprotein metabolism. It has been recognized that vascular damages in diabetic patients could be initiated through ligation of TLRs, and this initiates a self-perpetuating inflammatory process that is maintained even upon return to normal glucose homeostasis [91]. Based on this recognition, the development of complications could be affected through regulating one or more TLR signaling cascades. Among others, the use of TLR4, TLR2, TLR3, and MyD88 inhibitors as well as statins and thiazolidines have been suggested in diabetes and obesity-associated cardiovascular disorders. By interfering with TLR signaling, these agents may turn off inflammatory process that sustains diabetic complications and reduces the extent to which complications occur [91]. As shown in TLR-deficient mice, TLR4 is a main molecular link among nutrition, lipids, and inflammation [92,93]. TLR4 participates in the regulation of energy balance and insulin resistance in response to changes in the nutritional environment. Certain dietary lipids activate TLR4 and thereby promote insulin resistance. Development of insulin resistance in response to lipid infusions did not seem to occur in TLR4-deficient mice [92,93]. Similarly, MyD88 is thought to play a role in the overproduction of cytokines during obesity and thereby influences the development of type 2 diabetes. To study the effects of genetic MyD88 deficiency on obesity induced inflammation, attempts were made to make MyD88-deficient animals obese using a high fat diet and results showed that MyD88 deficient mice are resistant to developing diet-induced obesity [94]. Furthermore, TLR3 knockout mice fed a high fat diet are also protected from developing impaired glucose tolerance as a feature of insulin resistance, demonstrating that the absence of TLR3 signaling protects mice against type 2 diabetes [95]. TLR3 knockout mice on a high fat diet have lower levels of total cholesterol, LDH and HDL cholesterol as well as HDL-c/LDL-c ratio compared to wild type mice on a high fat diet [95]. Inhibition of TLR3 signaling increase circulating levels of insulin either via direct protective effects on pancreatic islet cells or by affecting the lipid profile. In type 2 diabetics, TLR3 inhibition by monoclonal TLR3 antagonist antibodies is suggested to postpone the introduction of insulin treatment and avoid side effects associated with insulin treatment [95].

Plasma protein and lipoprotein distribution and activity of TLR4 antagonist eritoran were examined during an i.v. infusion in healthy volunteers [96]. Eritoran binds principally to lipoproteins in the blood. Within 24 hours, eritoran

partitions predominantly into HDL (50 to 70%), LDL (15 to 20%), VLDL (2 to 10%), with 6 to 12% partitioning into lipoprotein-deficient plasma [96,97]. Eritoran is inactivated following binding to HDL, thus plasma lipoprotein levels may affect the distribution and pharmacological activity of the compound.

Other examples of TLR-based therapeutic compounds are the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or statins. Statins form a class of hypolipidemic agents, used as pharmaceuticals to lower cholesterol levels in people at risk for cardiovascular disease because of hypercholesterolemia. Their actions, however, go far beyond mere cholesterol reduction. Statins appear to confer broad anti-inflammatory and antiatherogenic effects, and they seem to improve blood flow in the peripheral circulation via a number of pathways [98,99]. In normocholesterolemic patients with nonischemic dilated cardiomyopathy, short-term statin therapy improved ventricular function [100]. Given the potential of genomics to identify people in a target population who are most likely to benefit from specific drugs or who are most at risk of side effects, it is notable that the efficacy of pravastatin treatment in preventing cardiovascular events was higher in carriers of TLR4 Asp299Gly polymorphism, compared with noncarriers [71]. These findings could allow tailoring of drug dosages for people based on their genotype, as well as contribute to overall knowledge of disease mechanisms [30]. Statins now seems to be molecularly configured to interact with TLR4 or MyD88 signaling pathways [101]. Fluvastatin directly inhibits TLR4 and TLR2 on circulating CD14⁺ monocytes in patients with heart failure, possibly through mechanisms unrelated to HMG-CoA reductase inhibition and cholesterol-lowering effects [102]. Simvastatin and atorvastatin influence TLR4 expression and signaling in CD14⁺ monocytes via inhibition of protein prenylation [101]. High-dose simvastatin pretreatment blunts monocyte TLR4 and TLR2 expression in a human endotoxemia model in vivo [103]. The inhibition of monocyte TLR4 expression attenuates LPS responsiveness, and provides new insight into the favourable pleiotropic actions of statins.

Similar to statins, peroxisome proliferators activated receptor (PPAR)- agonists, like thiazolidinediones, have also demonstrated broad anti-inflammatory properties in addition to their other beneficial effects on metabolism. Recent data shows that activation of PPAR- by thiazolidinediones results in a reduced stimulation of dendritic cells via TLR2, 3, 4, and 7 ligands. This effect is characterized by reduced secretion of cytokines and downregulation of adhesion and co-stimulatory molecules [104].

SEPSIS-ASSOCIATED MYOCARDIAL DYSFUNCTION

Systemic bacterial infection may culminate in an often fatal septic shock syndrome. Despite improved treatment regimens and the advent of antibiotics, its prognosis remains poor and the mortality ranges between 30 and 50% [105]. One particularly severe complication of sepsis is the corresponding evolution of myocardial dysfunction [106,107]. Microorganisms such as *Chlamydia* may play a role in the pathogenesis of some forms of heart disease [108]. This together with data from studies of LPS-induced myocardial

inflammation could lead to novel therapeutic strategies for cardiac diseases [109]. LPS causes myocardial dysfunction with direct activation and depression of myocytes, immune cells including heart tissue macrophages, mast cells, and infiltrating blood leukocytes. All of these respond to LPS and depress myocyte function [110,111]. Human genetic studies showed that mutations in human TLR genes increase progression to end-organ dysfunction during sepsis. Constitutively active human TLR4 interacts with the lipid A portion of endotoxin from Gram-negative bacteria, which is responsible for LPS-mediated syndromes [109,112]. TLR2 Arg753Gln polymorphism correlates with the incidence of sepsis in a Caucasian population. Of note, the 9.4% of the study population is found to be heterozygous for this polymorphism [113].

An increased understanding of the mechanism underlying the septic response led to test various TLR signal inhibitors that were aimed at interrupting specific steps in the septic cascade and may be useful targets for adjunctive therapies in septic shock [114]. Administration of non-peptide TLR4 antagonists are suggested to patients who have pulmonary bacterial infection or symptomatic pulmonary exposure to LPS [115]. Examples of such disorders include immunocompromise due to anti-rejection therapy after heart transplant, congestive heart failure with pulmonary edema and medical treatments that result in LPS release. In particular, TLR4 inhibitors may be useful in the therapy of symptoms due to increased inflammatory cytokine such as TNF- levels [116]. Novel synthetic antagonists of TLR4 were developed based on the structure of the lipid A portion of LPS. An injectable TLR4 antagonist eritoran is under development for the potential treatment of sepsis [96,117]. In healthy volunteers, eritoran completely inhibited stimulation of TNF-release by LPS (1 ng/ml) *ex vivo* during infusion and more than 72 h after ending infusion [117]. Apart from these safety studies, in a phase II trial for endotoxic complications following a coronary artery bypass graft surgery, a total of 1018 patients received a 4 hours intravenous (iv) infusion of eritoran (2, 12 or 28 mg). Results showed that although significant efficacy was not achieved between those receiving therapy and placebo in new organ dysfunction, a lower incidence of new organ dysfunction was seen in the 28-mg arm compared to placebo, as well as a lower 28-day all-cause mortality rate [118]. Eritoran at high doses (100 and 200 µg) prevents the drop in systolic and diastolic blood pressure following the administration of LPS. Close examination of the data shows that with 250 µg of eritoran, both the systolic and diastolic pressures were increased 15 hours after administration of the drug by 5 to 10 mmHg, an increase that persisted to the end of the 24 hours period. The drug ameliorated the fall in blood pressure induced by LPS [119]. TAK-242 is an other TLR4 signal inhibitor for the potential treatment of severe sepsis.

When tested in a mouse model of Gram-negative sepsis in vivo, TAK-242 administered at 0.1 to 3 mg/kg iv 1 hour before LPS administration (7 mg/kg ip) in mice improved cardiovascular performance, reduced serum cytokine TNF- and IL-6 and NO levels. Similar to eritoran, TAK-242 produces a 100% survival rate 7 days after LPS administration at a dose of 3 mg/kg [120]. Phase I clinical studies of TAK-242 are underway.

TLRs depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires CD14 and LPS binding protein [38,121,122]. The CD14 pathway inhibitors are antibodies that bind to and inhibit LPS, LPS binding protein, CD14, TLR4, and MD2 for Gram-negative sepsis and CD14, TLR2 [122,123], and TLR6 for Gram-positive sepsis [124,125]. The recombinant anti-CD14 neutralizing monoclonal antibodies include 4C1 [126] and IC14 [24]. IC14 was shown to decrease LPS-induced responses in endotoxaemia [127] and *ex vivo* [128, 129]. Intravenous infusion of IC14 is well-tolerated in both healthy subjects and severe sepsis patients and does not increase the incidence of secondary infections [24]. Administration of IC14 in healthy volunteers led to a reduction in the release of TNF, IL6, and IL10. Recent phase I study in patients with severe sepsis had disappointing results, as plasma levels of various proinflammatory cytokines stayed unchanged in response to intravenous IC14 compared with baseline or placebo [130]. A soluble CD14 protein antigen is found to be useful as a marker for diagnosing sepsis. It may serve as an effective marker in diagnosing sepsis, as this soluble CD14 protein exists at a higher content in the blood of a sepsis patient compared to the blood of a normal donor [131].

HEART FAILURE

Chronic heart failure (CHF) continues to represent an enormous clinical challenge. Despite the addition of angiotensin-converting enzyme inhibitors and β -blockers to current heart failure treatment regimens, the quality of life and survival of affected individuals remains unacceptably poor [132]. Therefore, novel therapeutic approaches that may confer incremental prognostic benefits in CHF are urgently needed. Considerable data now exist linking innate immunity effector proteins to the pathophysiology of myocardial dysfunction in heart failure [133]. Studies have suggested that in certain scenarios, TLR4 on cardiac myocytes is functional in failing heart and can play a role in the pathology of disease [134]. LPS, ligand of both TLR4 and TLR2, is recognized as an important factor in the development of immune activation in CHF [135]. Myocardial TLR4 mRNA levels are upregulated in patients with end-stage CHF requiring left ventricular assist device [136]. In hearts of patients with CHF, the accumulation of TLR4-regulated cytokines and TLR4 have been reported to be increased [134,136]. In acute heart failure after myocardial infarction and in CHF, monocyte TLR4 levels are upregulated [65]. Rapid activation of the TLR-regulated cytokines such as TNF- α in the myocardium and peripheral tissues have been shown to be an important marker of the progression of myocardial dysfunction [137]. Other member of TLRs, myocardial TLR2 was shown to contribute to cardiac dysfunction in murine models [21,44]. Novel inventions suggest methods of treating or ameliorating heart failure by administering LPS binding protein or antibody, CD14 binding antibody, soluble CD14, or TLR inhibiting compounds [138].

MYOCARDITIS

Studies have shown that myocyte TLR4 is functional in myocarditis, where TLR4 correlates to enteroviral replication

and cardiac dysfunction [139]. MyD88 may become another target for prevention of heart-specific autoimmunity and cardiomyopathy. Indeed, Coxsackie B virus-induced chronic inflammatory response has been shown to be mediated through MyD88-dependent TLR8 and to a lesser extent through TLR7 in viral myocarditis [140]. Autoimmune myocarditis induction depends on MyD88 signaling in self-antigen presenting cells in the peripheral compartments [141]. It has been shown that dendritic cells loaded with a myocardium-specific self peptide induce autoimmune myocarditis. Of note, dendritic cell mediated autoimmunity and heart disease only occurs when dendritic cells are activated through TLRs [142,143]. These results provide an experimental concept as to how tissue damage and multiple infectious triggers can induce autoimmune heart disease and dilated cardiomyopathy. The presentation of self-antigen together with stimulation of TLRs on dendritic cells might explain cardiac dysfunction in sepsis or the link between a worse clinical prognosis after myocardial infarction and the magnitude of the systemic inflammatory response. Exacerbations and relapses in autoimmune cardiac diseases may occur in genetically susceptible humans that experience unspecific stimulation of TLRs *in vivo* [144].

TLR-BASED CARDIOPROTECTIVE AND ANTI-APOPTOTIC DRUG THERAPY

Novel substances with cardioprotective effects have been developed. Cardioprotective effect of a random heterocopolymer of L-glutamic acid and L-tyrosine (poly-Glu, Tyr) has been proven in animal models of myocardial infarction. It has been shown that poly-Glu, Tyr causes down-regulation of T regulatory cells via the TLR5 and TLR9. Poly-Glu, Tyr triggers proliferation of T regulatory cells and increases expression of TLR5 and TLR9 after 24h or 48h of activation [145]. These results might point to a role of TLRs in recognition of poly-Glu, Tyr by the T regulatory cells and a subsequent decrease in their inhibitory activity. Protectans compounds CBLB-501 and CBLB-502, recombinant flagellin proteins isolated from *Salmonella typhimurium* flagella, activate NF κ B via TLR5 for the protection against tissue injury in conditions involving high levels of apoptosis, such as radiotherapy, other radiation exposure and hypoxia [146]. With support from National Aeronautics and Space Administration (NASA), CBLB-501 is under development as an anti-radiation antidote in space missions. Their potential role in cardiovascular therapy is suggested by the findings that this new class of inhibitors of cell death may be used for the treatment or prevention of other life-threatening injuries that involve massive cell death, including stroke, myocardial infarction and acute inflammation, as well as cancer treatment (even cardiac) side effects caused by chemo- and radiotherapy [147].

CURRENT & FUTURE DEVELOPMENTS

There is currently a strong belief that selectively targeting TLRs might be a fruitful approach to combat a variety of diseases. The results underscore the potential for interfering with TLR signaling in the context of drug development for conditions such as inflammatory or autoimmune cardiovascular diseases. The complex nature of the inflammatory response however poses significant challenges to the

development of efficient treatments. To date, development efforts are focused primarily on compounds targeting specific TLRs such TLR4, TLR2, TLR3, TLR5, TLR7 and TLR9. Because of multiple molecular links between chronic infections, inflammation, cardiovascular disease and TLRs, modulating just one receptor might not produce a complete immune response. Effective treatment and prevention of cardiovascular disease will therefore require an integrated approach that utilizes a combination of strategies to target the underlying inflammatory processes. Based on this idea, there are first attempts to combine therapies targeting two or multiple TLRs [148]. As TLR-based therapeutic strategies to treat cardiovascular disease may entail treatment for an extended period of time ranging from months to years, long-term modulation of TLR can cause debilitating side effects. Indeed, recent work indicates that a chronic TLR modulation is not bereft of toxicity [149]. Future studies need to answer these uncertainties remain regarding long-term interventional immune-based therapies. In summary, progress towards therapeutics designed to target TLR signaling is already well underway. Despite improvements in survival with previous therapies, it is now clear that major cardiovascular event rates for patients with cardiovascular diseases remain high. Thus, these novel approaches may confer incremental prognostic benefits and substantially decrease the problem of this significant unmet medical need.

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CONFLICT OF INTEREST

No financial contribution to the work has been declared. No patents are reported in any stage of legal litigations.

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