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Toll-Like Receptor 4 Stimulation Initiates an Inflammatory Response That Decreases Cardiomyocyte Contractility

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Abstract

Toll-like receptors (TLRs) have been identified as primary innate immune receptors for the recognition of pathogen-associated molecular patterns by immune cells, initiating a primary response toward invading pathogens and recruitment of the adaptive immune response. TLRs, especially Toll-like receptor 4 (TLR4), can also be stimulated by host-derived molecules and are expressed in the cardiovascular system, thus acting as a possible key link between cardiovascular diseases and the immune system. TLR4 is involved in the acute myocardial dysfunction caused by septic shock and myocardial ischemia. We used wild-type (WT) mice, TLR4-deficient (TLR4-knockout [ko]) mice, and chimeras that underwent myeloablative bone marrow transplantation to dissociate between TLR4 expression in the heart (TLR4-ko/WT) and the immunohematopoietic system (WT/TLR4-ko). Following lipopolysaccharide (LPS) challenge (septic shock model) or coronary artery ligation, myocardial ischemia (MI) model, we found WT/TLR4-ko mice challenged with LPS or MI displayed reduced cardiac function, increased myocardial levels of interleukin-1β and tumor necrosis factor-α, and upregulation of mRNA encoding *TLR4* prior to myocardial leukocyte infiltration. The cardiac function of TLR4-ko or WT/TLR4-ko mice was less affected by LPS and demonstrated reduced suppression by MI compared with WT. These results suggest that TLR4 expressed in the cardiomyocytes plays a key role in this acute phenomenon. *Antioxid. Redox Signal.* 15, 1895–1909.

Introduction

Impaired myocardial contractile function is a well-documented feature in myocardial ischemia (MI) and septic shock (13, 17, 31, 37). In response to MI and systemic inflammatory stimuli such as sepsis, cardiomyocytes express proinflammatory cytokines, which are able to initiate a local inflammatory response (10, 18). Cardiomyocytes have properties analogous in some respects to innate immune dendritic cells, so that cardiomyocytes can respond to danger signals with a complex inflammatory and functional response. Cardiomyocytes express chemotactic cytokines (chemokines) such as keratinocyte-derived chemokine, monocyte chemoattractant proteins, and macrophage inflammatory protein-2, which stimulate polymorphonuclear leukocytes and monocytes (88, 89). These cytokines play an important role in the propagation of the postoperative myocardial inflammatory response and the development of the systemic inflammatory response (8). The anti-inflammatory cytokines recruit and activate appropriate inflammatory cell subsets necessary for response and repair (27, 49, 104, 111, 118, 119, 131). Cardiomyocyte inflammatory response involving cytokines (111), chemokines, and the subsequently recruited leukocytes (45, 131) and cell surface adhesion molecules (27, 131) lead to decreased cardiomyocyte contractility, which may impact repair processes. Analogous to that of dendritic cells, cardiomyocytes also have an initial response directed by Toll-like receptors (TLRs) against the danger signals (17).

TLRs are a class of molecules that were first discovered to play a role in body development (70) and later found to play a role in body maintenance (11, 12, 35, 42, 58, 75). The TLR family has been shown to be of importance in the innate immune system for the recognition of pathogen-associated molecular patterns (PAMPs) by immune cells, initiating a primary response toward invading pathogens and recruitment of the adaptive immune response (28, 52, 57, 58, 60, 75, 78, 79, 85, 86, 95, 107, 134, 143). Functional TLRs are also expressed in adult mesenchymal stem cells (MSCs) and their activation by specific ligands regulates MSC functions (112). Thus, TLRs are factors controlling MSC renewal and

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differentiation (112). TLRs can be activated not only by pathogen components but also by mammalian endogenous molecules such as heat-shock proteins (HSPs) and extracellular matrix breakdown products (25, 105, 106).

Cardiomyocytes express a wide array of TLRs including TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9. By signaling *via* nuclear factor kappa B (NF-κB), these TLRs initiate the expression of proinflammatory cytokines (*e.g.*, IL-6), chemokines (*e.g.*, KCMIP-2), and cell surface adhesion molecules (*e.g.*, ICAM-1). Thus, a pleiotropic immunologic response to danger signals, either endogenous molecules such as HSPs or exogenous molecules such as PAMPs, is initiated. This is associated with an NF-κB-dependent rapid downregulation of cardiomyocyte contractility (17). We and others have recently found that the immediate inflammatory responses following myocardial ischemia, as well as sepsis, are modulated *via* TLR4 (10).

This article reviews the experimental evidence of (i) TLR signaling pathways and (ii) the role of TLRs, mainly TLR4 signaling, in modulating the cardiac response to danger signals such as ischemia and sepsis.

Innate and Adaptive Immunity

The mammalian immune system consists of two types of immunity: innate and adaptive. Innate immunity constitutes the first-line host defense system, the components of which are encoded by DNA rather than expressed by clonal cells after antigenic exposure, as in the case of adaptive immunity. The innate immune system destroys many pathogens, determines the localization and extent of the challenge, and facilitates the adaptive immune response. Innate immunity is mediated by genes that remain in the germ line and encode for proteins that recognize conserved structural patterns on microorganisms. Specific components of microbial cell walls are strong activators of innate immune responses. These PAMP molecules are all essential, conserved microbial components that are recognized as foreign by specific pattern recognition receptors, which are preferentially expressed in monocytes and macrophages and also in other cell types. The receptors can be structurally divided into those containing a leucine-rich repeat domain, a calcium-dependent lectin domain (Ca^{+2}), or a scavenger receptor protein domain. Functionally, they are divided into secreted or endocytotic proteins or signaling molecules (92). Secreted receptors usually activate the complement cascade, whereas endocytotic receptors move the pathogen from the surface of the phagocyte into its intracellular lysosomes for destruction. In mammals, the PAMPs activate the production of, for example, bioactive lipids (*e.g.*, platelet-activating factor), reduced oxygen species, and proteins, such as the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α), which all are important in the response to infection (93, 154).

The adaptive immunity is clonal expansion of lymphocytes in response to a particular antigen and the ability to evoke an immunologic memory. The response to a specific antigen rises through specific B- and T-cell receptors for each clone of cells. These receptors are structurally unique, not predestined to recognize any particular antigen and not encoded by the germ line. Therefore, they must be established by every generation (47, 64).

The TLRs

The Toll protein was first characterized in the fruit fly *Drosophila* while studying the genetic mechanisms that control early embryonic development (6, 63, 94). In 1995, it was found that the TLR mammalian protein is an 80-kDa cell membrane receptor that binds lipopolysaccharide (LPS) in the presence of certain serum proteins (5, 63). However, the first-characterized mammalian TLR (first named hToll and later renamed TLR4) was found in 1997 (93) and a minimum of 10 TLRs have been identified (23, 126).

Mammalian TLRs are pattern-recognition receptors (PPR) that function as a cluster of differentiation (CD)-14–associated signal transducers, help cells to recognize and distinguish between pathogens, and initiate appropriate signaling cascades. They also help to bridge innate and adaptive immunity by inducing various costimulatory and effector molecules (154). TLR1, -2, -4, -5, and -6 are expressed on the cell surface. TLR3, -7, and -9 are found in intracellular endosomal compartments. TLR8 appears to be localized primarily intracellularly with a small portion expressed on the cell surface. This allows recognition of plasmatic as well as intracellular ligands. TLRs share the same structure: a large (550–980 amino acids) extracellular domain consisting of leucine-rich repeats, a transmembrane domain, and a Toll/IL-1 receptor (TIR)-like cytoplasmic domain of $\sim$200 amino acids long. The extracellular domain has ligand-binding capacity, and the TIR domain mediates the signal. TIR regions have also been found in vaccinia virus, and they are used by the virus to suppress host IL-1 and TLR signaling (24). Despite these similarities, TLRs are differentially expressed and regulated in many tissues and cell types (15, 16, 101, 152). Different TLRs are capable of activating distinct cellular responses despite their shared capacities to signal through the activation of NF-κB, activator protein-1 (AP-1), and mitogen activated protein (MAP) kinases (15). This may be explained by differential use of adapter proteins (66). Ligands for many TLRs have been characterized: TLR4 mediates the innate immune response to LPS. TLR2 has been shown to mediate the response to yeast and Gram-positive bacteria (40, 83, 108). TLR1 functions as an accessory protein (135), TLR3 recognizes viral double-stranded RNA (4, 148), TLR5 is activated by bacterial flagellin (29, 51), TLR6 functions to assist TLR2 (133, 134), and TLR9 responds to unmethylated CpG dinucleotide motifs (19). Similar to TLR1 and TLR6, TLR10 is highly homologous to TLR2 and is probably another TLR2-associated receptor, but its function is still unknown (53). TLR3, TLR7, TLR8, and TLR9 are intracellular receptors for nucleic acids (48). There is some lack of distinction between the ligands of TLR4 and TLR2, and it thus seems quite possible that some of the TLRs can replace each other under certain circumstances (Table 1). TLR4 is also activated by HSP60 and 70, heparan sulfate, hyaluronan, fibronectin extra domain A, fibrinogen, and surfactant protein (21).

TLRs have the capacity to oligomerize in their cytoplasmic domains (110). *TLR4* acts as a homodimer, and a recent study has also implicated the formation of a *TLR5/TLR4* heterodimer in the signaling of bacterial flagellin (71). *TLR2* can form functional pairs with *TLR6* and *TLR1*, but also functions alone (110).

TLR Signaling

Each TLR activates similar, general signaling pathways, but also triggers its specific pathways. This differential

Table 1. Toll-Like Receptor 4 and Its Ligands

Receptor	*Ligand*	*Origin of ligands*
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Fusion protein respiratory	Virus protein F
	Taxol	Plant taxol
	HSP60/70	Fibroblasts
	Fibronectin EDA domain	Host
	Hyaluronan	Dendritic cells
	Heparan sulfate	Dendritic cells
	Fibrinogen	Macrophages

Modified from the work by Chao (21).
TLR, Toll-like receptor; HSP, heat-shock protein.

induction pattern mainly depends on cytoplasmic adaptor molecules that can associate with the intracytoplasmic portion of TLRs (99). There are different TLR adaptor molecules: MyD88 was first found to be critical for TLR signaling (MyD88-dependent pathways). MyD88 can associate with all TLRs except TLR3. TLRs are type-1 transmembrane receptors involved in microbial recognition. TLR4 has been shown to function as the LPS signaling receptor, whereas TLR2 recognizes peptidoglycans from Gram-positive bacteria and lipoproteins. Although various microbial cell wall components are recognized by different receptors, all of these responses are abrogated in MyD88-deficient cells. These results show that different TLRs recognize different microbial cell wall components and that MyD88 is an essential signaling molecule shared among IL-1 receptor/Toll family members. However, in LPS signaling, the MyD88-independent pathway is present in addition to the MyD88-dependent pathway (1). After association of the TLRs with the adapter protein MyD88, IL receptor-associated kinase (IRAK-1 and -4), and tumor necrosis factor receptor-activated factor-6 (TRAF6) are recruited. The IRAK-4/IRAK-1/TRAF6 complex then interacts with another membrane complex involving transforming growth factor β-activating kinase (TAK1), TAK1-binding protein (TAB1), and TAB2/3. This induces the phosphorylation of TAB2 and TAK1, and their translocation to the cytosol, together with TRAF6 and TAB1. TAK1 subsequently phosphorylates IκB kinase-β and MAP kinase kinase 6, which results in the activation of MAP kinases and phosphorylation of IκB, thereby promoting NF-κB translocation to the nucleus and gene transcription (69, 99). Subsequently, MyD88-independent pathways were discovered. TLR4 and -3 can also activate TIR domain-containing adaptor protein (TRIF)-inducing interferon-β (IFN-β)-dependent pathways without MyD88 association. TRIF then recruits TBK1 and mediates phosphorylation of the transcription factor IFN-regulating factor 3, which leads to the production of IFN and costimulatory molecules (150). The TLR class-specific signaling cascades allow different TLRs to trigger distinct signaling pathways. TLR4 responses include secretion of IL-10, IFN β, and IL-12, whereas TLR2 responses involve IL-8, IL-12, and IL-23 (3, 94). Taking into account tissue-specific expression of TLRs as well as the possibility of heterodimer or homodimer formation for TLR activation, TLR-dependent innate immunity signaling pathways do trigger responses with some degree of specificity, allowing distinct immune responses for different ligands.

TLR and Sepsis

Sepsis is the leading cause of mortality in critically ill people (7). The development of sepsis occurs as a result of a systemic inflammatory response to a severe bacterial infection (113). Under normal conditions, a controlled cellular response to bacterial products protects the host from infection. In sepsis, hyperactivation of the immune response leads to the excessive production of various proinflammatory cytokines and cellular injury (113). In mammals, the innate immune system is the first line of host defense involved in detecting the wide variety of invading microbial pathogens (91). Receptors of the innate immune system are activated by microbial components such as LPS, which is a key molecule involved in the initiation of the sepsis syndrome (91).

The signaling pathways for TLR4, the receptor for LPS, are similar to the signaling of IL-1 (Fig. 1) (2). LPS has been shown to bind LPS-binding protein, MD-2, and CD14, thus enabling association with TLR4 (142). This interaction can result in both MyD88-dependent and MyD88-independent signaling events. MyD88-dependent signaling includes the activation of TLR4 TIR domain, which results in the recruitment of an adapter protein MyD88 *via* its own TIR domain (94). This association will then result in autophosphorylation of IRAK *via* death domain–death domain homophilic interactions. Subsequent acquisition of TNF receptor-associated factor 6 will signal to activate the protein kinase NF-κB–inducing kinase, which then activates IkB kinase to phosphorylate I-kB. This phosphorylation in turn promotes the translocation of the transcription factor, NF-κB, to the nucleus (147). Signaling of the MyD88-independent pathway involves activation of translocation-associated membrane protein 1 and TIR domain-containing adaptor-inducing IFN-h (TIR-containing adapter molecules), resulting in a variety of responses, including the production of type 1 IFN and similar but delayed NF-κB responses (36).

Activation of TLR4 induces the NF-κB–dependent expression of proinflammatory cytokines, such as TNF-α and IL-1β (39). LPS lowers the contractile function of the heart (140), and as TLR4 is the only LPS receptor, it could be concluded that TLR4 plays a role in the heart function. TLR4 levels in the heart are detectable by polymerase chain reaction and flow cytometry and the myocytes have similar levels of TLR4 as endothelium, a cell that responds to LPS (137, 138). Functional TLR4 has been reported in failing myocardium (41), myocarditis (125), and cytokine production, including TNF-α within the heart of endotoxemic mice (10). LPS-induced cardiac depression was largely prevented in C3H/HeJ mice with nonfunctional TLR4 (102). Langendorff perfused isolated heart preparations demonstrated that LPS-treated TLR4-deficient hearts were resistant to LPS, exhibiting contractile responses similar to saline-treated wild-type controls (13). There is no doubt that LPS does reduce the contractile function of the heart, leading to the reduction of left ventricular pressure, rates of pressure generation, and rates of relaxation (139, 140).

Controversy surrounds the role of myocardial TLR4 in the depression of cardiac function following LPS challenge. On the one hand, it has been demonstrated that the suppression of heart function by LPS is mediated directly by TLR4 expressed on the cardiomyocytes (13, 115). On the other hand, TLR4-mediated suppression of cardiac function has been attributed to leukocytes (76, 138). We have investigated the impact of TLR4 activation following LPS on cardiac

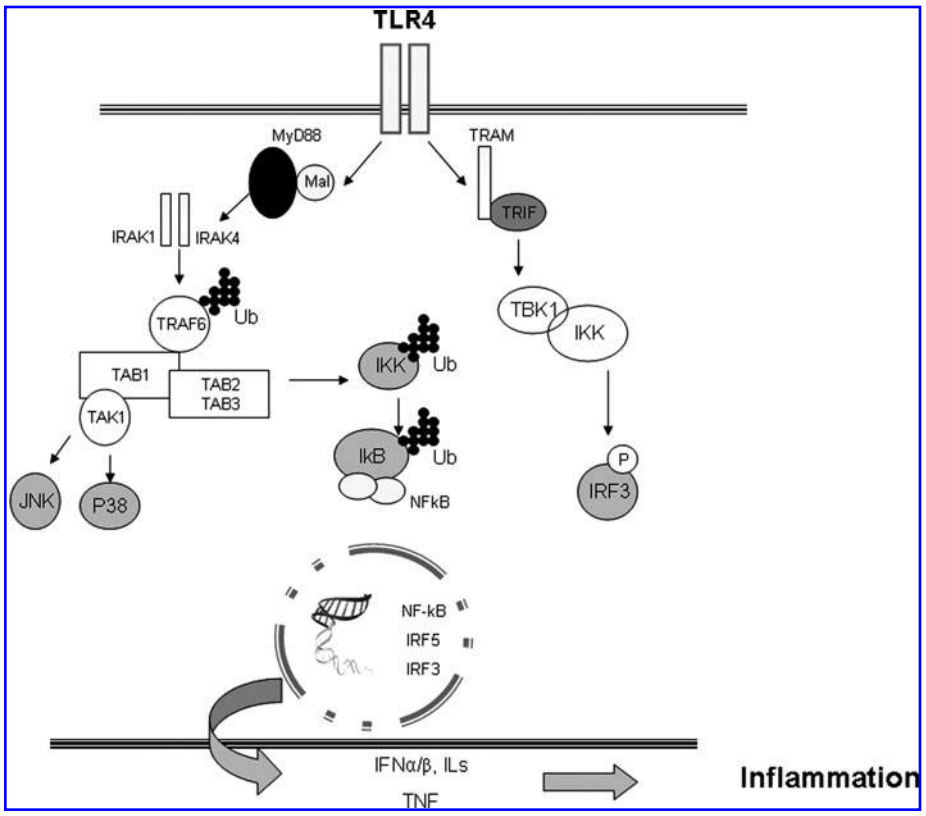

FIG. 1. Toll-like receptor (TLR) 4 signaling. All TLRs are transmembrane proteins with a large extracellular domain containing leucine-rich repeats and a unique cytoplasmic Toll/IL-1 receptor (TIR) domain. TLRs exist in dimmers, and all TLR family members, except TLR3, signal through the key adaptor myeloid differentiation primary-response gene 88 (MyD88) to recruit downstream interleukin (IL)-1 receptor-associated kinases (IRAKs) and the stress-activated protein kinases p38 and JNK1/2. In TLR2 and TLR4, MyD88 adaptor-like protein (Mal) is required for recruiting MyD88 to their receptors. In addition to Mal/MyD88-dependent pathway, TLR4 can also signal through an MyD88-independent pathway that activates TBK1 *via* Trif-related adaptor molecule (TRAM)-Trif-dependent mechanism. These kinases ultimately activate transcription factors such as nuclear factor kappa B (NF-κB) and IFN regulatory factor (IRFs), which result in the production of various proinflammatory cytokines such as tumor necrosis factor (TNF), ILs, and IFNs. IFN, interferon.

performance (32). Wild-type (WT) mice developed diarrhea, eye exudates, and lethargy within 4 h post-LPS treatment, with 25% mortality at 24 h, whereas TLR4-knockout (ko) mice displayed no signs of LPS toxicity. To define the critical time point of cardiac dysfunction, *in vivo* hemodynamic parameters of the left ventricle were determined at 4, 24, and 72 h after treatment. Left ventricular end systolic pressure (Fig. 2A) showed significant cardiac depression in WT mice at 4 h after LPS challenge, when compared with (saline-injected) controls, and TLR4-ko mice did not display suppressed cardiac function after LPS challenge. Maximal suppression in cardiac function was observed at 4 h after LPS challenge in WT mice. Lactate dehydrogenase leakage to the serum was highest in WT mice treated with LPS, whereas TLR4-ko mice displayed no signs of LPS biochemical marker of damage (Fig. 2D). To determine whether immune cells infiltrated the heart at this time point, hearts were evaluated by hematoxylin and eosin (H&E) histology staining (Fig. 2B). No neutrophil infiltration to the myocardium was observed in WT mice at 4 h after LPS challenge, but neutrophils were observed in the tissue at 24 h. Neutrophil infiltration was diffused after LPS challenge. In contrast, in TLR4-ko mice, no neutrophil infiltration was observed at all time points by H&E staining procedure. Immunohistochemistry staining using anti-mouse neutrophils recognizing polymorphic 40-kDa antigens expressed by polymorphonuclear cells was also performed (Fig. 2B). Significant neutrophil infiltration was observed in WT mice hearts only at 24 h post-LPS challenge using the neutrophil marker MCA771G (Fig. 2B). These data demonstrated that the functional suppression of the myocardium preceded the chemotaxis of neutrophils to the heart. Further, in TLR4-ko mice, neutrophil migration to the heart was delayed. Finally, TNF and IL-1β were significantly lower ($p < 0005$) in serum (Fig. 2E, F) and in the heart tissues at 4 h following LPS challenge of mice deficient in TLR-4, indicating that this receptor indeed mediates cytokine release. Using the isolated hearts in a Langendorff system, LPS administration ameliorated cardiac function of TLR4-ko hearts compared with WT (data not shown). These data demonstrated that the functional suppression of the myocardium is due to TLR4 activation and intracellular signaling (32).

TLR Signaling Mediates Myocardial Ischemic Injury

The innate immune response to I/R is the most common cause of myocardial inflammation (120). Molecular and cellular mechanisms underlying I/R injury involve reactive oxygen species (ROS) generation, activation of endothelial cells and complement, increased vascular permeability, and a rapid accumulation of neutrophils (37, 38, 55, 103, 114, 151). Several hours after the onset of myocardial reperfusion, neutrophils accumulate in the infracted myocardial tissue in response to the release of chemoattractants: ROS, cytokines, and the activated complements. The upregulated cell-adhesion molecules (ICAM-1, VCAM-1, and P-selectin), likely mediated by NF-κB activation (81) in response to myocardial ischemia, facilitate the migration of neutrophils into the myocardial tissue. Neutrophils mediate cardiomyocyte death by causing vascular plugging and releasing degradative enzymes and ROS (145), which causes myocardial injury by

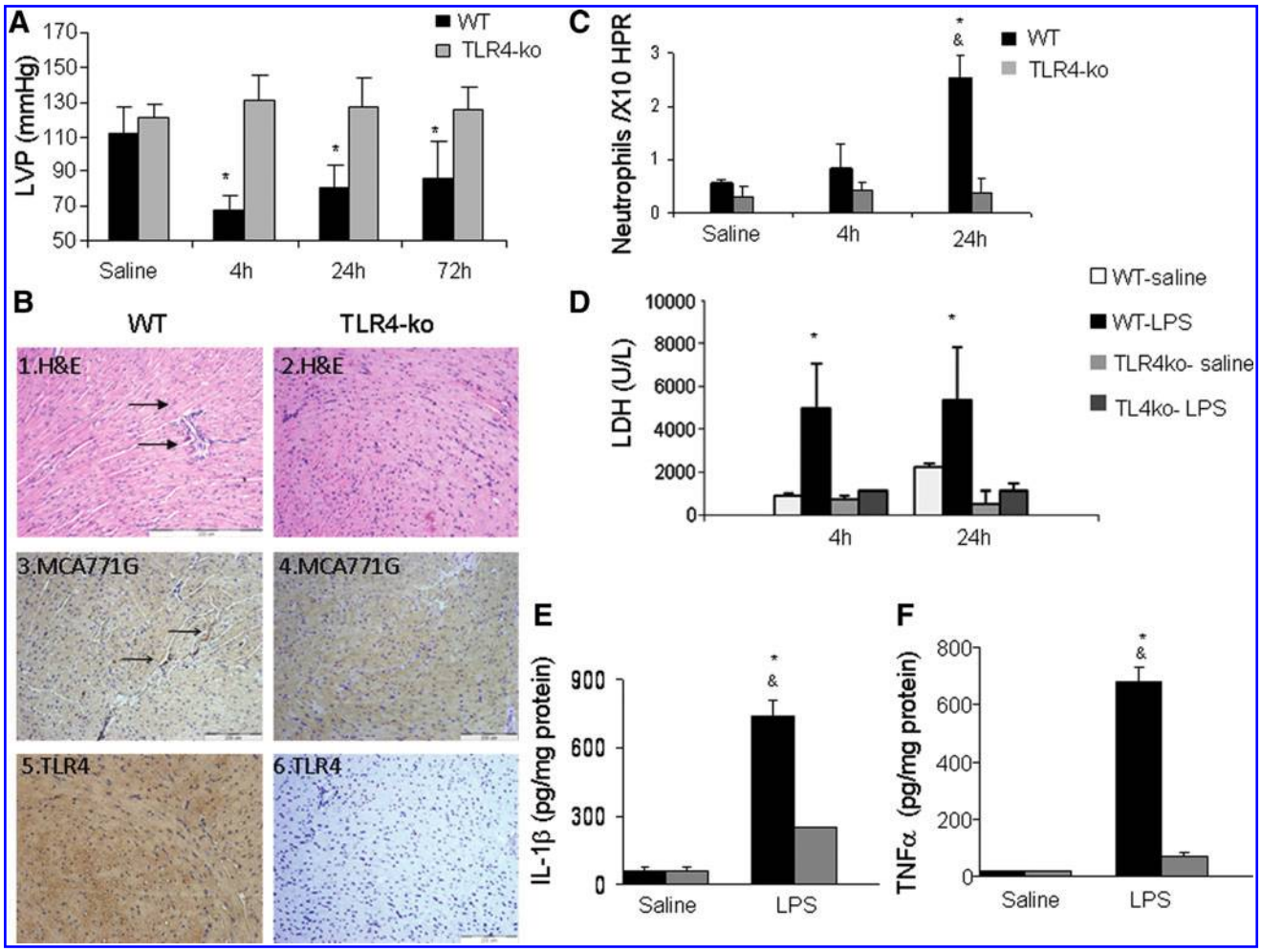

FIG. 2. Myocardial function following LPS treatment. (A) The critical time point of cardiac dysfunction following LPS treatment, *in vivo*. Hemodynamic measurements of the left ventricle of WT (black bars) *versus* TLR4-ko (gray bars) mice were determined at 4, 24, and 72 h after challenge using a Millar microtip transducer ($n = 6$ per group). Mice were subjected to LPS injection (*vs*. saline injection) (*vs*. sham control). Data are mean ± SD. **(B)** Hematoxylin and eosin (H&E) staining shows that 24 h following LPS there is diffused leukocyte infiltration in the WT hearts (indicated by *arrows*) (1. H&E). In the TLR4-ko mice, there is no leukocyte infiltration (2. H&E). Immunohistochemistry for neutrophils with rat anti-mouse neutrophil antibody (MCA771GA) shows a high level of neutrophil infiltration in WT mice hearts at 24 h post-LPS challenge (indicated by *arrows*) (3. MCA771G). In TLR4-ko mice, minor infiltration was observed at 24 h posttreatment (4. MCA771G). TLR4 protein expression was analyzed by immunohistochemical staining for TLR4 in WT (5. TLR4) and TLR4-ko (6. TLR4) mice at 24 h post-LPS treatment. **(C)** Quantification of immunohistochemical staining for neutrophils by counting 10 high power field (HPF)/slide; $n = 5$ per group. Data are mean ± SD. Significant neutrophil infiltration was observed in WT mice hearts at 24 h post-LPS (fourfold) compared with the control groups. $^{*}p < 0.05$, saline control *versus* LPS or sham control *versus* MI in WT mice; $^{\&}p < 0.05$, saline control *versus* LPS or sham control *versus* MI in TLR4-ko mice. **(D)** Enzyme leakage of LDH to the serum was higher in WT mice treated with LPS compared with TLR4-ko. **(E, F)** A higher level of TNFα and IL-1β in the serum of WT and TLR4-deficient mice treated with LPS is presented ($p < 0005$). ko, knockout; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; WT, wild type; SD, standard deviation. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

inducing a mitochondrial permeability transition pore opening and a subsequent ATP depletion and cell death (50). Inflammation is an important functional contributor to the pathogenesis of ischemic myocardial injury. Interventions such as antibodies to the proinflammatory cytokine IL-1 targeted at a specific inflammatory mediators have demonstrated benefits in I/R injury (61).

TLRs mediate myocardial inflammation and injury during I/R. Enhanced TLR4 expression was documented in patients with idiopathic dilated cardiomyopathy remodeling, in murine myocardium remote from sites of ischemic injury, and in heart tissue (41). TLR signaling has different roles in different injury organs, such as lung (65), liver (116), brain (136), and heart (20, 22, 67, 68, 73, 109, 141). In the lung, hyaluronan, produced in response to the acute lung injury, induces a proinflammatory and an antiapoptotic effect *via* both TLR2- and TLR4-MyD88–dependent mechanisms (65). Similarly, in a hyperoxic lung injury model (155), activation of TLR4 confers resistance to hyperoxia-induced pulmonary apoptosis (117). The heart with an inactive TLR4 gene or genetically

TLR4 deficient (22, 73, 109) exhibited reduced MI sizes compared with WT animals, suggesting that TLR4 signaling contributed to ischemic injury in the heart. MyD88-deficient mice (34) or mice pretreated intravenously with a TLR4 antagonist (Eritoran) demonstrated reduced MI injury and markers of an inflammatory response (129). Using isolated hearts, it was demonstrated that myocardial tissue TLR4, rather than neutrophil TLR4, is the determinant of myocardial neutrophil infiltration after global ischemia reperfusion (8). Moreover, TLR4 signaling on both donor and recipient cell types plays a central role in mediating the robust early inflammatory response that occurs after cold I/R, as in the setting of solid organ transplantation (67, 68). A remarkable dependency on TLR4 has also been reported in a model of hepatic injury after cold preservation and transplantation (128). Together, these studies strongly implicate TLR4 as a mediator of inflammation and organ injury after cold I/R during transplantation. Isolated cardiomyocytes from C3H/HeJ hearts showed resistance to hypoxia-induced contractile dysfunction compared with those from C3H/HeN hearts, which are associated with greater hypoxic activation of AMPK and ERK signaling—better intracellular Ca^{2+} handling in C3H/HeJ *versus* C3H/HeN cardiomyocytes. These findings suggest that the cardioprotective effects against ischemic injury of hearts with deficiency in TLR4 signaling may be mediated through modulating AMPK and ERK signaling pathway during ischemia (156).

Endothelial Cells and TLR4

The endothelium plays a central role in maintaining vascular health by its vital anti-inflammatory and anticoagulant properties (82). Endothelial dysfunction is the earliest detectable manifestation of atherothrombosis (62). Endothelial cells respond to pharmacologic or hemodynamic stimuli by modulating the induction and/or repression of several genes. TLR4 is markedly augmented in the endothelial cells of human atherosclerotic lesions. However, TLR4 was expressed at low levels by endothelial cells in normal arteries (30). Further, cultured human vascular endothelial cells express little TLR4 under baseline conditions, and they express high levels of TLR4 on stimulation with proinflammatory cytokines (33). Although these data suggested that TLR4 in endothelial cells was associated with the initiation of atherosclerosis, it remains unclear how TLR4 in endothelial cells play roles in atherosclerosis. The endothelium plays a central role in maintaining vascular health by virtue of its vital anti-inflammatory and anticoagulant properties (62).

Circulating monocytes from patients with arterial disease exhibit increased expression of TLR4 and TLR2 compared with healthy controls (26, 43, 77, 98, 130). However, such increases in expression do not always result in enhanced TLR signaling (9, 84, 144). Analogous with human coronary artery disease, $ApoE^{-/-}$ mice with advanced atherosclerotic disease also display increased surface expression of TLR2 and TLR4 on circulating monocytes (127). TLR4 expression in atherosclerotic lesions in $ApoE^{-/-}$ mice has been shown to colocalize with macrophage staining (149). Increased expression of TLR2 and TLR4 in lesions may be a consequence of exposure to oxidized low-density lipoproteins in the plaque as expression of both receptors has been shown to increase *in vitro* following oxidized LDL stimulation and foam cell formation in monocyte-derived macrophages (59, 149). Oxidized LDL can also act as a ligand engaging TLR-4, inducing a vicious circle of cell activation. Lipids are also putative ligands for TLR-2 and -4. Saturated fatty acids display the capacity of delivering a TLR4 signal and to induce inflammatory gene expression, whereas polyunsaturated fatty acids block the activation of TLR4 (80).

The first cardiac cells that can come in contact with the circulating LPS may be the coronary endothelial cells. Human coronary artery endothelial cells were shown to respond to LPS in a TLR4-dependent manner, leading to inflammatory cytokines release of IL-6, IL-8, and monocyte chemoattractant protein-1, and enhanced expression of adhesion molecules such as ICAM-1, VCAM, and ELAM-1. Therefore, it was proposed that human coronary artery endothelial cell activation could exert negative effects on the contractile function of a failing heart (153).

LV remodeling is strongly associated with an inflammatory response. The lower ischemic injury during the acute phase is accompanied with improved survival and left ventricular remodeling (121). TLR4-ko-MI mice showed better survival and LV function and reduced LV remodeling as indexed by reduced levels of atrial natriuretic factor, total collagen, and heart weight-to-body weight ratio, when compared with WT-MI mice. This was associated with a reduction of protein levels of the intracellular TLR4 adapter protein MyD88 and enhanced protein expression of the antihypertrophic JNK in TLR4-ko-MI mice when compared with wild-type (WT)-MI mice. There is direct evidence of a causal role of TLR4 in postinfarct maladaptive LV remodeling, probably *via* inflammatory cytokine production and matrix degradation (141). Increased collagen density following MI and fewer macrophages in the peri-infarct area together with reduced inflammation-regulating cytokine expression levels and matrix metalloproteinase activity were found in TLR4-ko mice.

Cardiomyocyte Apoptosis Following Ischemia or LPS Challenge

Cardiomyocyte apoptosis is particularly prominent in reperfusion injury and mainly localized in the border zone of the histological infarction area and a few in remote noninfarcted myocardia (44, 54, 56, 123, 124). HSP60 induced apoptosis in cardiac myocytes and the apoptosis was decreased by anti–TLR4-blocking antibodies but not by blocking antibodies to TLR-2 or CD14 (74). These findings may imply that HSP60 released during cardiac injury can have a paracrine effect on neighboring myocytes, leading to cell death. In contrast, prolonged LPS treatment in rats activated proapoptotic and survival pathways and induced very modest cardiomyocyte apoptosis (90). The low levels of apoptosis appeared insufficient to account for the LPS-induced cardiomyocyte dysfunction. A recent study has indicated that *in vivo* administration of LPS actually reduced myocardial apoptosis induced by I/R injury (46).

Gain of Function Following Myocardial Infarction in TLR4-Deficient Mice

It has been shown that a deficiency of TLR4 signaling reduces myocardial infarction and inflammatory pathways including neutrophil accumulation (109, 156). It was also reported that TLR4 deficiency results in reduced infarct size

with no gain in heart function (73). To define the critical time point of cardiac dysfunction following LAD ligation in WT *versus* TLR4-ko mice, *in vivo* hemodynamic parameters of the left ventricle were determined at 4, 24, and 72 h after treatment. Left ventricular end systolic pressure (Fig. 3A) showed significant cardiac depression in WT mice at 4 h after MI, when compared with sham-operated mice. In contrast, TLR4-ko mice showed superior myocardial function compared with WT mice after MI. Myocardial suppression was accompanied with higher biochemical marker of tissue injury in the WT *versus* TLR4-ko mice (Fig. 3D). Maximal suppression in cardiac function was observed at 4 h after MI in WT mice. To determine whether immune cells infiltrated the heart at this time point, hearts were excised and evaluated by H&E histology staining. Although there was no neutrophil infiltration in WT mice at 4 h after MI, neutrophils were observed in the tissue at 24 h (Fig. 3B). The neutrophil infiltration was massive at the peri-infarct area after MI. In TLR4-ko mice, low neutrophil infiltration was observed at all time points using H&E staining procedure. To be more specific, immunohistochemistry staining using anti-mouse neutrophils recognizing polymorphic 40-kDa antigens expressed by polymorphonuclear cells was performed. Significant neutrophil infiltration was observed in WT mice hearts at 24 h post-MI (40-fold),

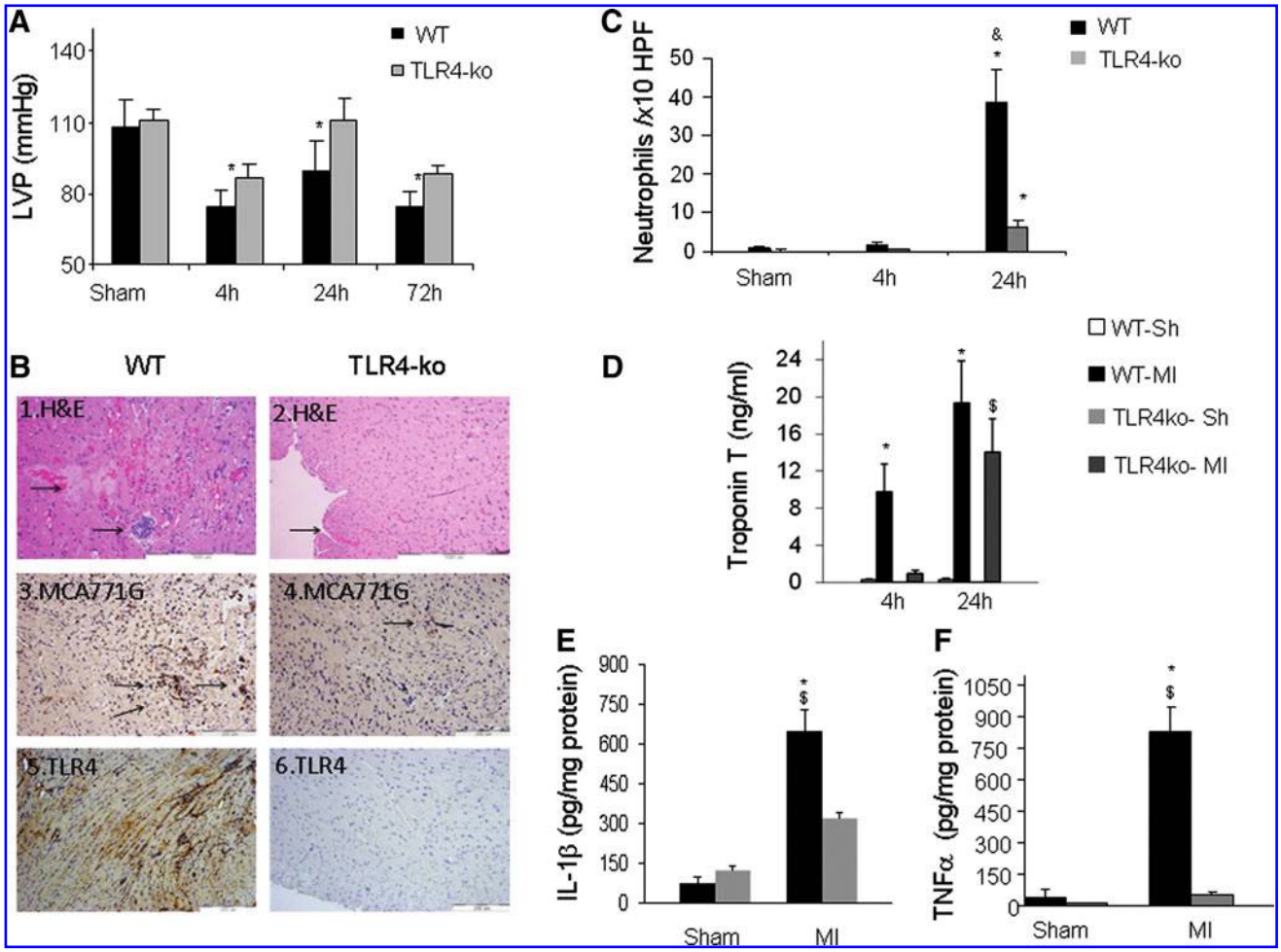

FIG. 3. Cardiac function following MI. (A) The critical time point of cardiac dysfunction following MI, *in vivo.* Hemodynamic measurements of the left ventricle of WT (black bars) *versus* TLR4-ko (gray bars) mice were determined at 4, 24, and 72 h after challenge using a Millar microtip transducer ($n = 6$ per group). Mice were subjected to MI (*vs.* sham operated). Data are mean ± SD. **(B)** H&E staining shows that at 24 h there is a massive infiltration in the peri-infarct area following MI in the WT hearts (1. H&E). In the TLR4-ko mice, there is no leukocyte infiltration (indicated by *arrows*) (2. H&E). Immunohistochemistry for neutrophils with rat anti-mouse neutrophil antibody (MCA771GA) shows a high level of neutrophil infiltration in WT mice hearts at 24 h post-MI (3. MCA771G), compared with the TLR4-ko group (indicated by *arrows*) (4. MCA771G). TLR4 protein expression was analyzed by immunohistochemistry staining for TLR4 in WT (5. TLR4) and TLR4-ko (6. TLR4) mice at 24 h post-MI. **(C)** Quantification of immunohistochemical staining for neutrophils by counting 10 HPF/slide; $n = 5$ per group. Data are mean ± SD. Significant neutrophil infiltration was observed in WT mice hearts at 24 h post-MI (40-fold, compared with the control groups. $^{*}p < 0.05$, saline control *versus* LPS or sham control *versus* MI in WT mice; $^{\$,\&}p < 0.05$, saline control *versus* LPS or sham control *versus* MI in TLR4-ko mice. **(D)** Troponin T leakage to the serum was higher in WT mice following MI, compared with TLR4-ko mice. **(E, F)** A higher level of TNFα and IL-1β in the serum of WT and TLR-4–deficient mice following MI ($p < 0005$). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

respectively, compared with the control groups (Fig. 3B). In TLR4-ko mice, minor infiltration was observed in the MI group at 24 h posttreatment (Fig. 3C). These data demonstrated that the functional suppression of the myocardium preceded the chemotaxis of neutrophils to the heart. Further, in TLR4-ko mice, neutrophils migration to the heart is delayed. Despite demonstrating equal areas at risk, TLR4-deficient hearts had a 2.3 times smaller infarct size normalized to the area at risk compared with WT hearts ($p < 0.05$). TNF and IL-1β were significantly lower ($p < 0005$) in serum (Fig. 2E, F) and in the heart tissues at 4 h following MI of mice deficient in TLR4, indicating that this receptor mediates cytokine release (Fig. 3E, F).

Myocardial Function in Chimera Hearts Following LPS or MI Challenge

To exclude that resident macrophages in the heart play a role in the acute (4 h) cardiac depression following LPS or MI challenge, we produced chimera mice in which TLR4 was expressed in either the heart or the leukocytes. Control chimera mice were WT (C57Bl) with WT bone marrow (B6b) to eliminate the effects of radiation and the process of bone marrow transplantation. Chimerism was confirmed 8–10 weeks following transplantation only when at least 95% of the transplanted bone marrow was present.

To determine TLR4 expression in blood following LPS challenge, white blood cells were stained with anti-mouse TLR4. As in TLR4-ko/WT, the hematopoietic system lacks TLR4, because its expression was not observed, but both the control group (WT/WT) and the WT/TLR4-ko, wherein the hematopoietic system expresses TLR4, a positive TLR4 cell count was noted. The administration of LPS to TLR4-ko mice transplanted with bone marrow cells of WT (WT/TLR4-ko) mice was similar to WT/WT (Fig. 4B_1–B_4).

Figure 4C represents LV hemodynamic pressure measurements *in vivo*: following LPS or MI challenge, no change was observed in the end diastolic pressure, which was 0–10 mmHg in all the tested chimera groups at 4 h posttreatment. Cardiac contractile depression of chimera mice whose hearts expressed TLR4 (TLR4-ko/WT) was similar to the control chimera WT/WT mice. Chimera mice deficient in

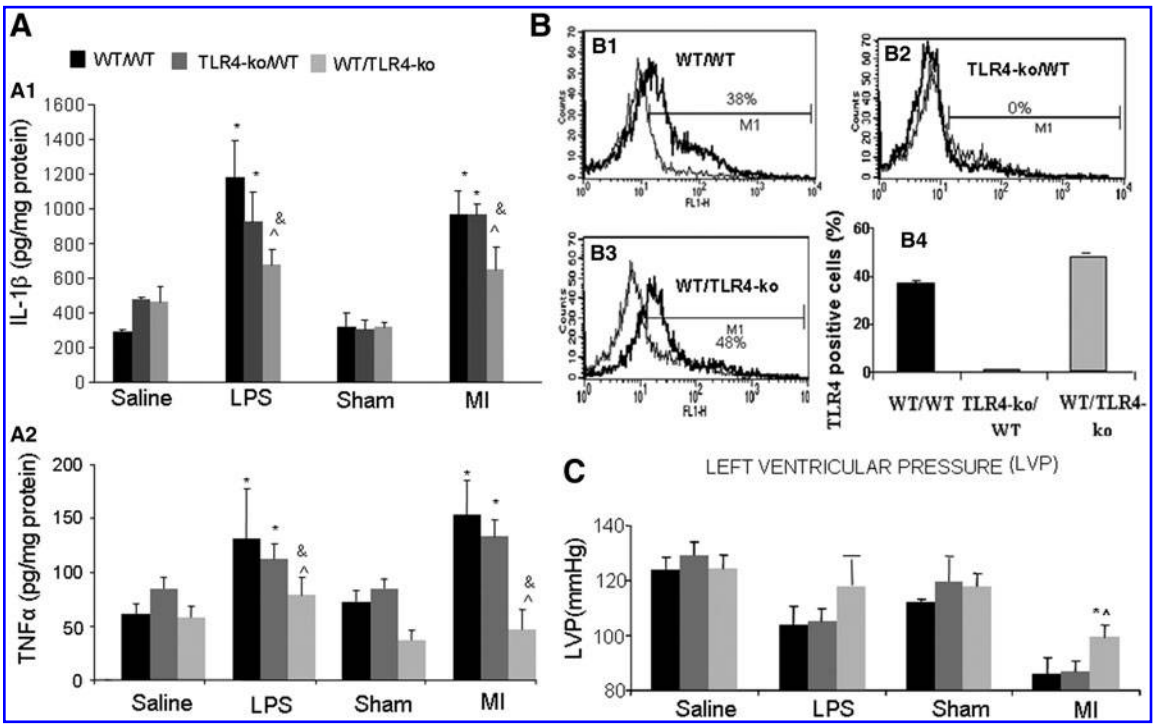

FIG. 4. Myocardial cytokines expression and function in chimeric mice. (A) Cytokine expression of TNF-α and IL-1β in the myocardial tissue ($n = 4$ per group; mean ± SD.) TNF-α expression in the heart was upregulated only in the chimeric groups that express TLR4 in the heart (WT/WT, TLR4-ko/WT). IL-1β expression in the heart was upregulated following LPS or MI challenge in all the chimeric groups. $^*p < 0.05$, LPS *versus* saline control or MI *versus* sham control. $^{\&}p < 0.05$, WT/TLR4-ko *versus* WT/WT. **(B)** Expression of TLR4 in the blood following LPS challenge in all the chimeric groups by flow cytometry. The percentage of positive WBC cells expressing TLR4 is shown in each representative histogram. WBC from 3 different chimeric groups were stained with mouse anti-TLR4 (Alexa Fluor 488). No events of TLR4 expression were observed in TLR4-ko/WT in total viable cells of the blood because the hematopoietic system lacks TLR4 (B2). Both the control group WT/WT and the WT/TLR4-ko demonstrated positive TLR4 expression in the WBC (B1 and B3). TLR4 expression for isotype control is presented as a regular line compared with the bold TLR4 histogram. Mean positive count for TLR4 expression in the different groups is expressed as mean ± SD; $n = 3$ in each group (B4). **(C)** Hemodynamic measurements of LV of chimera mice LVP was tested at 4 h post-LPS or MI challenge ($n = 6$ per group). Data are mean ± SD. Chimera mice hearts deficient in TLR4 (WT/TLR4-ko) were resistant to LPS injection and significantly less depressed following MI. $^*p < 0.05$, WT/TLR4-ko *versus* WT/WT. $^{\wedge}p < 0.05$, WT/TLR4-ko *versus* TLR4-ko/WT. WBC, white blood cells; LVP, left ventricular end systolic pressure.

TLR4 in the heart (WT/TLR4-ko) were resistant to LPS injection and significantly less depressed following MI, similar to TLR4-ko mice, suggesting that myocardial TLR4 plays a key role in the acute phase of cardiac depression following both insults.

As TLR-4 has been associated with the secretion of proinflammatory cytokines, TNF-α and IL-1β protein expression in the heart were assessed in WT, TLR4-ko, and chimeric mice after MI or LPS administration. In WT hearts, both IL-1β and TNF-α peaked significantly at 4 h post-LPS or MI challenge. Cytokine values then returned to normal in the LPS-administered mice, whereas in MI mice, they remained high throughout the observation period. TNF-α and IL-1β were significantly lower ($p < 0005$) in the heart of TLR4-ko, compared with WT, indicating that this receptor indeed mediates cytokine release. Moreover, in TLR4-ko mice, we found no elevation of these cytokines following both treatments at any time point. In the chimeric mice, cytokine levels were tested only at 4 h (the peaking time point) post-LPS or MI challenge. These proinflammatory cytokines levels in the chimeras expressing the receptor only in the immunohematopoietic system (WT/TLR4-ko) were found to be significantly lower than the control group (WT/WT) and the chimeras expressing the receptor in the heart (TLR4-ko/WT). TNF-α in the heart did not elevate after LPS or MI challenge in the chimeras expressing the receptor only in the immunohematopoietic systems and not in the heart (WT/TLR4-ko) (Fig. 4A1, A2), indicating that deficiency of TLR4 in the heart prevents TNF-α secretion in the acute phase. However, IL-1β was found to be elevated in this group (Fig. 4A1), but remained significantly lower than the other two chimera groups, demonstrating that the presence of TLR4 in the immunohematopoietic system might be responsible for this IL-1β secretion in the heart. In this study, we demonstrated that TLR4 is involved in cytokine production within the heart following LPS or ischemia challenge. There is a controversy on the source of the inflammatory cytokines. It was previously shown that TLR4 was involved in cytokine production within the heart of endotoxemic mice, and in contrast to our study, TLR4 on leukocytes is the key player in myocardial dysfunction associated with increased cytokine production following LPS challenge (10, 138). We did not detect significant infiltration of cells into the heart in the acute phase (4 h), which corresponded to maximal depression of heart contraction. Therefore, we can conclude that the myocytes can be the source of the cytokines and not the infiltrating leukocytes. Moreover, the absence of leukocytes infiltration in TLR4-ko mice at longer time points after the LPS and MI challenge suggests that the activity of this receptor or related molecular pathways might be involved in the cells homing to the heart. Supporting our findings, it was demonstrated in isolated hearts (8) that TLR4 in the myocardial tissue, rather than neutrophil TLR4, is the determinant of myocardial neutrophil infiltration after global ischemia reperfusion. It seems possible that the reduction in myocardial inflammation may have contributed to reduced MI sizes in TLR-4–deficient animals.

Pharmacological Preconditioning

Sepsis/bacteremia induces myocardial dysfunction but also stimulates the heart to develop protection from a second injury, such as ischemia reperfusion damage. The protection developed by sepsis/bacteremia is very efficient so that the recovery of left ventricular developed pressure is complete after 30–40 min of global ischemia in the isolated heart (97). Isolated rat hearts pretreated with a low dose of LPS at 24 h before had a better preserved myocardial function after I/R compared with their control hearts (96). The results suggest that LPS-induced cardiac functional protection against ischemia is a delayed and long-lasting protective response that may involve *de novo* protein synthesis. LPS administration induces iNOS production in the heart *via* TLR4 (146, 157). Similar to ischemic preconditioning (14), the cardioprotection conferred by LPS seems to be mediated by iNOS (146) and through a PI3K/Akt-dependent mechanism (46). These studies are important because they demonstrate a clear link between the activation of systemic TLR4 innate immune system and cardioprotection against ischemic myocardial injury.

Conclusion

The mechanisms of LPS-induced, ischemia, or artherosclerosis signaling events mediated by TLR4 have been extensively studied over recent years. Much work has been carried out to elucidate the role of specific molecules comprising the signaling pathways and to identify the negative regulators of the LPS, ischemia, or artherosclerosis signaling cascades such that therapeutic approaches may be derived from these discoveries. TLR4 deficiency in the heart was found to lead to improved survival and LV function after LPS or MI challenge, suggesting that inhibition of this pathway may be beneficial in overcoming the acute phase. Anti-TLR4 antibodies were shown to prevent death caused by septic shock, even when treatment was given up to 13 h after endotoxic shock (122). An anti-TLR4 treatment strategy is supported by data obtained with Eritoran (E5564), a synthetic LPS antagonist that binds to MD-2 (72, 87), and TAK-242, a cyclohexene derivative that inhibits TLR4-mediated signal transduction, which prevented lethality in experimental models of LPS shock or bacterial sepsis in rodents (100, 132). Our findings provide key evidence of the importance of myocardial TLR4 in the acute phase as a mediator of septic shock and MI-induced cardiac dysfunction. Therefore, myocardial TLR4 may become a therapeutic target in septic shock or MI cardiac dysfunction.

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Abbreviations Used

CD = cluster of differentiation
H&E = hematoxylin and eosin
HSP = heat-shock protein
IFN-β = interferon-β
IL = interleukin
IRAK = IL receptor-associated kinase
IRF = IFN regulatory factor
Ko = knockout
LDH = lactate dehydrogenase
LPS = lipopolysaccharide
LVP = left ventricular end systolic pressure
MI = myocardial ischemia
MSCs = mesenchymal stem cells
MYD88 = myeloid differentiation primary response protein
NF-$\kappa\beta$ = nuclear factor kappa β
PAMPS = pathogen-associated molecular patterns
ROS = reactive oxygen species
TAB1 = TAK1-binding protein
TAK1 = transforming growth factor β-activating kinase
TIR = Toll/IL-1 receptor
TLR = Toll-like receptors
TLR4 = Toll-like receptor 4
TNF = tumor necrosis factor
TRAF6 = tumor necrosis factor receptor-activated factor-6
TRAM = Trif-related adaptor molecule
TRIF = TIR domain-containing adaptor protein
WBC = white blood cells
WT = wild type

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