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TLR3 exaggerated sepsis induced cardiac dysfunction via activation of TLR4-mediated NF-κB and TRIF/IRF signaling pathways

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Abstract

Cardiovascular dysfunction is a major consequence of septic shock and contributes to the high morbidity and mortality of sepsis. Groups of proteins that comprise the Toll or Toll-like family of receptors detect the pathogen and mount a rapid defensive response in vertebrate and invertebrate organisms, through induction of innate immune and inflammatory responses. The engagement of TLR4 homodimers by LPS or other protein cognate the ligands initiates a signaling cascade and thus induces genes involved in the immune response against pathogens. TLRs have been implicated in cardiac dysfunction in several important disease states, including ischemia/reperfusion (I/R) injury. MyD88 contains an N-terminal death domain and a C-terminal TIR domain. When stimulated, MyD88 is recruited and, in the early phase, interacts with the cytoplasmic TIR domain of TLR4. Although TLR3 is known to respond to RNA from damage cells, the importance of this response in vivo during acute inflammatory processes has not been fully understood. Our result shows that TLR3$^{-/-}$ rat significantly attenuated myocardial NF-κB binding activity both the levels of phosphorylated IκBα/IκBα after LPS administration, and improved cardiac function and reduce the inflammatory response. Further, LPS increased levels of TLR4, TRIF and IFN-β in the myocardium. Interestingly the TLR4-activation signaling was significantly prevented by TLR3 deficiency. We concluded that the use of antibody directed against TLR3 might serve as a therapeutic clinical option in the treatment of cardiac dysfunction induced by sepsis.

Key words: TLR3, TLR4, TRIF, IFN-β, LPS, Cardiac dysfunction

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Introduction

Toll-like receptors are transmembrane glycoproteins, which recognize many PAMPs with extracellular domains and aggravate the exaggerated inflammatory response to bacterial infection through activating nuclear factor (NF)-κB [1]. TLR4 is the most studied member in the SIC study among the TLRs family. A research from TLR4-deficient mice confirmed the essential role of TLR4 in mediating neutrophil migratory phagocytic functions, attenuating inflammation, reducing reactive oxygen species generation, and enhancing bacterial clearance [2]. Other TLR-

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related genes (TLR2, 3, and 9) were demonstrated to be involved in sepsis-induced cardiac dysfunction from recent studies. TLR2 increased the myocardium and serum cardio-depressant cytokines level and weakened the neutrophil migratory function, which sharpened the SIC [3]. TLR3 played a deleterious role in mediating cardiac dysfunction in sepsis by increasing cecal ligation and puncture (CLP)-induced cardiomyocytes apoptosis and Fas and Fas ligand expression in the myocardium [4]. CpG oligodeoxynucleotide, the TLR9 ligand, through activating both phosphoinositide 3 kinase/Akt and extracellular signal-related kinase signaling, attenuated cardiac dysfunction in polymicrobial sepsis [5]. However, a recent research demonstrated that eritoran, an anti-TLR4 to terminate MD2/TLR4-mediated signaling, did not significantly improve outcome for patients with severe sepsis and septic shock [6]. Additional studies are needed to explain the detailed mechanisms of SIC regulated by TLRs. TLR3 is located in intracellular endosomes and recognizes double-stranded RNA (dsRNA) and polyinosinic-polycytidylic acid (Poly I:C, a synthetic analog of dsRNA), resulting in induction of antiviral immune responses [7]. TLR3 also recognized byproducts from apoptotic and necrotic cells [8]. More significantly, a recent study demonstrated that TLR3 deficient ($TLR3^{-/-}$) mice showed an increased survival rate in CLP-septic mice [9]. Administration of anti-TLR3 antibody to wild type (WT) mice increased the survival rate in CLP-septic mice [10]. This study suggests that TLR3 contributes to the pathophysiology of sepsis/septic shock. However, TLR3 exaggerated sepsis induced cardiac dysfunction via activation of TLR4-mediated NF-κB and TRIF/IRF has not been investigated.

Materials and Methods

Experimental animals

TLR3 knockout mice ($TLR3^{-/-}$), that were crossbreed with C57BL/6, and age-and weight-matched male C57BL/6 mice were obtained from Jackson Laboratory (Indianapolis, IN). The mice were maintained in the Division of Laboratory Animal Resources at Mississippi Medical Center. The experiments outlined in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No.85-23, Revised 1996). All aspects of the animal care and experimental protocols were approved by the University of Mississippi Committee on Animal Care.

CLP polymicrobial sepsis model

Cecal ligation and puncture (CLP) was performed to induce sepsis in mice as previously described [11]. Briefly, the mice were anesthetized by 5.0% Isoflurane. A midline incision was made on the anterior abdomen and the cecum was exposed and ligated with a 4-0 suture. Two punctures were made through the cecum with an 18-gauge needle and feces were extruded from the holes. The abdomen was then closed in two layers. Sham surgically operated mice served as the surgery control group. Immediately following surgery, a single dose of resuscitative

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fluid (lactated Ringer's solution, 50 ml/kg body weight) was administered by subcutaneous injection [12]

Echocardiography

Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Aplio 80 Imaging System (Toshiba Medical Systems, Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously [13]. M-mode tracings were used to measure left ventricular (LV) wall thickness, LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) and percent ejection fraction (%EF) were calculated as described previously [14]. All measurements were made by one observer who was blinded with respect to the identity of the tracings. All data were collected from 10 cardiac cycles.

Statistical analysis

Survival trends were compared with the Cox regression proportional hazards procedures. All other data were expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA) and Tukey's procedure for multiple range tests was performed. $P< 0.05$ was considered to be significant.

Results

TLR3 deficiency attenuated cardiac dysfunction and increased survival outcome following CLP-induced sepsis

CLP significantly induced cardiac dysfunction as evidenced by decreased ejection fraction (%EF) by 25.7% and fractional shortening (%FS) by 29.8%, respectively, compared with baseline (Table 1). In contrast, $TLR3^{-/-}$ mice maintained the levels of %EF and %FS at baseline levels following CLP. Figure 1A shows representative images of M-model images of parasternal short-axis view at the papillary muscle level at base line and after CLP. CLP WT mice exhibited marked ventricular dilatation and poor left ventricular (LV) wall motion compared with base line. There was no significant change in LV dilatation and LV wall motion in $TLR3^{-/-}$ CLP mice.

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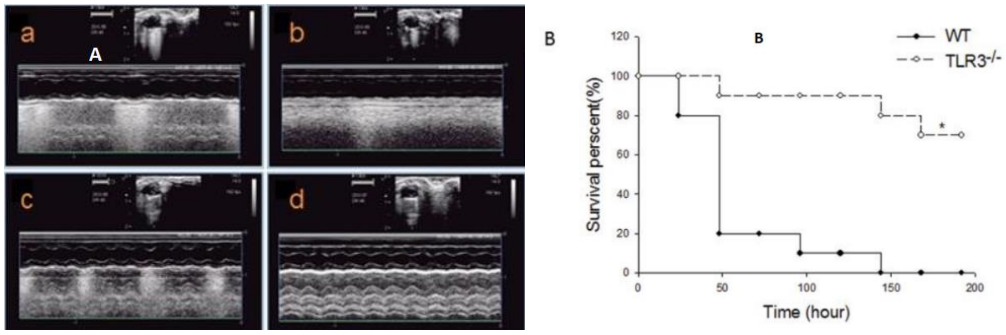

Figure 1.

TLR3 deficiency attenuated cardiac dysfunction and increased survival outcome following CLP-induced sepsis in mice

TLR3$^{-/-}$ and age-matched WT mice (n=12/group) were subjected to CLP and cardiac function was measured before and 6 hrs after CLP. **(A)** Representative images generated by echocardiography. a: WT mice base line (before CLP); b: WT mice after CLP; c: TLR3$^{-/-}$ mice base line (before CLP); d: TLR3$^{-/-}$ mice after CLP. **(B)** TLR3 deficiency increases survival outcome in CLP-induced septic mice. TLR3$^{-/-}$ and age-matched WT mice (10/group) were subjected to CLP and the survival was carefully monitored daily. * P<0.05 compared with indicated groups.

Table 1

TLR3 deficiency attenuated cardiac dysfunction in CLP-induced sepsis

Group	Heart rate	%EF	%FS	LVESD (mm)	LVEDD (mm)	SV (mmHg*µl)	CO (µl)
WT pre-CLP	440.9 ± 21.72	61.2 ± 5.49	32.4 ± 3.99	2.2 ± 0.34	5.5 ± 0.33	33816.9 ± 3279.01	15170.2 ± 1609.34
WT CLP	368.1 ± 40.52*	45.5 ± 7.32*	22.8 ± 8.15*	0.6 ± 0.13*	1.3 ± 0.51*	6696.6 ± 3787.75*	2483.9 ± 1241.00*
TLR3$^{-/-}$ pre-CLP	454.2 ± 19.34	61.8 ± 6.93	33.0 ± 5.25	2.1 ± 0.39	5.6 ± 0.61	34270.8 ± 5730.02	17249.0 ± 3648.22
TLR3$^{-/-}$ CLP	392.8 ± 6.55 $^{\#}$	62.6 ± 6.08$^{\#}$	33.2 ± 4.58$^{\#}$	1.5 ± 0.58$^{\#}$	4.4 ± 0.65$^{\#}$	28924.5 ± 6411.69$^{\#}$	12682.1 ± 3525.39$^{\#}$

TLR3 deficiency prevented sepsis-induced activation of TLR4 mediated NF-κB and TRIF/IRF signaling pathways

TLR4-mediated signaling activates both MyD88-dependent NF-κB and TRIF/IRF-dependent IFN pathways. Figure 2 shows that CLP significantly increased myocardial NF-κB binding activity (**A**) and the levels of phosphorylated IκBα/IκBα (**B**); however, the levels of phosphorylated IRF3 were significantly lower in both TLR3$^{-/-}$ sham and CLP mice compared with respective WT groups.

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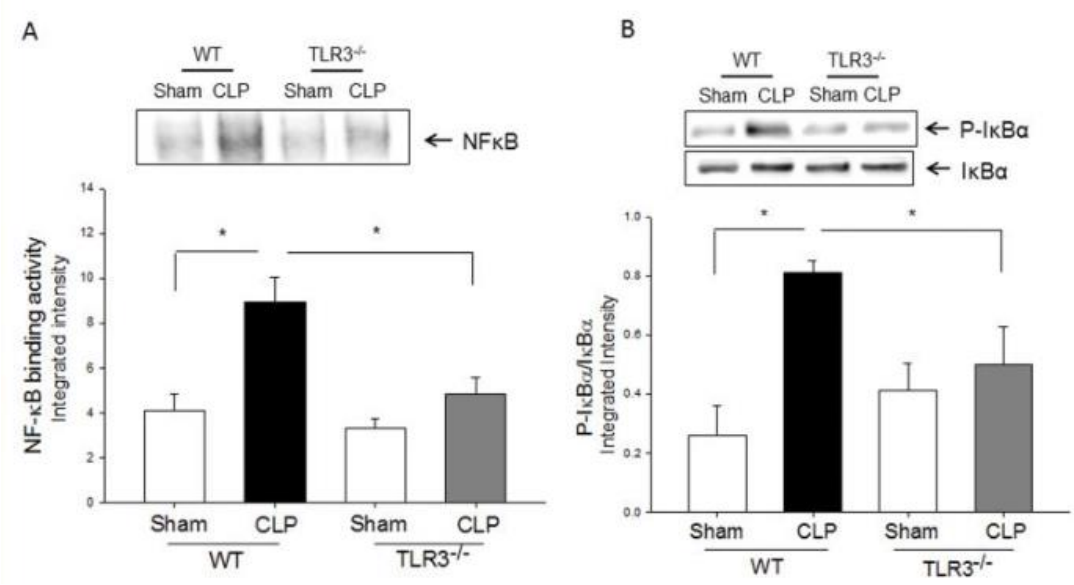

Figure 2.

TLR3 deficiency prevented sepsis-induced activation of TLR4-mediated NF-κB activation and TRIF/IRF-dependent IFN signaling pathways

TLR3$^{-/-}$ and WT mice were subjected to CLP (5-6/group). Sham operation served as sham control (5-6/group). Hearts were harvested six hrs after CLP and the nuclear and cytoplasmic proteins were prepared. (**A**) NFκB binding activity was determined by EMSA with nuclear proteins. The levels of p-IκBα/IκBα (**B**)

TLR3 deficiency prevented sepsis-induced expression of adhesion molecules in the myocardium

Increased expression of adhesion molecules, such as VCAM-1 mediate neutrophil and macrophage infiltration into the myocardium during sepsis/septic shock [15]. Figure 3, show that CLP significantly increased both VCAM-1 (↑63.8%) expression in the myocardium of WT mice compared with sham control. However, TLR3 deficiency prevented sepsis-induced increases in VCAM-1 expression. Specifically, the levels of VCAM-1 in septic TLR3$^{-/-}$ mice were not significantly different from TLR3$^{-/-}$ sham control. Immunohistochemistry staining demonstrated that increased expression of VCAM-1 is predominantly located in endothelium. There was less staining of VCAM-1 in myocardial tissue sections of septic TLR3$^{-/-}$ mice compared with that of septic WT mice.

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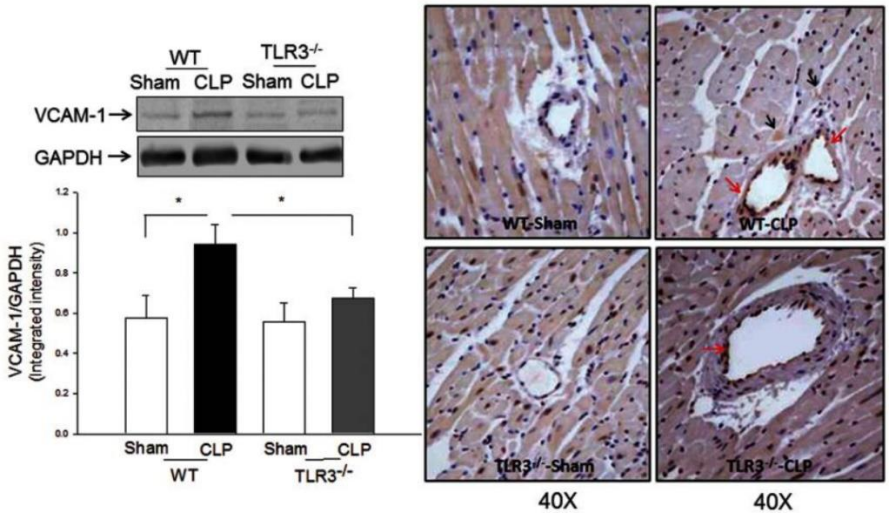

Figure 3.

Sepsis-increased expression of myocardial VCAM-1 was attenuated by TLR3 deficiency
$TLR3^{-/-}$ and WT mice were subjected to CLP (6/group). Sham operation served as sham control (6/group). Hearts were harvested six hrs after CLP and sectioned. * $p<0.05$ compared with indicated groups.

Attenuation of chemokine secretion by $TLR3^{-/-}$ macrophages following stimulation with TLR ligands

We examined the effect of TLR3 deficiency on the response to TLR ligand stimulation by macrophages. Figure 4 shows that both LPS and Poly I:C stimulation significantly increased the levels of MCP-1 and KC in WT macrophages but not in $TLR3^{-/-}$ macrophages. Both LPS and PGN significantly increased the levels of IP-10 in WT mice, however, the increased IP-10 by LPS and PGN was attenuated in $TLR3^{-/-}$ macrophages. CpG-ODN, a ligand for TLR9, did not stimulate the secretion of chemokines in WT and $TLR3^{-/-}$ macrophages.

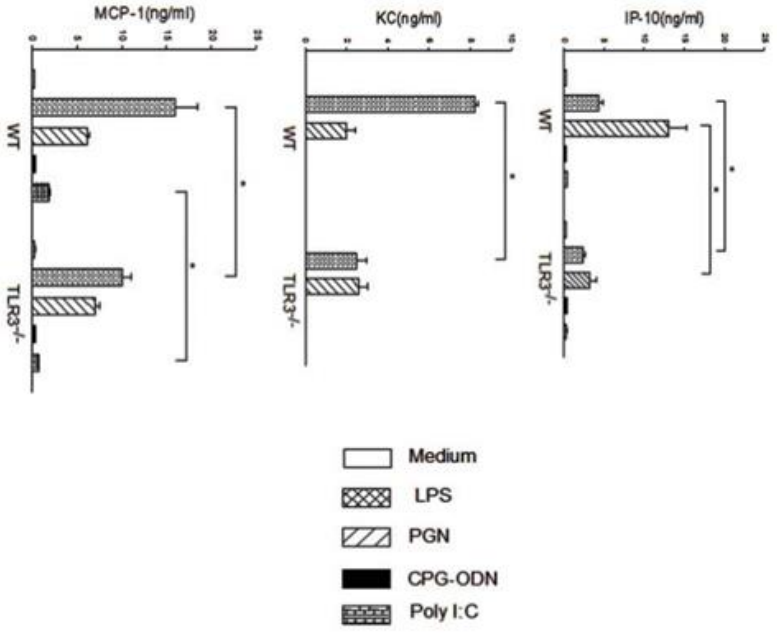

Figure 4.
Attenuated chemokine secretion by $TLR3^{-/-}$ macrophages following TLR ligand stimulation
Peritoneal macrophages were isolated from $TLR3^{-/-}$ and WT mice and stimulated with LPS, PGN, CpG-ODN and Poly I:C, respectively. Secretion of chemokines into the supernatants was measured using commercial kits. There were four replicates in each group (4/group). * $p<0.05$ compared with indicated groups.

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Sepsis-induced neutrophil and macrophage infiltration into the myocardium was prevented by TLR3 deficiency

Neutrophil and macrophage infiltration into the myocardium contribute to cardiac dysfunction during the development of sepsis/septic shock. Figure 5A shows that CLP increased MPO activity by 52.6% ($p<0.05$) in WT mice compared with sham control, indicating increased neutrophil infiltration into the myocardium. There were more neutrophils staining in WT CLP heart tissues compared with WT sham. In contrast, MPO activity was not significantly increased and there was less neutrophil staining in TLR3$^{-/-}$ mice following CLP. Figure 5B shows that CLP significantly increased the number of macrophages in the myocardium of WT mice compared with WT sham control. However, the numbers of macrophages in the myocardium of TLR3$^{-/-}$ mice were not significantly increased following CLP.

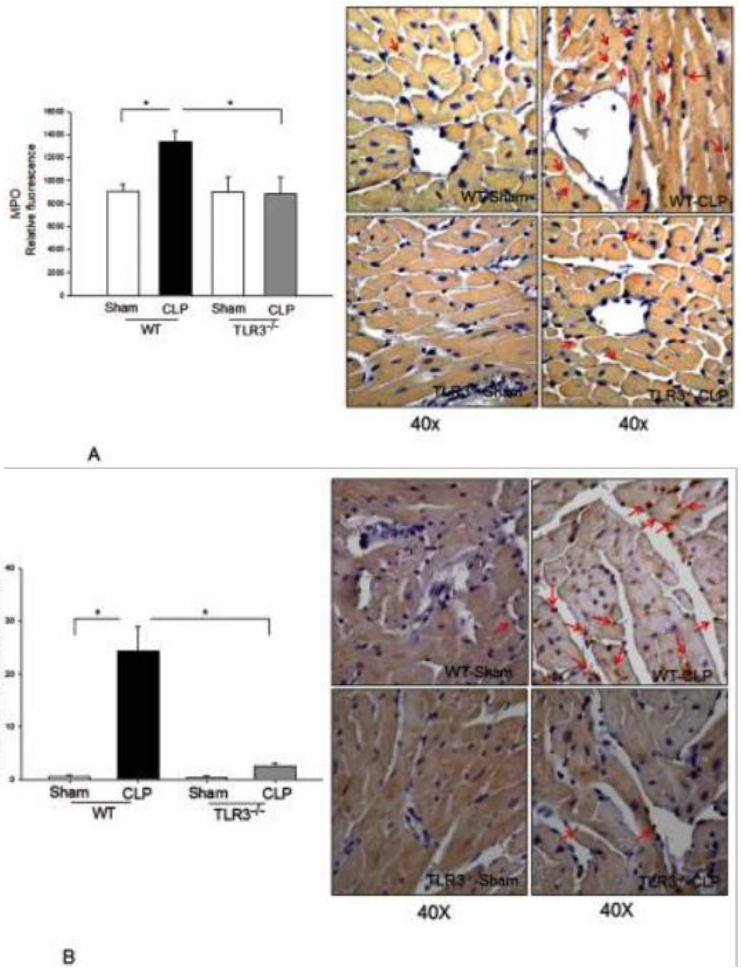

Figure 5.

Neutrophil and macrophage myocardial infiltration was attenuated in TLR3 deficiency
TLR3$^{-/-}$ and WT mice were subjected to CLP (6/group). Sham operation served as sham control (6/group). Hearts were harvested six hrs after CLP and sectioned. **(A)** MPO activity was measured in cellular protein preparations by a kit (left). Neutrophil accumulation (pink color) was marked by red arrows (n=3/group). **(B)** Macrophages in the myocardium were examined by immunohistochemistry (right) with a specific antibody (n=3/group). The dark brown color indicates macrophage infiltration marked by red arrows. Quantitated data are shown on the left. * $p<0.05$ compared with indicated groups.

Adoptive transfer of wild type bone marrow stromal cells abolished the cardioprotective effect of TLR3 deficiency in sepsis

To examine the role of peripheral cells in TLR3 deficiency-induced protection against sepsis-induced cardiac dysfunction, we isolated bone marrow stromal cells (BMSCs) from WT mice and transplanted them into TLR3$^{-/-}$ mice immediately prior to induction of CLP. Cardiac function was assessed 6 hrs after CLP. As shown in Figure 8, adoptive transfer of WT BMSCs into WT mice did not affect the CLP-induced decrease in cardiac function. However, TLR3$^{-/-}$ mice that received WT-BMSCs showed a loss of cardioprotection 6 hrs after sepsis. Both %EF (61.8%) and %FS (33.0%) values in TLR3$^{-/-}$ mice that received BMSCs were similar to that of WT CLP mice (46.1%, and 22.3%) and significantly lower than TLR3$^{-/-}$ mice (66.6%, 36.2%) that did not receive BMSCs.

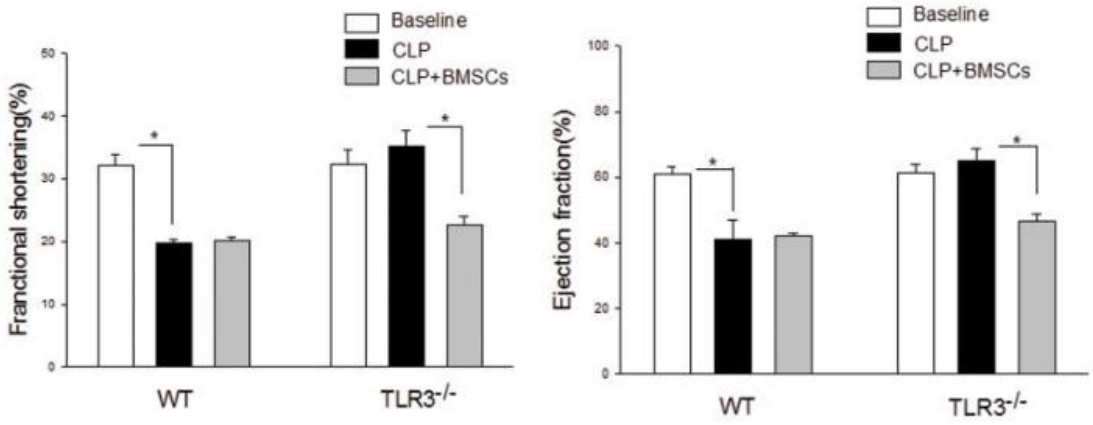

Figure 6.

Adoptive transfer of WT bone marrow stromal cells abolished the cardioprotective effect of TLR3 deficiency

Bone marrow stromal cells were isolated from WT mice and transplanted to TLR3$^{-/-}$ mice and WT mice, immediately before the mice were subjected to CLP (5/group). Cardiac function was measured by echocardiography before and 6 hrs after CLP. * $p<0.05$ compared with indicated groups.

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Discussion

The major finding in this study was that the TLR3 plays a key role in the pathophysiology of sepsis-induced cardiac dysfunction. Study reported that TLR3$^{-/-}$ mice were resistant to CLP-induced lethality [16]. We also observed that TLR3 deficiency significantly improved survival outcome in CLP-sepsis/septic shock. Our current study provides insights into the mechanisms by which TLR3 mediates septic morbidity and mortality.

Clinical and experimental studies have shown that myocardial dysfunction is an early and fatal complication of septic shock [17]. The current study demonstrated that sepsis-induced myocardial dysfunction was dramatically attenuated in the absence of TLR3. In addition, TLR3 deficiency reduced myocardial apoptosis in response to sepsis. We have previously shown that cardiac myocyte apoptosis contributes to cardiac dysfunction during the development of sepsis/septic shock [18]. The mechanisms by which TLR3 deficiency attenuated CLP-induced cardiac myocyte apoptosis is by inhibition of Fas/FasL-mediated apoptotic signaling. It is unclear how TLR3 deficiency attenuates sepsis-induced Fas/FasL-mediated apoptotic signaling. However, activation of TLR3 is known to stimulate the extrinsic and intrinsic apoptotic pathways by up-regulating TNF-related apoptosis-inducing ligand (TRAIL) and its receptors and by down-regulating the anti-apoptotic protein Bcl2 [19].

Previously reported that CLP significantly increased the levels of TLR4 and NF-κB binding activity in the myocardium [20]. In the present study, we observed that CLP sepsis markedly increased TLR4-mediated MyD88-dependent NF-κB activation and TRIF-dependent IRF3-mediated IFN-β signaling pathways. Importantly, TLR3 deficiency prevented CLP sepsis activation of both TLR4-mediated MyD88-dependent NF-κB activation and TRIF-dependent IFN-β signaling pathways. Indeed, the levels of inflammatory cytokines, such as TNFα, IL-1β and myocardial IFN-β, were markedly lower in TLR3$^{-/-}$ septic mice than in WT septic mice [21]. At present we do not understand why the levels of IL-6 were significantly higher in TLR3$^{-/-}$ mice than in WT mice after CLP. It could be possible that increased IL-6 levels in TLR3$^{-/-}$ mice may serve as an anti-inflammatory effect [22].

Neutrophil and macrophage infiltration play a critical role in mediating cardiac dysfunction during sepsis/septic shock [23]. Infiltrated neutrophils release inflammatory cytokines, including TNF-α and IL-1β which are the important suppressors of cardiac function. Activated macrophages also release chemokines such as MCP-1 and KC, which attract neutrophils into the myocardium. In addition, macrophages release macrophage inhibitory factor (MIF) which contributes to cardiac dysfunction and correlates with sepsis severity [18]. In the present study, we observed that CLP sepsis resulted in significantly increased numbers of neutrophils and macrophages in the myocardium of WT mice. However, neutrophil and macrophage infiltration into the myocardium was markedly attenuated in septic TLR3$^{-/-}$ mice. It is well known that increased expression of adhesion molecules, such as ICAM-1 and VCAM-1 plays a critical role

in recruitment of neutrophils and macrophages into the myocardium during sepsis/septic shock [24] and are associated with myocardial dysfunction induced by LPS [25-27]. We observed that CLP-sepsis significantly increased expression of ICAM-1 and VCAM-1 in the myocardium of WT mice. In contrast, TLR3$^{-/-}$ deficiency blunted CLP-increased expression of adhesion molecules in the myocardium. When taken together, these data indicate that TLR3 plays a crucial role in sepsis-induced adhesion molecule expression, and neutrophil and macrophage infiltration into the myocardium during sepsis/septic shock [28].

To more critically evaluate this conclusion, we isolated peritoneal macrophages from WT and TLR3$^{-/-}$ mice and examined chemokine secretion following stimulation with TLR ligands. We observed that the TLR3 ligand, Poly I:C markedly increased secretion of MCP-1 and KC in WT macrophages but not in TLR3$^{-/-}$ macrophages, suggesting that TLR3 mediates activation of chemokines following ligand stimulation. Interestingly, LPS, a TLR4 ligand and PGN, a TLR2 ligand, significantly increased secretion of MCP-1 and IP-10 in WT macrophages. However, increased secretion of MCP-1 and IP-10 by LPS and PGN was also significantly attenuated in TLR3$^{-/-}$ macrophages. This may be due to cross talk within the TLR signaling network. Other researcher reported that the levels of peritoneal and tissue cytokine (TNFα) and chemokines (CCL5, CCL3, CXCL10, and MIP-2) were significantly increased in WT mice following CLP, but markedly decreased in TLR3$^{-/-}$ mice [29-31].

We have observed a similar phenomenon in TNFα and IL-1β levels in WT and TLR3$^{-/-}$ mice after CLP. In addition, other study has shown that TLR3$^{-/-}$ macrophages responded normally to the other TLR ligands but did not respond to RNA from necrotic neutrophils [32]. Their observation suggests that RNA released from necrotic neutrophils could be an endogenous ligand that triggers activation of TLR3-mediated signaling.

Competing interests

The authors declare that they have no competing interests.

Ethics Statement

The study was approved by the ethics committees at the University of Mississippi Medical Center in Jackson, USA.

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