Immune Cell Toll-Like Receptor 4 Is Required for Cardiac Myocyte Impairment During Endotoxemia

Samantha A. Tavener, Elizabeth M. Long, Stephen M. Robbins, Krista M. McRae, Holly Van Remmen, Paul Kubes

Abstract—The aim of this study was to investigate the importance of Toll-like receptor 4 (TLR4) signaling on cardiac myocytes versus immune cells in lipopolysaccharide (LPS)-induced cardiac dysfunction. Cardiac myocytes isolated from LPS-treated C57Bl/6 mice showed reduced shortening and calcium transients as compared with myocytes from untreated mice. In addition, LPS-treated C57Bl/6 mice showed impaired cardiac mitochondrial function, including reduced respiration and reduced time of induction of permeability transition. All of the aforementioned cardiac dysfunction was dependent on TLR4, because LPS-treated TLR4-deficient mice did not have reduced myocyte shortening or mitochondrial dysfunction. To evaluate the role of cardiac myocyte versus leukocyte TLR4, LPS was injected into chimeric mice with TLR4-positive leukocytes and TLR4-deficient myocytes. These mice showed reduced myocyte shortening in response to LPS. Myocytes from chimeric mice with TLR4-deficient leukocytes and TLR4-positive myocytes had no response to LPS. In addition, isolated myocytes from C57Bl/6 mice subsequently treated with LPS and serum for various times did not have reduced shortening, despite the presence of TLR4 mRNA and protein, as determined by reverse-transcription polymerase chain reaction and fluorescent-activated cell sorting. In fact, cardiac myocytes had equivalent amounts of TLR4 as endothelium; however, only the latter is responsive to LPS. Furthermore, signaling pathways downstream of TLR4 were not activated during direct LPS treatment of myocytes. In conclusion, TLR4 on leukocytes, and not on cardiac myocytes, is important for cardiac myocyte impairment during endotoxemia.

Key Words: inflammation ■ sepsis ■ neutrophils ■ heart ■ contractility

Gram-negative septicemia continues to elude effective treatment with 50% mortality, translating into the deaths of ≈400 000 North Americans per year.1 One consistent result during the development of sepsis is the corresponding evolution of myocardial dysfunction.2–3 Reduced cardiac contractile function has been observed in septic patients4–5 and experimental animal models of lipopolysaccharide (LPS)-induced sepsis.6 LPS, a cell membrane component shed from Gram-negative bacteria, is vital for the development of septicemia, but how LPS causes myocyte dysfunction remains largely unclear. Two paradigms are possible; the first involves direct activation and depression of myocytes via LPS, whereas the second would involve immune cells (non-myocyte sources) including heart tissue macrophages, mast cells, and infiltrating blood leukocytes (neutrophils and monocytes) responding to LPS and depressing myocyte function. To date, most studies have examined cardiac responses after mice were treated with LPS, with clear evidence that LPS does have myocardial depressive properties. However, whether these were direct effects on the myocyte or indirect effects via nonmyocyte cells remains unclear. Finally, stimulation of cardiac myocytes directly with LPS has resulted in variable results including reduced myocyte shortening and no myocyte impairment.7–10 Clearly, a systematic assessment of the effect of LPS on myocytes versus the immune system in endotoxemia is warranted.

The discovery of the LPS receptor, Toll-like receptor 4 (TLR4), has provided major advances in the field of LPS signaling. TLR4 belongs to the family of Toll receptors that can be activated by LPS, resulting in the activation of multiple signaling pathways. Under septic conditions in which LPS is abundant, TLR4 inappropriately activates the immune system, thereby causing extensive injury. The profound importance of TLR4 as an LPS receptor is highlighted by TLR4-deficient mice being completely resistant to LPS effects. These TLR4-deficient animals do not demonstrate characteristic signs of endotoxic shock on exposure to
LPS;\textsuperscript{11,12} no early neutropenia, no leukocyte infiltration into organs, and, most importantly, no detectable mortality is noted.

TLR4 is expressed on the leukocytes and cardiac myocytes; therefore, it is uncertain which cell type is truly responsible for the myocyte dysfunction. Because resident macrophages and mast cells, and any infiltrating leukocytes, express TLR4 and are equipped with cytotoxic mediators, these cells may play a key role in myocyte impairment. However, because cardiac myocytes also express TLR4, LPS could directly alter myocyte function. Studies have shown that myocyte TLR4 is functional in failing myocardium,\textsuperscript{13} myocarditis, where TLR4 correlates to enteroviral replication and cardiac dysfunction,\textsuperscript{14} and cytokine production within the heart of endotoxemic mice.\textsuperscript{15} Interestingly, none of these studies has addressed which TLR4-positive cell types are responsible for the observed cardiac dysfunction. Even more surprising is the fact that the importance of TLR4 in LPS-induced myocyte impairment as it pertains to myocytes versus immune cells has not been elucidated. Determining the importance of both cell types, leukocytes and cardiac myocytes, may clarify the complex attack bestowed on cardiac myocytes during sepsis.

This study systematically addresses the importance of TLR4 in LPS-induced myocyte impairment. LPS-treated C57BL/6 mice demonstrate a profound reduction in myocyte shortening and mitochondrial function as compared with saline-treated C57BL/6 and LPS-treated TLR4-deficient mice. In vitro LPS treatment of cardiac myocytes did not reduce myocyte shortening in the same time period. Generation of chimeric mice, with either TLR4-positive myocytes or TLR4-positive leukocytes, demonstrated that TLR4-positive leukocytes are responsible for the myocyte dysfunction observed during sepsis.

**Materials and Methods**

**Animals and Experimental Protocol**

Male C57BL/6 mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada), and male TLR4-deficient mice (C57Bl/10ScN) and a limited number of C3H/HeJ mice (point mutation in the TLR4 gene) were purchased from The Jackson Laboratory (Bar Harbor, Me). All 185 mice were maintained in a pathogen-free facility until 6 to 16 weeks old, at which time the mice were used. Mice received 10 mg/kg LPS intraperitoneally to induce endotoxemia, a dose demonstrated to ensure cardiac dysfunction without any mortality.\textsuperscript{16} After 4 hours, mice were anesthetized and hearts excised for myocyte isolation. All experimental protocols were reviewed and approved by the University of Calgary Animal Resource Center and conform to the guidelines established by the Canadian Council for Animal Care.

**Bone Marrow Transplants for Development of TLR4 Chimeric Mice**

Briefly, bone marrow chimeras were generated following a standard protocol previously described by our laboratory.\textsuperscript{17,18} Bone marrow was isolated from mice euthanized by spinal cord dislocation. Recipient mice were irradiated with 2 doses of 500 rad (Gammacell 40 \textsuperscript{137}Cs γ-irradiation source), with an interval of 3 hours between the first and second irradiations (previously shown to destroy 99% of existing bone marrow cells). Next, 8×10^{6} donor bone marrow cells were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 8 weeks to allow full humoral reconstitution. This protocol has previously confirmed that ~99% of leukocytes in the recipient were from donor bone marrow as assessed using Thy1.1 and Thy1.2 congenic mice.\textsuperscript{17} In addition, we recently reported that tissue macrophages were also entirely of donor origin.\textsuperscript{18}

**Murine Ventricular Myocyte Isolation**

Ventricular myocytes were isolated as previously described\textsuperscript{20} and, unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Briefly, 6- to 16-week-old male septic (LPS, *Escherichia coli* serotype 0127:B8, 10 mg/kg intraperitoneally, 4 hours), wild-type (C57Bl/6), TLR4-deficient, and TLR4 chimeric mice were anesthetized with methoxyflurane (Metafane; Janssen Pharmaceuticals). Mice were then cervically dislocated and hearts were removed and placed into ice-cold Tyrodes buffer (in mmol/L: 139.9 NaCl, 5.4 KCl [BDH Inc], 1.0 NaHPO₄ [BDH Inc], 5.0 HEPES, 10.0 glucose, 1.0 MgCl₂ containing 1 mmol/L CaCl₂). Hearts were then cannulated via the aorta for retrograde perfusion of the coronary arteries with Tyrodes buffer containing 1 mmol/L CaCl₂ at 2 mL/min for 5 minutes at 37°C and subsequently perfused with buffer containing no CaCl₂ for 5 minutes. Final perfusion buffer contained 40 µmol/L CaCl₂, 20 µg/mL collagenase (Yakult Pharmaceuticals Co), and 4 µg/mL protease (type XIV bacterial) for 8 to 10 minutes. Ventricles were then minced and placed in Tyrodes buffer containing 500 µmol/L collagenase, 100 µg/mL protease, and 2.5% bovine serum albumin and placed in a shaking water bath for 10 to 20 minutes to obtain a suspension of individual myocytes. Myocytes were placed in Dulbecco modified Eagle medium (Gibco) and were used within 4 hours.

**Cell Shortening**

The mechanical properties of the myocytes were measured using a video-based edge-detection system (IonOptix).\textsuperscript{20} Isolated myocytes were allowed to adhere to a glass coverslip in a Warner chamber, which was mounted on the stage of an inverted microscope. Myocytes were perfused with 1 mL/min Tyrodes buffer and field stimulated at 1 Hz using a threshold voltage +10%. Shortening/relenmenthing was recorded with the soft-edge software (IonOptix Corporation). Nons spontaneously contracting myocytes were selected randomly but had to respond to electrical stimulation and have increased shortening in response to a β-adrenergic agonist, isoproterenol (0.1 µmol/L, Sigma).

**Calcium Transient Measurements**

Isolated cardiac myocytes were labeled with 0.5 µmol/L Fura-2 AM (Molecular Probes) for 15 minutes. Perfusion and pacing of cardiac myocytes were performed as described previously and measurements recorded with the Ionoptix Soft Edge and Fluorescence System.

**Mitochondrial Isolation From the Heart**

Mitochondria were isolated as described by Van Remmen et al.\textsuperscript{21} Hearts were homogenized in ice-cold homogenization buffer containing (in mmol/L) 225 mannitol, 75 sucrose, 1 EGTA, and 10 HEPES, and 0.02% protease (type 1: crude), with the use of a glass homogenizer and glass pestle (Glas-Col). After homogenization, 0.5% bovine serum albumin was added to the homogenate and centrifuged at 1000g for 5 minutes to remove cell debris. The resulting supernatant was centrifuged at 1000g for 5 minutes to isolate the mitochondrial pellet. This pellet was washed twice by resuspending in homogenization buffer without EGTA and centrifugation at 10 000g for 5 minutes. Protein concentrations were determined by using the Bradford assay from Bio-Rad.

**Mitochondrial Respiration**

Oxygen consumption was measured using an oxygen monitor (YSI Incorporated) equipped with an electrode as described by Van Remmen.\textsuperscript{21} Experiments were conducted at 30°C in a 2-mL chamber containing 500 µg of mitochondrial protein in a respiration buffer composed of (in mmol/L) 250 sucrose, 10 K,HPO₄, 1 EGTA, and 10 Tris-HCl (pH 7.4), containing different substrates. Respiration rates were measured using substrates that enter the electron transport chain.
Mitochondrial Permeability Transition

Mitochondrial permeability transitions are characterized by an increase in the permeability of the mitochondrial inner membrane to small ions and molecules, which can result in the collapse of mitochondrial membrane potential (Δψm). A consequence of permeability transitions is the loss of matrix components from the mitochondria, resulting in mitochondrial swelling. Induction of the permeability transitions can be measured by a decrease in mitochondrial absorbance at 540 nm as described by Van Remmen. Mitochondrial protein (1 mg) was resuspended in 1 mL of buffer containing (in mmol/L): 215 mannitol, 71 sucrose, 3 HEPES (pH 7.4), and 5 succinate. The solution was maintained at 25°C and allowed to equilibrate for 30 seconds. The following inducers were added to the aforementioned buffer to induce the permeability transition: 400 μmol/L calcium chloride or 75 μmol/L t-butylhydroperoxide (t-BuOOH; generates oxidative stress). The decrease in absorbance was observed until the absorbance value stabilized. The time in seconds at which one-half of the absorbance (1/2) is lost was used to measure the induction of the permeability transition, as described previously.

TLR4 Identification With Reverse-Transcription Polymerase Chain Reaction

Reverse-transcription polymerase chain reaction (PCR) was used to determine whether isolated myocytes expressed TLR4 RNA. RNA was extracted using RNeasy kit (Qiagen Inc) and total RNA (100 ng) was used as template for one-step reverse-transcription PCR reaction (Qiagen Inc.). TLR4 primers used for the reaction were (5′→3′): sense ATTCCTGCAATGGTCAAAGG and anti-sense ACAATTCCACCTGGTGCCCTC. GAPDH cDNA was co-amplified as an internal control using the following primer sequences (5′→3′): sense and anti-sense, respectively, CGGAGTCAACGGATTTGGTCGTAT and AGCCCTTCTCATTGTTGGTAGAACG. The conditions for one-step reverse-transcription PCR reaction were as follows: cDNA synthesis 50°C for 30 minutes, 95°C for 15 minutes; PCR reaction 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and repeated for 35 cycles. PCR products were run on a 2% agarose gel containing 0.5 μg/mL ethidium bromide. The bands were visualized and printed using FluorS Multiimager gel documenting system (BioRad).

Flow Cytometry

Fluorescent-activated cell sorting (FACS) was used to measure the expression of TLR4 on cardiac myocytes. Myocytes were isolated and fixed with Optylse B solution according to manufacturers’ directions. IgG1a-PE was used as an isotype control to TLR4-PE (MTS510, Santa Cruz). After 30 minutes, cells were washed and fluorescence was measured on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). In additional experiments, endothelial cells were isolated from mice as previously described, and stained for TLR4. This was performed as a positive control because the endothelial cells have similar levels of TLR4 as myocytes, and endothelial cells are well-known to respond robustly to LPS.

Statistical Analysis

All results are expressed as mean±SEM. A 2-tailed t test was used for all analysis. Statistical significance was set at P<0.05.
membrane becomes damaged, then the function of the electron transport chain within this membrane may be interrupted. Figure 3 illustrates the effect of LPS treatment on mitochondrial permeability transition. Saline-treated wild-type and TLR4-deficient mice have mitochondria with a $t_{1/2}$ of 1015±70 seconds when exposed to CaCl$_2$ (Figure 3, first bar). LPS-treated wild-type mice have altered mitochondrial inner membranes as indicated by a $t_{1/2}$ of 670±117 seconds in response to CaCl$_2$. LPS-treated TLR4-deficient mice have mitochondria with a $t_{1/2}$ of 980±76 seconds in response to CaCl$_2$, demonstrating that these mitochondria have intact inner membranes. Figure 3 also shows that addition of the

Figure 1. A, Ventricular myocyte shortening of saline and LPS-treated C57Bl/6 (wild-type [WT]) and TLR4-deficient (TLR4$^{-/-}$) mice. Control mice represent both saline-treated WT and TLR4-deficient mice, because there was no difference between these groups. B, LPS responses in myocytes from C3H/HeN or C3H/HeJ mice. C, Calcium flux in myocytes from mice treated with either saline or LPS. *$P<0.05$ relative to saline controls. n=minimum of 2 mice or 5 myocytes.

Figure 2. Respiratory control ratio (RCR) in saline and LPS-treated C57Bl/6 (WT) and TLR4-deficient (TLR4$^{-/-}$) mice. Complex I (A) and complex II (B) of the electron transport chain were examined. Control is represented as both untreated C57Bl/6 and TLR4-deficient mice, because there was no difference between these groups. *$P>0.05$ as compared with saline-treated. n=minimum number of 3 mice.
oxidant, t-butyl hydroperoxide (a TLR4-independent cytotoxic agent) as a positive control, caused similar levels of dysfunction in both wild-type and TLR4-deficient mice (bars 4 and 5). Noteworthy is the fact that optimal levels of t-butyl hydroperoxide caused a similar decrease in the permeability transition as LPS (in wild-type mice), suggesting that LPS is causing potent mitochondrial dysfunction.

**LPS Does Not Directly Affect Myocytes**

All of the aforementioned cardiac dysfunction was dependent on TLR4, because LPS-treated TLR4-deficient mice do not exhibit reduced myocyte shortening or increased mitochondrial dysfunction (Figures 1 through 3). TLR4 is found on myocytes\(^1\) and resident leukocytes and leukocytes that infiltrate the heart during endotoxemia. However, it remains unknown whether the cardiac dysfunction is a direct effect of TLR4 on cardiac myocytes or an indirect effect of the immune cells. To evaluate if TLR4 on cardiac myocytes could reduce myocyte shortening, a series of in vitro experiments were performed. Myocytes were isolated from wild-type mice and subsequently treated with LPS and serum for various times (Figure 4). The control serum-treated myocytes have shortening of 4.2±0.7%, whereas LPS-treated myocytes have comparable shortening of 5.4±0.7%. At no time during up to 4 hours of LPS treatment was myocyte shortening depressed. Deterioration of both LPS-treated and untreated myocyte function was noted. Clearly, 4 hours was the limitation of time that we could perform these experiments. Evidently, LPS does not directly impair cardiac myocyte function. Tumor necrosis factor (TNF)-α treatment was selected as a positive control (Figure 4). Within only 1 hour of TNF-α treatment, myocyte shortening was reduced by >50% (Figure 4A). Figure 4B shows that TNF-α could also decrease myocyte shortening in TLR4-deficient mice. Furthermore, addition of TNF-α to myocytes that were obtained from mice treated with LPS caused further myocyte dysfunction, suggesting some difference in mechanism of action of LPS versus TNF-α (data not shown).

Although dysfunction per se was not noted, we sought to determine whether any signaling downstream of TLR4 could be detected in these in vitro LPS-treated myocytes. However, phospho-p38 MAPK, phospho-JNK, and phosph-P44/42 kinase were not detected to be increased in LPS-treated myocytes via Western blotting during the first hour (data not shown).

**Leukocytes Mediate TLR4-Dependent Myocyte Dysfunction**

To further investigate the role of TLR4 on cardiac myocytes versus leukocytes, mice that were chimeric for TLR4 were generated by bone marrow transplantation and myocyte shortening was measured (Figure 5). These chimeric mice either express TLR4 exclusively on leukocytes or are missing TLR4 on their leukocytes. The mice that have TLR4-deficient leukocytes have TLR4-positive myocytes. The saline-treated group includes data from wild-type and both chimeric mice because no differences were noted. LPS-treated wild-type mice and mice that expressed TLR4 on their leukocytes (but not their myocytes) demonstrated significantly reduced myocyte shortening to very similar levels. Clearly, myocyte contractile impairment occurred regardless of the presence or absence of TLR4 on myocytes. Importantly, the LPS-treated chimeric mice having TLR4-deficient leukocytes (but TLR4 on myocytes) have contractile function that was not significantly reduced from the saline-treated group (Figure 5). These data illustrate that TLR4 must be present on leukocytes to cause myocyte dysfunction during endotoxemic conditions.
Expression of TLR4 mRNA and Protein on Cardiac Myocytes

The presence of TLR4 RNA and protein on cardiac myocytes was confirmed using reverse-transcription PCR and FACS analysis, respectively. RNA was extracted from wild-type, TLR4-deficient, and chimeric myocytes, and subsequent reverse-transcription PCR for TLR4 was performed (Figure 6). The expression of TLR4 was noted in murine leukocytes (lane 1), wild-type myocytes (lane 2), and myocytes from chimeric mice having TLR4-positive myocytes (lane 4). The absence of a band for TLR4 RNA was observed in TLR4-deficient mice (lane 3) and myocytes from chimeric mice having TLR4-deficient myocytes (lane 5). A FACS analysis illustrates positive expression of TLR4 protein on the cell surface of cardiac myocytes (Figure 7A). Although the argument could be made that a reasonably low expression of TLR4 on myocytes was the reason for the lack of response, we demonstrated that endothelial cells have similar low levels of TLR4 expression (Figure 7B), despite well-characterized responses to LPS.\(^{22,23}\) In fact, investigators have reported that dendritic cells also known to respond to LPS have TLR4 expression below the level of detection.\(^{24}\) We permeabilized myocytes to examine intracellular staining of TLR4 and performed Western blots on cardiac myocytes with the antibody H-80 under many different conditions. However, both results were ambiguous with intense nonspecific binding and numerous nonspecific bands (data not shown).

Discussion

Cardiac dysfunction contributes significantly to the morbidity associated with sepsis; however, the molecular mechanisms involved in the myocyte impairment are not well-identified. LPS has been identified as a very important component of the septic response. In this study, administration of LPS alone effectively mimicked the cardiac depression in septic patients and was used as a model to study the mechanisms that may contribute to the myocardial depression. Our data demon-
strated that TLR4 was an essential molecule for LPS-induced myocyte impairment. Interestingly, our results also reveal a profound impairment in mitochondrial function assessed in three separate ways including reduced respiratory control ratios and rapid permeability transitions. The mitochondrial dysfunction was downstream of TLR4 activation because mice lacking TLR4 had no impairment in mitochondrial function. Although TLR4 is expressed on both cardiac myocytes and leukocytes, our data suggest that all of the myocardial impairment observed occurred indirectly via bone marrow-derived leukocytes rather than myocytes per se.

Although there is little doubt that LPS does reduce the contractile function of the heart leading to reduced left ventricular pressure, rates of pressure generation, and rates of relaxation, whereas a direct effect of LPS on cardiac myocytes is more ambiguous. A group has reported that LPS does not directly decrease myocyte shortening, whereas other groups reported that LPS does not directly decrease myocyte shortening. Although at first glance these results may appear to be discrepant, a closer look reveals that there may be a simple explanation. For example, Yasuda and Lew reported that 6 hours of LPS treatment can directly reduce rabbit myocyte shortening. However, the myocyte impairment was only ∼3%, and the authors examined ∼100 cells to demonstrate this difference. It is conceivable that if we had used higher numbers of myocytes, then we, too, would have identified a subtle direct effect of LPS on cardiac myocytes. However, in vivo, in the presence of the immune system (neutrophils, mast cells, monocytes, and macrophages), the contractility of myocytes was impaired by a magnitude of >30% to 50%. These data would suggest that bone marrow-derived cells play the major role in endotoxin-induced myocyte dysfunction. A second study that also reported a direct effect of LPS on myocytes used very high concentrations of the endotoxin (1 g/mL) for 18 hours. Although it is difficult to know whether such high levels of LPS would still only function via TLR4, the longer time point could certainly activate TLR4 to cause cytokine synthesis (including TNF-α) and potentially induce myocyte dysfunction. In our preliminary data, the effect of LPS seems to be, at least in part, distinct from TNF-α, because this cytokine further reduced the myocyte dysfunction in response to LPS. Because we were using primary myocytes, our in vitro assay was only viable for the first 4 hours; however, direct myocyte dysfunction may occur with longer exposure times.

Our data would suggest that bone marrow-derived leukocytes, either infiltrating or resident, are responsible for the LPS-induced myocyte dysfunction. This conclusion is based on a number of observations. First, wild-type mice and chimeric mice containing only TLR4-positive leukocytes demonstrated reduced myocyte contractility to the same degree. These chimeric mice had absolutely no TLR4 on their myocytes (Figure 6) or any other parenchymal cells, and yet they had profound contractility impairment. The opposite experiment, wherein only leukocytes were negative, resulted in no response of myocytes to LPS despite ample TLR4 on the myocytes (Figure 6). An argument could be made that resident macrophages were not replaced by the bone marrow procedure and retained their recipient phenotype. However, macrophages of the heart and other organs typically have a mean turnover time of 7 days, and chimeric mice are used 6 to 8 weeks after bone marrow transplantation, ensuring complete turnover. We recently documented complete turnover of macrophages in these chimeric mice. Thus, the chimeric mice containing TLR4-positive leukocytes would almost certainly contain macrophages in the heart, and either or both cell types responded to LPS and cause myocyte dysfunction. Even if the resident immunocytes had retained the recipient phenotype, it would not affect the conclusion that the leukocytes not myocytes were responsible for the cardiac depression. Actually, we could not distinguish which leukocytes (neutrophils, macrophages, etc) were affecting the myocytes. Although other literature suggests that blocking neutrophil infiltration preserves cardiac function in LPS-induced sepsis models, our own preliminary data using neutrophil depletion are not consistent with the view that infiltrating leukocytes and, more importantly, neutrophils affect myocyte function (Kubes and Tavener, unpublished observation). Clearly, a systematic assessment of the resident cells is warranted.

Surprisingly, no one to our knowledge to date has directly examined the importance of TLR4 on LPS-induced myocyte impairment. In fact, other functions for TLR4 have been identified in the myocardium unrelated to LPS per se. Interestingly, TLR4 has been implicated in a number of nonseptic cardiac diseases, including failing myocardium and myocarditis, in which enteroviral replication and TLR4 correlates to cardiac dysfunction. These studies suggested that in certain scenarios, TLR4 on cardiac myocytes can play a role in the pathology of disease. To date, a single study has shown that TLR4 was involved in cytokine production within the heart of endotoxemic mice. Whether the cytokines affected myocyte biology was not addressed. Moreover, it remained unclear which cell type within the heart was responsible for increased cytokine production during endotoxic shock. Therefore, our study, for the first time to our knowledge, specifically identifies TLR4 on leukocytes as an important player in myocardial dysfunction associated with endotoxin.

It would make sense that all cells would be able to detect infection to recruit the immune system. However, our own data suggest that the major intracellular signaling pathways downstream of TLR4 on myocytes (p38 MAPK, JNK, and P44/42 kinase), were not activated by LPS. One possibility is that the sophistication and complexity of the mammalian immune system with sentinel cells (mast cells, macrophages) has made the need for cardiomyocytes as detectors obsolete. Alternatively, the myocyte system could be a back-up system that is activated only after either prolonged exposure (ie, >4 hours) or higher concentrations of LPS. Finally, negative regulatory molecules like Tollip might inhibit TLR4 signaling in these cells.

In conclusion, this study is the first to our knowledge to identify the importance of TLR4 in myocyte impairment during endotoxemia. In addition, this work also demonstrates that TLR4 on the leukocytes is more important than TLR4 on cardiac myocytes in LPS-induced myocyte dysfunction. We show that TLR4 is required to cause myocyte and mitochondrial dysfunction in endotoxemic mice, but direct LPS treat-
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ment of myocytes does not reduce myocyte shortening, despite TLR4 expression on cardiac myocytes. This work clearly demonstrates that leukocytes, and not cardiac myocytes, are responsible for the LPS-induced myocyte dysfunction.

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