Toll-like Receptor 4 Signaling Confers Cardiac Protection against Ischemic Injury via Inducible Nitric Oxide Synthase- and Soluble Guanylate Cyclase-dependent Mechanisms

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ABSTRACT

Background: Prior administration of a small dose of lipopolysaccharide confers a cardiac protection against ischemia–reperfusion injury. However, the signaling mechanisms that control the protection are incompletely understood. We tested the hypothesis that Toll-like receptor 4 (TLR4) mediates the ability of lipopolysaccharide to protect against cardiac ischemia–reperfusion injury through distinct intracellular pathways involving myeloid differentiation factor 88 (MyD88), TIR–domain-containing adaptor protein-inducing interferon-β–mediated transcription factor (Trif), in-

What We Already Know about This Topic

• Emerging evidence indicates that Toll-like receptors (TLRs) may play an important role in tissue injury in various organs, including the heart, lung, brain, and liver

What This Article Tells Us That Is New

• In mice, TLR4 activation confers a potent cardiac protection against ischemia–reperfusion injury via a myeloid differentiation factor 88 (MyD88)-dependent, but TIR–domain-containing adaptor protein-inducing interferon-β–mediated transcription factor (Trif)-independent mechanism
• The inducible nitric oxide synthase/soluble guanylate cyclase (iNOS/sGC) pathway is critical for the TLR4-elicited cardiac protection

Emerging evidence indicates that Toll-like receptors (TLRs) may play an important role in tissue injury in various organs, including the heart, lung, brain, and liver. In mice, TLR4 activation confers a potent cardiac protection against ischemia–reperfusion injury via a myeloid differentiation factor 88 (MyD88)-dependent, but TIR–domain-containing adaptor protein-inducing interferon-β–mediated transcription factor (Trif)-independent mechanism. The inducible nitric oxide synthase/soluble guanylate cyclase (iNOS/sGC) pathway is critical for the TLR4-elicited cardiac protection.

Methods: Wild-type mice and genetically modified mice, that is TLR4-deficient (TLR4−/−), TLR2 knockout (TLR2−/−), MyD88−/−, Trif−/−, iNOS−/−, and sGCα1−/−, were treated with normal saline or 0.1 mg/kg lipopolysaccharide intraperitoneally. Twenty-four hours later, isolated hearts were perfused in a Langendorff apparatus and subsequently subjected to 30 min global ischemia and reperfusion for as long as 60 min. Left ventricular function and myocardial infarction sizes were examined.

Results: Compared with saline-treated mice, lipopolysaccharide-treated mice had markedly improved left ventricular developed pressure and dP/dt max (P < 0.01) and reduced myocardial infarction sizes (37.2 ± 3.4% vs. 19.8 ± 4.9%, P < 0.01) after ischemia–reperfusion. The cardiac protective
effect of lipopolysaccharide was abolished in the TLR4−/− and MyD88−/− mice but remained intact in TLR2−/− or Trif−/− mice. iNOS−/− mice or wild-type mice treated with the iNOS inhibitor 1400W failed to respond to the TLR4-induced nitric oxide production and were not protected by the lipopolysaccharide preconditioning. Although sGCα1−/− mice had robust nitric oxide production in response to lipopolysaccharide, they were not protected by the TLR4-elicited cardiac protection.

Conclusions: TLR4 activation confers a potent cardiac protection against ischemia–reperfusion injury via a MyD88-dependent, but Trif-independent, mechanism. iNOS/sGC are essential for the TLR4-induced cardiac protection.

Toll-like receptors (TLRs) represent the first line of host defense against microbial infection and play a pivotal role in both innate and adaptive immunity. TLRs recognize invading pathogens through the “molecular pattern recognition,” transduce the signals via distinct intracellular pathways involving a unique set of adaptor proteins and kinases, and ultimately lead to activation of transcription factors and inflammatory responses. All TLRs, except TLR3, signal through the common myeloid differentiation factor 88 (MyD88)-dependent pathway. TLR3 signals exclusively, and TLR4 partly via MyD88-independent but TIR–domain-containing adaptor protein–inducing interferon−β–mediated transcription factor (Trif)–dependent pathway. Animal studies have indicated that these receptors are in part responsible for cardiac dysfunction in certain pathologic conditions characterized in either Gram-negative or Gram-positive bacterial infection, such as endotoxemia, peptidoglycan-associated lipoprotein, Staphylococcus aureus, and in polymicrobial peritonitis. In addition to their role in mediating cardiac dysfunction in septic conditions, emerging evidence indicates that TLRs may also play an important role in noninfectious tissue injury in various organs, including the heart and liver. For example, in the heart systemic deficiency of TLR2, TLR4, or MyD88 leads to attenuated myocardial inflammation, smaller infarction size, and better preserved ventricular function after transient ischemic injury. These loss-of-function studies suggest that the intrinsic TLR signaling may contribute to myocardial inflammation and injury during ischemia–reperfusion (I/R).

Intriguingly, a large body of evidence in several animal models demonstrates that previous administration of sublethal dose of lipopolysaccharide confers a “preconditioning-like” effect (review: ref. 5), similar to the well-characterized ischemic preconditioning or anesthetic preconditioning, protecting the heart against subsequent lethal I/R injury. The cardioprotective effect of lipopolysaccharide usually occurs between 12 and 24 h after administration of lipopolysaccharide and is abolished by cycloheximide, a protein synthesis inhibitor that blocks translation elongation, suggesting a mechanism involving de novo synthesis of cardioprotective proteins. Lipopolysaccharide is known to enhance production of inducible nitric oxide synthase (iNOS) in the heart, a process mediated via TLR4. We have previously demonstrated, in an in vitro system, that TLR4 activation by lipopolysaccharide leads to a survival benefit in isolated cardiomyocytes through MyD88- and iNOS-dependent mechanisms. iNOS has also been proposed to be the trigger for the lipopolysaccharide-induced preconditioning. However, given the multiple systemic reactions in response to in vivo administration of lipopolysaccharide in these animal studies, it is unclear whether the observed cardiac benefits are the direct results of TLR4 stimulation or attributable to other events secondary to systemic TLR4 activation. Therefore, the critical role of TLR4 and its downstream signaling events in the cardiac protection in vivo remain unclear.

Here, we tested the hypothesis that TLR4 specifically mediates the lipopolysaccharide-elicited cardiac protection against I/R injury through distinct intracellular pathways involving MyD88, Trif, iNOS, and soluble guanylate cyclase (sGC). Using genetically modified mice and an ex vivo model of I/R injury, we demonstrate that TLR4-MyD88 signaling confers a potent cardiac protection against I/R injury via iNOS- and sGC-dependent mechanisms.

Materials and Methods

Animals

C57BL/6J, C57BL/10ScSn, iNOS−/−/C57BL/6J, and TLR4−/− mice (C57BL/10ScCr) were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/10ScCr is also referred to as C57BL/10ScNJ (Stock No. 003752) with wild-type (WT) Il12rb2 allele. C57BL/10ScCr mice have a deletion of the Tlr4 gene that results in absence of both mRNA and protein and thus in defective response to lipopolysaccharide stimulation. Tlr4−/− differs from the Tlr4−/− mutant of C3H/HeJ mice, a point mutation of Tlr4 gene that causes an amino acid substitution. C57BL/10ScSn mice were used as the appropriate WT controls for the TLR4−/− mice. TLR2−/− mice were generated by Takeuchi et al., MyD88−/− mice were generated by Kawai et al. and had been backcrossed more than 10 generations into the C57BL/6J strain. Trif−/− mice were generated by Yamamoto et al. and sGCα1−/− mice were generated on the 129S6 background (sGCα1−/−/S6) and were backcrossed at least eight generations into a C57BL/6J background (sGCα1−/−/B6). sGCα1−/−/B6 mice carry a targeted deletion of the sixth exon of the gene encoding sGCα1, resulting in the expression of a mutant, catalytically inactive protein. All mice used in the study were 8–12-week-old, male (except sGCα1−/−/B6) and weighed between 20 and 30 g. Mice were fed with the same bacteria-free diet (Prolab Isopro RMH 3000, LabDiet, Brentwood, MO) and water, and housed in accordance with guidelines from the American Association for Laboratory Animal Science (Memphis, TN). All animal protocols used in the study were approved by the

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Subcommittee on Research Animal Care of the Massachusetts General Hospital (Boston, Massachusetts).

**Lipopolysaccharide Treatment**

WT and the genetically modified mice were treated with normal saline or lipopolysaccharide (Escherichia coli 0111:B4; Sigma, St. Louis, MO) at the dose of 0.1 mg/kg by intraperitoneal injection 24 h before the I/R protocol. Lipopolysaccharide was dissolved in normal saline and diluted to a final concentration of 0.02 mg/ml for intraperitoneal injection. Baseline left ventricular (LV) function was examined with echocardiography and catheter-based LV pressure measurement in both normal saline- and lipopolysaccharide-treated mice before the *ex vivo* I/R protocol. To test the effect of iNOS inhibition, WT C57BL/6J mice were intraperitoneally injected with 1400W (20 mg/kg) (Enzo Life Sciences International, Plymouth Meeting, PA) 2 h before lipopolysaccharide treatment. 1400W or N-(3-amino)-omethylbenzyl)acetamidine is a slow, tight-binding, and highly selective inhibitor of iNOS.29 It is at least 5,000- and 1,000-fold selective for iNOS versus endothelial NO synthase (eNOS) *in vitro* assay and in isolated rat aortic rings, respectively. Inhibition of neuronal NO synthase (nNOS) and eNOS by 1400W was weaker and rapidly reversible. In the original *in vivo* study, 1400W at 5 mg/kg inhibited lipopolysaccharide-induced vascular leakage by 94%.29

**Echocardiographic Measurements**

Twenty-four hours after lipopolysaccharide administration, mice were lightly anesthetized with ketamine (20 mg/kg). Transthoracic echocardiographic images were obtained and interpreted by an echocardiographer blinded to the experimental design using a 13.0-MHz linear probe (Vivid 7; GE Medical System, Milwaukee, WI) as described previously.10,16 M-mode images were obtained from a parasternal short-axis view at the midventricular level with a clear view of papillary muscle. LV end-diastolic internal diameter (LVID _ED_ ) and LV end-systolic internal diameter (LVID _ES_ ) were measured. Fractional shortening (FS) was defined as [(LVID _ED_ − LVID _ES_ )/LVID _ED_ ] × %. Ejection fraction (EF) was calculated by the formula (EDV − ESV)/EDV × %, in which EDV was defined as 7 × LVID _ED_ 3/(2.4 + LVID _ED_ ) or area-length method in two-dimensional long axis view, ESV was defined as 7 × LVID _ES_ 3/(2.4 + LVID _ES_ ) or area-length method in two-dimensional long axis view. The values of three consecutive cardiac cycles were averaged.

**Ex Vivo Model of Myocardial Ischemia–Reperfusion Injury**

The aorta was cannulated and the hearts perfused in a Langendorff apparatus as described previously with minor modifications.10,16 Isolated hearts were perfused at a constant pressure of 80 mmHg with modified Krebs-Henseleit buffer containing 118 mM NaCl, 24 mM NaHCO₃, 10 mM glucose, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, and 2 mM pyruvate, pH 7.4, at 37°C. The perfusate was saturated with continuous gas flow consisting of 95% O₂ and 5% CO₂. A balloon was made with wrapped plastic film connected to a PE-50 polyethylene tube. The neck-to-tip distance of the balloon was approximately 6–7 mm. The balloon was inserted into the LV chamber through the mitral valve with an incision in the left atrium and was connected to a pressure transducer (APT300; Harvard Apparatus, Holliston, MA) for continuous measurement of LV pressure. The balloon was inflated with deionized water (15–20 µl) to adjust LV end-diastolic pressure (LVEDP) to 6–10 mmHg. The perfused hearts were then immersed in the perfusate maintained at 37 ± 0.5°C and paced at 420 beats/min with pacing electrodes placed on the right atrium. After 30 min of constant pressure perfusion, the hearts were subjected to 30 min of zero-flow ischemia, followed by reperfusion for 60 min. Pacing was interrupted during ischemia and resumed 3 min after the start of reperfusion. LV function data were recorded continuously on a data acquisition system (PowerLab; AD Instruments, Colorado Springs, CO). LV developed pressure (LVPD) was calculated as the difference between peak systolic pressure and LVEDP. The dP/dt max was calculated as the maximum rate of increase of LVPD. The recovery of the LV function during the reperfusion was expressed as the percentage of the baseline LVPD and dP/ dt max, which were recorded before the ischemic phase.

**Determination of Myocardial Infarction Sizes**

Infarct sizes were determined by triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO) staining as described previously.10,16 The hearts were removed from the Langendorff device at the end of reperfusion. Each heart was sliced into four sections. The myocardial sections were incubated in 1% TTC in phosphate-buffered saline for 15 min at 37°C. The infarct area (white) and noninfarct area (red) from each section were measured using an image analysis program. Ratios of infarct area/left ventricle were calculated and expressed as the percentage of LV (MI/LV × 100%).

**Measurement of Plasma Nitrate/Nitrite**

The plasma nitrate/nitrite was measured according to the manufacturer’s instructions of the NOx fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI). Briefly, the plasma was filtered through 30-kDa molecular weight cutoff Amicon ultracentrifugal filters (Millipore, Billerica, MA); 10 µl appropriately diluted samples were incubated with nitrate reductase and cofactor at room temperature for 2 h to convert nitrate to nitrite. The assay mixture was then incubated with 10 µl DAN reagent in the dark for 10 min. The reaction was stopped by adding 20 µl NaOH solution to the mixture. The fluorescence was measured in a SpectraMax M5 ( Molecular Devices, Sunnyvale, CA) with an excitation of 360 nm and an emission of 430 nm. Nitrite concentrations in the plasma samples were calculated from a standard curve.

**Statistical Analysis**

Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, La Jolla, CA). The distributions of the
continuous variables were expressed as mean ± SE. For LVDP and dP/dt_max data analysis, statistical significance of the difference between two groups (e.g., normal saline vs. lipopolysaccharide or WT vs. knockout) at the different time points was measured by two-way ANOVA with Bonferroni post hoc tests and repeated measurements. Of note, these specific comparisons were made based on a priori hypotheses rather than pure statistical considerations. Paired Student t test was used for echocardiographic data analysis, myocardial infarction (MI) size, and nitrate/nitrite measurements. The null hypothesis was rejected for \( P < 0.05 \).

**Results**

**Low Dose (0.1 mg/kg) of Lipopolysaccharide Pretreatment Improves Left Ventricular Function and Reduces Myocardial Infarction Sizes after Ischemia–Reperfusion Injury Through a TLR4-dependent Mechanism**

Lipopolysaccharide is known for inducing cardiac dysfunction during endotoxemia. In mice, at the doses of 5 mg/kg and 25 mg/kg, lipopolysaccharide induces significant LV contractile dysfunction through a TLR4-dependent mechanism.\(^7\) To test if at a low dose (0.1 mg/kg) lipopolysaccharide induces any LV dysfunction, mice were treated with either normal saline or lipopolysaccharide by intraperitoneal injection. Cardiac function was assessed before and 24 h after the injection by echocardiography (fig. 1 and table 1) and by LV pressure measurement (table 2). As indicated in figure 1 and table 1, 0.1 mg/kg lipopolysaccharide did not affect the LV function, nor did it change the LV chamber sizes. The saline- and lipopolysaccharide-treated mice had similar EF, FS, and LVID before and 24 h after the treatment. We also calculated the EF with the end diastolic/systolic volumes as measured by area-length method in a two-dimensional long-axis view and confirmed that there was no difference between the saline and LPS groups (data not shown).

However, pretreatment with the low dose of lipopolysaccharide conferred a marked cardiac protection against I/R injury. As indicated in figure 2, \( A \) and \( B \), after 30 min of ischemia and 60 min of reperfusion, LV function, as measured by LVDP and dP/dt_max, recovered to 47.6 ± 2.8% and 55.8 ± 4.4% of the baseline, respectively, in WT mice treated with saline (WTBL/10-saline) (\( n = 9 \)/group). In comparison, the hearts isolated from the lipopolysaccharide-treated mice (WTBL/10-lipopolysaccharide) exhibited significantly better LVDP (65.2 ± 5.6% at 60 min, \( P < 0.01 \)) and dP/dt_max (89.8 ± 10.2% at 60 min, \( n = 8, P < 0.05 \))

**Table 1.** Serial Echocardiographic Measurements before and 24 h after Normal Saline or Lipopolysaccharide Administration

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>LPS (0.1 mg/kg)</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>24 h</td>
</tr>
<tr>
<td>HR, beats/m</td>
<td>686 ± 12</td>
<td>698 ± 16</td>
</tr>
<tr>
<td>FS, %</td>
<td>49.8 ± 1.4</td>
<td>52.8 ± 1.9</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>82.4 ± 1.3</td>
<td>85.1 ± 1.7</td>
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<tr>
<td>LVID&lt;sub&gt;ED&lt;/sub&gt;, mm</td>
<td>3.1 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>LVID&lt;sub&gt;ES&lt;/sub&gt;, mm</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.1</td>
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Values are mean ± SE; \( n = 5 \)/group.

FS = fractional shortening; HR = heart rate; LPS = lipopolysaccharide; LVEF = left ventricular ejection fraction; LVID<sub>ED</sub> = left ventricular end-diastolic internal diameter; LVID<sub>ES</sub> = left ventricular end-systolic internal diameter.

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compared with the WTBL/10-saline group. The significant difference between WTBL/10-saline and WTBL/10-LPS was seen as early as 10 min after reperfusion based on Bonferroni post hoc test. In addition, 30 min of ischemia and 60 min of reperfusion resulted in significant MI in WT mice (MI/LV: 37.2 ± 3.4%, n = 8). Lipopolysaccharide pretreatment led to a marked reduction in MI sizes (19.8 ± 4.9%, n = 7, P < 0.05) (fig. 2, C and D). To determine whether TLR4 mediates the lipopolysaccharide-elicited cardiac protection, we tested the lipopolysaccharide preconditioning in the TLR4-deficient mice (TLR4−/−). As indicated in figure 2, LVDP, dP/dt max, and MI sizes after 30 min of ischemia and 60 min of reperfusion were similar in saline and lipopolysaccharide-treated TLR4−/− mice, suggesting the inability of lipopolysaccharide to induce cardiac protection in the TLR4−/− mice. In contrast, similar to their WT controls (C57BL/6J), TLR2+/− mice treated with lipopolysaccharide had improved LV function and reduced MI sizes (fig. 3). It is noteworthy that the two strains of WT mice, TLR4−/− and TLR2+/− mice all had the same baseline LV function before I/R, as demonstrated by LVDP and dP/dt max in the Langendorff system (table 2). Taken together, these data demonstrate that TLR4, not TLR2, specifically mediates the lipopolysaccharide-induced cardiac protection against I/R injury.

**Myeloid Differentiation Factor 88, Not Interferon-β—Mediated Transcription Factor, Is Essential for the TLR4-mediated Cardioprotection against Ischemia–Reperfusion Injury**

MyD88 and Trif are two adaptors that are critical for TLR4 signaling and mediate activation of NF-κB and interferon regulatory factor 3 (IRF3), respectively. To determine whether the two signaling pathways control the TLR4-induced cardioprotection, we tested the lipopolysaccharide preconditioning effect in MyD88−/− and Trif−/− mice. Both MyD88−/− and Trif−/− mice had the same baseline LV function compared with WT mice before I/R (table 2). LV function was similarly impaired in the saline-treated MyD88−/− and Trif−/− mice (fig. 4). Compared with the saline-treated Trif−/− animals, lipopolysaccharide-treated Trif−/− mice had a significant improvement in LVDP and dP/dt max and reduced MI sizes after I/R, a protective effect similar to that seen in the WT controls (WTBL6-LPS) (fig. 4). In contrast, lipopolysaccharide pretreatment failed to improve the LV function or reduce MI size in MyD88−/− mice (fig. 4). These data suggest that MyD88, but not Trif, signaling mediates the lipopolysaccharide-induced cardioprotection against I/R injury.

**Toll-like Receptor 4 Activation Induces Nitric Oxide Production in a Myeloid Differentiation Factor 88-dependent Manner**

TLR4 stimulation induces iNOS induction and activates the nitric oxide-sGC signaling pathway. To determine whether iNOS-NO-sGC signaling mediates the TLR4-induced cardioprotection, we tested whether lipopolysaccharide, at the dose of 0.1 mg/kg, induced nitric oxide production. Nitric oxide has an extremely short life of a few milliseconds in vivo, making direct measurement of nitric oxide particularly challenging. Plasma nitrite and nitrate concentrations, as a measure of nitric oxide production, were measured at 24 h after saline or lipopolysaccharide injection. As indicated in figure 5, lipopolysaccharide induced a 2.7-fold increase in the plasma concentration of NOx of WTBL/6 mice (13.2 ± 1.0 μM vs. 36 ± 4.4 μM, P < 0.01) and a 2.5-fold increase in WTBL/10 mice (14.6 ± 5.0 μM vs. 36.1 ± 6.9 μM, P < 0.05). TLR4 or MyD88 deficiency resulted in an inability of lipopolysaccharide to increase the plasma concentrations of NOx, although the TLR4−/− mice had a higher concentration of baseline NOx than did the WT (C57BL/10) (P < 0.05). Moreover, iNOS−/− mice did not respond to lipopolysaccharide with the plasma NOx at the baseline concentration of the saline group (fig. 5). Importantly, 1400W, a highly selective and potent iNOS inhibitor, blocked nitric oxide induction in the lipopolysaccharide-treat WT mice. These data suggest that a low dose of...
lipopolysaccharide induces nitric oxide production in a TLR4- and MyD88-dependent manner and that either iNOS genetic deletion or inhibition blocks nitric oxide production in response to lipopolysaccharide in vivo.

**Inducible Nitric Oxide Synthase Is Critical for the TLR4-mediated Cardioprotection**

We next tested the impact of iNOS genetic deletion or pharmacological inhibition on the TLR4-mediated cardioprotection. As indicated in figure 6, iNOS genetic deletion (iNOS−/−) abolished the lipopolysaccharide-induced cardiac preconditioning. LV function and MI size were similar between the saline- and lipopolysaccharide-treated iNOS−/− mice (LVDP: 58.7 ± 6.4% vs. 61.3 ± 5.6%; dP/dt max: 68.6 ± 8.1% vs. 70.7 ± 7.0%; MI sizes: 30.4 ± 4.1% vs. 28.8 ± 5.1%, n = 6) (fig. 6). However, iNOS inhibition by 1400W significantly attenuated the ability of lipopolysaccharide to protect against I/R-induced LV dys-function. Although lipopolysaccharide treatment still resulted in a partial improvement in LV function in 1400W-pretreated mice (WTBL/6–1400W-LPS) compared with the saline control (WTB/6–1400W-saline), the LV function recovery was significantly less than that of the lipopolysaccharide-treated mice without 1400W pretreatment (WTBL/6–saline) (P < 0.01). Taken together, these studies demonstrate that iNOS genetic deletion or pharmacological inhibition blocks or inhibits the lipopolysaccharide-induced preconditioning, suggesting that iNOS and nitric oxide contribute to the lipopolysaccharide-induced and TLR4-mediated cardioprotection against I/R injury.

**TLR4-mediated Cardioprotection Is Soluble Guanylate Cyclase-dependent**

Nitric oxide elicits its biologic activity via either cGMP-dependent or cGMP-independent mechanisms. Soluble guanylate cyclase is the primary target of nitric oxide and a
The current study was designed to delineate the intracellular signaling pathway that leads to the lipopolysaccharide-induced cardiac preconditioning against I/R injury. A small dose of lipopolysaccharide (0.1 mg/kg) did not affect the baseline LV function as assessed by serial echocardiography and LV pressure measurements, but it elicited a marked cardiac protective effect against I/R. Using genetically modified mouse strains and an ex vivo model of cardiac I/R injury, we demonstrated that TLR4 mediated the lipopolysaccharide-induced cardiac protection, and MyD88, but not Trif, was essential for the TLR4-induced protection. The small dose of lipopolysaccharide also resulted in a significant increase in the plasma concentration of nitrate/nitrite. The lipopolysaccharide-induced NOx production and cardiac protection were abolished or significantly attenuated in iNOS−/− mice or in WT pretreated with an iNOS inhibitor. Although sGCα1−/− mice responded to lipopolysaccharide with a robust nitric oxide production, they were not protected from I/R injury. Taken together, these data suggest that TLR4 sGCα1−/− mice were not protected by the lipopolysaccharide-elicted preconditioning. As indicated in figure 7, sGCα1−/− treated with lipopolysaccharide had the same concentration of LVDP and dP/dt max and same MI size as did the saline-treated sGCα1−/− controls. It is noteworthy that, in a manner similar to other genetically modified mice, sGCα1−/− mice had the same baseline LV function before ischemia, as demonstrated by LVDP and dP/dt max (table 2).

**Discussion**

The long and short horizontal lines represent the mean ± SE. The number shown in each bar represents the sample size in the group.

*P < 0.05, **P < 0.01, ***P < 0.001.

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**Fig. 4.** Lipopolysaccharide-elicited preconditioning against ischemia–reperfusion (I/R) injury is MyD88-dependent, but interferon-β–mediated transcription factor (Trif)–independent. Wild-type C57BL/6J (WTBL/6), myeloid differentiation factor 88 knockout (MyD88−/−), and TIR–domain-containing adapter protein inducing interferon-β–mediated transcription-factor knockout (Trif−/−) mice were treated with normal saline or 0.1 mg/kg lipopolysaccharide (LPS) intraperitoneally. Twenty-four hours later, mouse hearts were isolated and perfused in a Langendorff system. After 30 min of perfusion, the hearts were subjected to 30 min of no-flow global ischemia, followed by 60 min of reperfusion. (A) Left ventricular developed pressure (LVDP). (B) dP/dt max is expressed as percentage of the baseline. Each data point and error bar in A–B represents the mean ± SE. The number of mice in each group is as follows: WTBL/6–saline: 10; WTBL/6–LPS: 11; Trif−/−–saline: 6; Trif−/−–LPS: 6; MyD88−/−–saline: 7; MyD88−/−–LPS: 7. *P < 0.01, NS is not significant. (C) Myocardial infarct (MI) size is expressed as the percentage of left ventricular area (LV). The long and short horizontal lines represent the mean ± SE. *P < 0.05, **P < 0.01, NS is not significant. (D) Representative heart slices after triphenyltetrazolium chloride staining from Trif−/− and MyD88−/− mice. Viable myocardium was stained red, and infarcted myocardium appeared white.

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**Fig. 5.** Effect of low-dose lipopolysaccharide on the plasma concentrations of nitrite/nitrate in different mouse strains. Mice were treated with normal saline or 0.1 mg/kg lipopolysaccharide (LPS) intraperitoneally. 1400W (20 mg/kg) was given to wild-type C57BL/6J (WTBL/6) mice 2 h before saline or LPS administration. Twenty-four hours later, blood was collected and plasma prepared. Nitrite and nitrate were measured using a fluorometric assay. Each error bar represents the mean ± SE. The number shown in each bar represents the sample size in the group. *P < 0.05, **P < 0.01, ***P < 0.001.

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**Fig. 7.** Concentrations of nitrite/nitrate in different mouse strains. WTBL/6 mice responded to lipopolysaccharide with a robust nitric oxide production, they were not protected from I/R injury. Taken together, these data suggest that TLR4 sGCα1−/− mice were not protected by the lipopolysaccharide-elicted preconditioning. As indicated in figure 7, sGCα1−/− treated with lipopolysaccharide had the same concentration of LVDP and dP/dt max and same MI size as did the saline-treated sGCα1−/− controls. It is noteworthy that, in a manner similar to other genetically modified mice, sGCα1−/− mice had the same baseline LV function before ischemia, as demonstrated by LVDP and dP/dt max (table 2).
activation confers a potent cardiac protection against I/R injury via a MyD88-dependent, but Trif-independent, mechanism. Moreover, iNOS and sGCα1 are proven to be essential for the TLR4-induced cardiac protection (fig. 8).

Cardiac preconditioning is a well-documented phenomenon in which a brief treatment triggers endogenous survival mechanisms that protect the heart against I/R injury. Classic ischemic preconditioning (e.g., a brief period of 5 min) of repeated coronary artery occlusion protects the myocardium against subsequent lethal I/R injury. Inhaled anesthetics also triggers cardiac preconditioning that improves cardiac function and reduced infarction sizes. Similarly, a few TLR ligands, including lipopolysaccharide, monophosphoryl lipid A (MLA), and lipoteichoic acid (a TLR2 ligand), can trigger cardiac protection that mimics the ischemic or anesthetic cardiac preconditioning. Although lipopolysaccharide-induced cardiac protection has been well documented, the exact mechanisms that lead to the protection are not fully understood. Administration of lipopolysaccharide leads to a robust systemic production of various cytokines and chemokines, such as TNFα, IL-1, and IL-6, and hemodynamic disturbance, which can affect cardiac function. Given the systemic responses after in vivo lipopolysaccharide administration, it is unclear whether the observed cardiac benefits in lipopolysaccharide-treated animals are the direct results of TLR4 signaling or attributable to other events secondary to systemic TLR4 activation. The current study has clearly established that TLR4 signaling mediates the lipopolysaccharide-induced cardioprotective effect.

We also demonstrate that the TLR4-mediated preconditioning is MyD88-dependent but Trif-independent. These findings appear consistent with our previous in vitro finding that in isolated cardiomyocytes, direct activation of TLR4 by lipopolysaccharide confers a marked survival benefit in cardiomyocytes against apoptotic cell death during hypoxia and serum deprivation. The TLR4-induced cell survival pathway is mediated via MyD88. MyD88 is a key adaptor protein that is critical for transducing signals from all TLR family mem-

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**Fig. 6.** Effect of inducible nitric oxide synthase (iNOS) deletion or inhibition on the lipopolysaccharide-elicited preconditioning against ischemia-reperfusion (I/R) injury. Wild-type C57BL/6J (WTBL/6), WTBL/6 pretreated with 1400W, and inducible nitric oxide synthase knockout (iNOS−/−) mice were treated with normal saline or 0.1 mg/kg lipopolysaccharide (LPS) intraperitoneally. 1400W was given at the dose of 20 mg/kg to WTBL/6 mice 2 h before saline or LPS administration. Twenty-four hours later, mouse hearts were isolated and perfused in a Langendorff system and subjected to the I/R protocol. (A) Left ventricular developed pressure (LVDP). (B) dP/dt max is expressed as percentage of the baseline. Each data point and error bar in A–B represents the mean ± SE. The number of mice in each group is as follows: iNOS−/−-saline: 7; iNOS−/−-LPS: 8; WTBL/6-1400W-saline: 7; WTBL/6-1400W-LPS: 7. **P < 0.01. NS is not significant. (C) Myocardial infarction (MI) size as expressed as the percentage of left ventricular (LV) area. The long and short horizontal lines represent the mean ± SE. ***P < 0.01. (D) Representative heat slices after triphenyltetrazolium chloride staining from iNOS−/− and WT mice pretreated with 1400W. Viable myocardium was stained red, and infarcted myocardium appeared white.

**Fig. 7.** Effect of soluble guanylate cyclase (sGC)-deficiency on the lipopolysaccharide-elicited preconditioning against ischemia-reperfusion (I/R) injury. sGC knockout (sGCα1−/−) female mice were treated with normal saline or 0.1 mg/kg lipopolysaccharide (LPS) intraperitoneally. Twenty-four hours later, mouse hearts were isolated and perfused in a Langendorff system. After 30 min of perfusion, the hearts were subjected to 30 min of no-flow global ischemia, followed by 60 min of reperfusion. (A) Left ventricular developed pressure (LVDP). (B) dP/dt max is expressed as percentage of the baseline. Each data point and error bar in A–B represents the mean ± SE. The number of mice in each group is as follows: WTBL/6-saline: 10; WTBL/6-LPS: 11; sGCα1−/−-saline: 10; sGCα1−/−-LPS: 11. NS is not significant. (C) Myocardial infarction (MI) size as expressed as the percentage of left ventricle area (LV). The long and short horizontal lines represent the mean ± SE. (D) Representative heart slices after triphenyltetrazolium chloride staining from sGCα1−/− mice. Viable myocardium was stained red, and infarcted myocardium appeared white.
cardiac protection. In contrast, TIR–domain-containing blocks LPS/TLR4-induced NO production and inhibits its soluble guanylate cyclase (sGC)- and cGMP/protein kinase G (PKG)-de-
ducible form of NOS, is considered essential for the late
stimulation and result in the production of type I interferons, and TLR4 signaling pathways that respond to viral and bacterial
However, Trif is a key adaptor protein responsible for TLR3
and interleukin-1 receptor family members. MyD88 signals via IRAK-1 and other downstream
kinases, including IKKβ and IκB, eventually leading to the ac-
tivation of NF-κB and inflammatory cytokine production.3
However, Trif is a key adaptor protein responsible for TLR3 and TLR4 signaling pathways that respond to viral and bacterial stimulation and result in the production of type I interferons, including IFNα, IFNβ, and other IFNs.4 Stimulation of type I interferon pathways leads to induction of a specific set of genes, including chemokines (e.g., CXCL10) and antimicrobial/antiviral response genes.44 The detailed mechanisms by which each of the signaling components lead to the cardioprotection seen in lipopolysaccharide-treated animals need to be further investigated. Nevertheless, the current finding strongly suggests that TLR4-MyD88 signaling, upon being activated, triggers a potent survival mechanism that protects the heart against I/R injury (fig. 8).

NOS and nitric oxide have been implicated as a key in-
tracellular mediator responsible for both ischemic precondi-
tioning and anesthetic preconditioning.45,46 iNOS, the in-
ducible form of NOS, is considered essential for the late
phase of ischemic preconditioning in an in vivo model of I/R injury.45 Moreover, overexpression of iNOS is found sufficient to protect myocardium from I/R injury.47 Interestingly, NOS1 or endothelial NOS, but not iNOS, has been proposed as the trigger for the isoflurane-induced preconditioning in a rabbit model of I/R injury.46 Our finding that TLR4-MyD88 signaling is required for nitrate/nitrite pro-
duction in response to a preconditioning dose of lipopolysaccharide and that iNOS genetic deletion as well as pharma-
ocological inhibition attenuate the TLR4-mediated cardiac
preconditioning strongly suggests that iNOS-nitric oxide is the downstream effector of TLR4 signaling leading to the lipopolysaccharide-elicited preconditioning benefit against I/R. This is consistent with a previous study by Xi et al., who reported that monophosphoryl lipid A-induced preconditioning was abolished in iNOS−/− mice.32 The notion that cardiac TLR4 is directly activated by lipopolysaccharide and subsequently leads to an iNOS-dependent cardiac protection against I/R injury is supported by our previous finding that TLR4 activation in isolated cardiomyocytes leads to a direct antiapoptotic survival pathway that is dependent on endoge-
nous iNOS.21,22 It is noteworthy that although the selective
iNOS inhibitor 1400W completely inhibited the increase in the nitrate/nitrite production in the lipopolysaccharide-treated mice, it only partially blocked the lipopolysaccharide-elicited LV functional improvement. These findings may suggest that in addition to nitric oxide, other factor(s) also contribute to the TLR4-induced and iNOS-dependent cardiac protection.

Nitric oxide may confer myocardial protection by cGMP-
dependent as well as cGMP-independent mechanisms. For example, nitric oxide inhibits caspase-3 activity via S-nitrosylation and thus may promote cell survival in a sGC/cGMP-
dependent independent manner.45 However, nitric oxide can bind to the heme moiety of sGC and activate the enzyme to generate cGMP. sGC is a main downstream effector of nitric oxide and responsible for many nitric oxide-elicited biologic effects. Here we demonstrate that the TLR4-mediated cardio-
protection against I/R injury is indeed dependent on sGCα1. Mice deficient in sGCα1 completely lost the protection con-
ferred by lipopolysaccharide, despite sGCα1−/− mice re-
sponding normally to lipopolysaccharide with a robust nitric
oxide production.

Another endogenous survival pathway, namely phosphatidylinositol-3 kinase (PI3 kinase)/Akt, has been proposed for the lipopolysaccharide-induced cardiac protective effect. We have previously demonstrated that PI-3 kinase/Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), and IkB
kinase β (IKKβ) pathways all contribute to the lipopolysaccharide-induced antiapoptotic survival effect in cardi-
omycocytes.22 Moreover, in vivo, the cardiac benefit of lipopolysaccharide against I/R injury is abolished by a PI-3 kinase inhibitor or in transgenic mice expressing inactive Akt mutant.39

Some limitations of the current study should be acknowl-
edged. First, using the in vivo model, we were not able to

![Fig. 8. Proposed signaling pathway for the Toll-like receptor 4 (TLR4)-mediated cardiac protection against ischemia–rep-
erfusion (I/R). TLR4 activation by its ligand, such as lipopoly-
saccharide (LPS), leads to inducible nitric oxide synthase (iNOS) induction and increased nitric oxide (NO) production through a myeloid differentiation factor 88 (MyD88)-de-
pendent mechanism. NO protects myocardium via soluble guan-
ylate cyclase (sGC) and cGMP/protein kinase G (PKG)-de-
pendent mechanisms. 1400W, a potent iNOS inhibitor, blocks LPS/TLR4-induced NO production and inhibits its cardiac protection. In contrast, Trif-domain-containing adaptor protein-inducing interferon-β-mediated transcription factor (Trif) is not required for the TLR4-mediated cardiac protection.](image-url)
determine whether the cardiac ischemic protection in the lipopolysaccharide-treated mice is result of direct myocardial TLR4 activation. Our previous in vitro data suggest a direct survival benefit of TLR4-MyD88 signaling in cardiomyocytes, but other approaches will be needed, such as cardiac-specific TLR4 knockout or chimera model, to define the specific role of cardiac TLR4 in myocardial protection against I/R injury. Second, lipopolysaccharide is a potent pyrogenic agent and thus clearly not a good candidate to test its therapeutic value in attenuating I/R injury. Nonpyrogenic TLR4 agonists will be needed to further test the efficacy of TLR4 signaling to protect against I/R injury. Finally, the I/R injury model in this study was developed in isolated hearts perfused in a Langendorff apparatus. Although the ex vivo system offers certain advantages, such as relative ease to perform, the ability to control LV preload and after-load, and the ability to continuously measure cardiac contractile function throughout the I/R period, the heart is perfused with cell- and serum-free buffer, so certain neurohormonal factors in response to I/R could have been omitted. In this regard, in vivo I/R injury model involving coronary artery occlusion offers a clear advantage. Both experimental models have been widely used by numerous laboratories including ours.

Nevertheless, the current study establishes that TLR4 activation confers a potent cardiac protection against I/R injury via a MyD88-dependent, but Trif-independent, mechanism. Moreover, iNOS and sGCα1 are proven to be essential for the TLR4-induced cardiac protection (fig. 8). Our study suggests that selective targeting TLR4-MyD88 signaling may represent a novel therapeutic strategy for cardioprotection against ischemic myocardial injury.

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References


