Reduced Myocardial Ischemia-Reperfusion Injury in Toll-Like Receptor 4–Deficient Mice

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**Background**—Myocardial ischemia and reperfusion-induced tissue injury involve a robust inflammatory response, but the proximal events in reperfusion injury remain incompletely defined. Toll-like receptor 4 (TLR4) is a proximal signaling receptor in innate immune responses to lipopolysaccharide of Gram-negative pathogens. TLR4 is also expressed in the heart and vasculature, but a role for TLR4 in the myocardial response to injury separate from microbial pathogens has not been examined. This study assessed the role of TLR4 in myocardial infarction and inflammation in a murine model of ischemia-reperfusion injury.

**Methods and Results**—Myocardial ischemia-reperfusion (MIR) was performed on 2 strains of TLR4-deficient mice (C57/Bl10 ScCr and C3H/HeJ) and controls (C57/Bl10 ScSn and C3H/OuJ). Mice were subjected to 1 hour of coronary ligation, followed by 24 hours of reperfusion. TLR4-deficient mice sustained significantly smaller infarctions compared with control mice given similar areas at risk. Fewer neutrophils infiltrated the myocardium of TLR4-deficient Cr mice after MIR, indicated by less myeloperoxidase activity and fewer CD45/GR1-positive cells. The myocardium of TLR4-deficient Cr mice contained fewer lipid peroxides and less complement deposition compared with control mice after MIR. Serum levels of interleukin-12, interferon-γ, and endotoxin were not increased after ischemia-reperfusion. Neutrophil trafficking in the peritoneum was similar in all strains after injection of thioglycollate.

**Conclusions**—TLR4-deficient mice sustain smaller infarctions and exhibit less inflammation after myocardial ischemia-reperfusion injury. The data suggest that in addition to its role in innate immune responses, TLR4 serves a proinflammatory role in murine myocardial ischemia-reperfusion injury. (*Circulation. 2004;109:784-789.*)

**Key Words:** inflammation • myocardial infarction • ischemia

Reperfusion therapies have substantially impacted the effective treatment of ischemic heart diseases.1,2 Therapeutic progress has been tempered, however, by a host of events termed postsischemic myocardial reperfusion injury (myocardial ischemia-reperfusion [MIR]). A robust local and systemic inflammatory response characterizes MIR that may expand tissue injury and adversely affect left ventricular (LV) recovery.3,4 Disrupting inflammatory processes such as recruitment of neutrophils, production of radical oxygen species, and activation of complement have all modulated MIR injury.4 However, the proximal events that initiate these proinflammatory pathways are not fully elucidated. Indeed, such antiinflammatory strategies have thus far proven disappointing in clinical practice,5,6 emphasizing the importance of additionally understanding the basic mechanisms of MIR-induced inflammation and injury.

The Toll-like receptors (TLRs) serve as pattern-recognition receptors at a proximal step in the innate immune response to microbial pathogens.7 Among these receptors, TLR4 specifies cellular responses to bacterial lipopolysaccharide (LPS) in conjunction with accessory molecules.8 Activation of TLR4 is linked to expression of proinflammatory cytokines and activation of nuclear factor-κB signaling pathways in several cell types.7,9 TLR4 is expressed by cells of myeloid lineage, which are central to innate immune responses, but TLR4 is also expressed in tissues without a recognized immune function, notably the heart and vasculature.10,11 Consistent with its role as a receptor for LPS, cardiac expression of TLR4 is essential for LPS-induced LV dysfunction and myocardial expression of tumor necrosis factor α, interleukin (IL)-1β, and inducible NO synthase.12,13 Expression of tumor necrosis factor α, IL-1, and adhesion molecules vascular cellular adhesion molecule 1 and intracellular adhesion molecule 1 on coronary endothelial cells exposed to LPS is similarly dependent on TLR4.14 These studies support a role for TLR4 in cardiovascular inflammation and dysfunction during bacterial sepsis, but there is no evidence that TLR4 participates in myocardial inflammatory responses...
with a nonmicrobial etiology, like the inflammatory response to ischemia-reperfusion injury. Such a role for TLR4 is suggested by several observations. First, the Toll/IL-1 signaling pathway, specifically TLR2, has been implicated in the proinflammatory response to necrotic cell death. Second, endogenous factors associated with tissue injury, such as heat-shock protein 60, may act as activating ligands for TLR4. Lastly, constitutive expression of TLR4 in the heart increases markedly after experimental ischemic injury. On the basis of these considerations, we investigated whether myocardial infarction and inflammation produced by ischemia-reperfusion differed in mice that lack a functional TLR4 signaling pathway.

Methods

Experimental Animals

The murine strains C57 BL/10 ScCr and C3H/HeJ do not express TLR4 because of naturally occurring mutations in the TLR4 gene. C57 BL/10 ScCr mice were obtained from Dr Peter Tobias of Scripps Research Institute, La Jolla, Calif. The C57 BL/10 ScSn, C3H/HeJ, and C3H/OuJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Animals were housed in accordance with the Harvard Medical Area Standing Committee on Animals in a specific pathogen-free environment.

Murine Myocardial Ischemia and Reperfusion

Mice (12 to 20 weeks old, 22 to 30 g) were subjected to 1 hour of myocardial ischemia and 24 hours of reperfusion, as described previously. Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg body wt). Additional doses were given as needed to maintain anesthesia. Mice were intubated and ventilated with 100% oxygen. Ischemia was achieved by ligating the left anterior descending coronary artery (LAD) using an 8-0 silk suture with a 100% oxygen. Ischemia was achieved by ligating the left anterior descending coronary artery (LAD) using an 8-0 silk suture with a

Assessment of Area at Risk and Infarct Size

To assess the ischemic area at risk, 1% Evans blue was infused into the aorta and coronary arteries in retrograde fashion. Hearts were excised and sliced into 5-mm sections with the aid of an acrylic matrix (Alto Inc). The heart sections were incubated with 1% acrylamide and cut into 10-mm sections. Sections were stained with 15 minutes. Viable myocardium stained red, and infarcted tissue appeared white. The infarct area (I, white), the area at risk (AAR, red and white), and the total LV area from each section were measured using NIH Image (version 1.62) and Spot software. Ratios of AAR/LV and of I/AAR were calculated and expressed as a percentage.

Lipid Peroxidation Assay

Hearts were collected 24 hours after reperfusion and disrupted by Dounce homogenization in 1.0 mL of 20 mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L butylated hydroxytoluene. Lipid peroxides (malondialdehyde and 4-hydroxynonenal) were measured as an index of oxidative stress. Aliquots were assayed using a commercially available kit (catalog No. 437634, Calbiochem).

Myocardial Myeloperoxidase Activity Assay

Myeloperoxidase activity was measured as a marker of myocardial neutrophil infiltration. Frozen myocardial tissues were placed in 0.5 mL of a 50-mmol/L potassium PBS (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide, homogenized, and centrifuged for 15 minutes at 14,000 rpm at 4°C. The supernatants were mixed 1:9 (vol/vol) with 50 mmol/L PBS (pH 6.0) containing 0.2 mg/mL o-dianisidine and 0.0006% hydrogen peroxide; the absorbance change was measured at 30 and 90 seconds at a wavelength of 460 nm. Murine myocardial myeloperoxidase (MPO) activity was normalized using human MPO as a standard.

Immunohistochemistry

Hearts were fixed in methyl Carnoy’s solution at 4°C for 5 hours and then placed in 70% ethanol overnight. Hearts were paraffin embedded and cut into 10-mm sections. Sections were stained with hematoxylin and eosin to assess morphology and evidence of injury. Histological stains included anti-mouse CD45 antibody (Ly-5; 1:1000, Pharmingen), the neutrophil-specific marker GR-1 (Ly-6g; 1:1000, Pharmingen), and goat anti-rat complement-3 (C3, 1:500, Cappel, ICN Pharmaceuticals). Secondary antibodies consisted of biotinylated anti-mouse or anti-goat antibodies (diluted 1:200, Vector Laboratories), followed by incubation with horseradish peroxidase–coupled streptavidin (ABC reagent, Vector Laboratories) and development with 3,3′-diaminobenzidine substrate. Specificity of the primary antibodies was checked by using species and isotype-matched nonimmune antibodies. Sections were counterstained with hematoxylin and mounted for light microscopy. Histological analysis was performed on hearts from 3 mice from each strain.

Serum Concentrations of Interleukin-12 and Interferon-γ

Blood was collected at the time of heart harvest, and serum was prepared and assayed for interleukin-12 and for interferon-γ by Quantikine sandwich ELISA (R&D Systems).

Thioglycollate-Induced Peritonitis

Mice were given a 2-mL intraperitoneal injection of 4% Brewer’s thioglycollate medium (Difco No. 0236-17-7). After 3 hours, mice were anesthetized with pentobarbital, and abdominal infiltrates were collected with 3 washes with PBS. Cells were centrifuged and resuspended in 1.0 mL PBS, and total cell numbers were determined with a hemocytometer. Ratios of granulocyte to monocyte populations were determined from the forward and side-scatter characteristics on flow cytometry.

Serum Endotoxin

Levels of serum endotoxin were measured with a chromogenic Limulus amebocyte lysate assay according to the manufacturer’s directions (BioWhittaker, QCL-1000). Sensitivity of the assay is 0.1 EU/mL.

Statistics

Data are expressed as mean±SEM of n observations. Comparisons of data between Sn versus Cr and between HeJ versus OuJ were made using a 2-sample t test assuming equal variances. Differences were considered statistically significant when P<0.05.

Results

Reduced Infarct Size in TLR4-Deficient Mice

Myocardial ischemia-reperfusion was performed on C57/BL10 ScCr mice, which lack expression of TLR4, and on C57/BL10 ScSn (Sn) mice, which express TLR4 normally. After 1 hour of ligation of the LAD and 24 hours of reperfusion, the extent of myocardial infarction was measured. The LV area affected by LAD ligation, referred to as the area at risk (AAR), was similar between Sn and Cr mice (51±5% versus 53±5%, respectively; P=NS; Figure 1). Despite sustaining equal areas at risk, TLR4-deficient Cr
mice had significantly smaller myocardial infarctions compared with Sn mice, whether normalized to the area at risk or to the LV area (Figure 1).

In addition to a lack of TLR4 expression, Cr mice harbor a point mutation in the IL-12 receptor resulting in impaired microbial-induced production of interferon-γ, a characteristic not shared by other TLR4-deficient murine strains.18 Serum levels of IL-12 are also reported to increase after myocardial infarction in humans.19 Thus, part of the myocardial protection observed in Cr hearts may be linked to defective IL-12 as well as TLR4 signaling. We determined that serum IL-12 levels in Cr mice were similar before and after 1 hour and 24 hours of MIR (Cr before MIR, 19.61±1 pg/mL; Cr after MIR, 23.9±1.9 pg/mL). In addition, IFN-γ, the primary response gene induced by IL-12, was not detected in serum from Cr or Sn mice after MIR (≤2 pg/mL). Although not exclusionary, these data suggest that IL-12/IL-12R signaling is not activated during the course of our experiments. To additionally address this concern, we performed MIR on C3H/HeJ (HeJ) mice, which harbor a distinct point mutation in the conserved TIR signaling domain of TLR4. As shown in Figure 1, HeJ mice also sustained smaller myocardial infarctions compared with the control strain C3H/OuJ (OuJ). Thus, both Cr and HeJ mice that harbor distinct TLR4 mutations sustained less myocardial damage after ischemia reperfusion, as measured by infarct size, than did mice expressing functional TLR4.

As depicted in the Table, body weights did not differ significantly between the murine strains tested. The TLR4-defective Cr mice showed an increased survival rate compared with the Sn control mice of 71.4% versus 56.8%, respectively. However, there was not a similar increase in survival of TLR4-defective HeJ compared with OuJ control mice.

### Body Weight and Survival Rate for Experimental Mice After 1 h/24 h Myocardial Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Murine Strain</th>
<th>No.</th>
<th>Body Weight, g</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sn</td>
<td>44</td>
<td>27±0.41</td>
<td>57</td>
</tr>
<tr>
<td>Cr</td>
<td>35</td>
<td>25±0.31</td>
<td>71</td>
</tr>
<tr>
<td>OuJ</td>
<td>12</td>
<td>24±0.61</td>
<td>58</td>
</tr>
<tr>
<td>HeJ</td>
<td>12</td>
<td>25±0.31</td>
<td>58</td>
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</tbody>
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Part of the response to MIR may be related to postoperative bacterial infections that flourish during the 24-hour period of reperfusion, despite observing sterile surgical techniques. However, differences in serum endotoxin were not detected between unoperated versus 1-hour/24-hour MIR mice (0.12±0.05 versus 0.11±0.06 EU/mL, P=0.90), nor between operated Sn versus Cr mice (0.11±0.013 versus 0.10±0.05, P=0.95). To ensure validity of the assay, 1.0 EU/mL LPS was added to murine serum, which was detected by the LAL assay (1.3±0.21 EU/mL).

### Reduced Myocardial Inflammation in Cr Mice After MIR

We next examined whether smaller infarctions in Cr mice corresponded with less myocardial inflammation, defined by neutrophil infiltration, lipid peroxidation, and complement deposition. Hearts from Sn mice showed evidence of leukocyte infiltration at sites of tissue injury after 1-hour/24-hour MIR, as shown by CD45-positive immunostaining (Figure 2A). The pattern of CD45-positive staining overlapped the pattern of GR-1–positive staining, the latter a specific marker for neutrophils. Fewer CD45+ / GR-1– infiltrating leukocytes were observed on sections of injured myocardium from Cr mice compared with Sn mice, suggesting less neutrophil trafficking (Figure 2A). Results from histological stains were supported by quantitatively less MPO activity in hearts from Cr compared with Sn mice after 1-hour/24-hour MIR (Cr, 0.60±0.05 U/100 mg; Sn, 0.94±0.07 U/100 mg; Figure 2B).

Reactive oxidative species are generated after MIR from intracellular oxidases present in the myocardium and from infiltrating leukocytes.20 Consistent with smaller infarctions and fewer neutrophils, hearts from Cr mice contained fewer lipid peroxides (malondialdehyde and 4-hydroxyalkenal) compared with Sn hearts (Figure 3), suggesting a lesser degree of oxidative stress in Cr hearts after MIR.

Neutrophils express TLR4 and respond to LPS.21 To determine whether the TLR4 mutation in Cr mice caused a general defect in neutrophil trafficking, we examined the recruitment of neutrophils in response to thioglycollate, a complement and leukotriene-dependent response.22 The number of peritoneal leukocytes 3 hours after intraperitoneal injection of thioglycollate increased to a similar extent in Sn and Cr mice (Figure 4), indicating a normal neutrophil response in Cr mice.
Complement is activated and component C3 is deposited on tissues in response to IR injury of several organs, including the heart. After 1-hour/24-hour MIR, a distinct pattern of C3 deposition was observed in areas of the left ventricle that also showed evidence of injury and neutrophil infiltration (Figure 5). Deposition of C3 appeared primarily on the myocardium and was not observed on leukocytes. Quantification of LV C3 deposition showed a significantly greater area of C3 staining on hearts from Sn mice compared with Cr mice after 1-hour/24-hour MIR (Sn, 12.7 ± 1.8% versus Cr, 2.2 ± 0.6%).

Discussion
This study demonstrates that a deficiency of TLR4 signaling reduces myocardial infarctions after a period of ischemia-reperfusion in mice. The data additionally demonstrate that TLR4 deficiency reduces inflammatory pathways linked to expansion of myocardial injury in MIR, including neutrophil accumulation, oxidative stress, and deposition of activated complement. Moreover, this study suggests that TLR4 is involved in inflammatory responses to ischemic tissue injury in addition to its role in inflammation triggered by microbial pathogens. Thus, these data establish an important role for TLR4 signaling in MIR-induced inflammation and injury in mice.

Myocardial ischemia-reperfusion elicits an intense inflammatory response that involves production of oxidants, activation of complement, and infiltration by polymorphonuclear neutrophils (PMNs).4 Experimental interruption of these pathways individually is reported to reduce MIR-induced infarctions, suggesting that the inflammatory response to MIR contributes cooperatively with ischemia to cause myocardial damage. Among these pathways, PMN infiltration is a prominent event after MIR. PMN infiltration was signifi-

Figure 2. A. Histochemical sections of hearts from TLR4-deficient (Cr) or wild-type control (Sn) mice after 1-hour/24-hour MIR. Sections were stained with hematoxylin and eosin (A and B), CD45 Ab (brown, C through F), or neutrophil-specific marker GR-1 Ab (G and H). Results are representative of 3 mice from each strain. B. MPO activity in heart homogenates from Cr (n=6) and Sn (n=6) mice after 1-hour/24-hour MIR. MPO values for sham-operated hearts were Cr, 0.37 ± 0.12; and Sn, 0.52 ± 0.085 U/100 mg (P=0.38, NS). Units of myocardial MPO activity are expressed as units per 100 mg tissue weight and are mean ± SEM. *Significant difference by Student’s t test at P<0.05.

Figure 3. Lipid peroxidation products malondialdehyde and 4-hydroxynonenal (MDA + 4-HNE) in hearts from TLR4-deficient (Cr) or wild-type control (Sn) mice after 1-hour/24-hour MIR. Units are nanomole per milligram of protein and represent the mean ± SEM from 6 observations per group. *Significant difference by Student’s t test at P<0.05.

Figure 4. Thioglycollate-induced peritonitis in TLR4-deficient (Cr) or wild-type control (Sn) mice. Animals were injected intraperitoneally with PBS (control, n=4 for Sn and n=4 for Cr) or with thioglycollate (n=6 for Sn and n=8 for Cr). The number of peritoneal leukocytes was determined 3 hours later. Granulocytes and macrophages were distinguished by FACS. Values represent mean cell number ± SEM in millions; the error bars refer to the standard error of the total leukocyte count. No significant differences from single-factor ANOVA; P=0.24.
fewer lipid peroxides, major byproducts of oxidative stress. It is possible that TLR4 activation increases ROS production in part by upregulating expression of inducible NO synthase and cyclooxygenase.\textsuperscript{28,29} It is more likely that reduced lipid peroxidation in Cr mice is secondary to fewer numbers of activated neutrophils, and thus less ROS generation, after MIR. Our experiments, however, cannot exclude a more direct link between TLR4 activation and generation of ROS in MIR. Nevertheless, TLR4 appears linked to oxidative stress in myocardial IR, which may contribute to inflammation and myocyte damage.

Inhibiting complement at the level of C3 or C5b-9 in experimental IR reduces myocardial infarct size and postischemic inflammation.\textsuperscript{30,31} The area of C3 deposition was significantly reduced in hearts from Cr mice compared with Sn mice, despite identical areas at risk of infarction. Reduced necrosis in the hearts from Cr mice might contribute to reduced myocardial C3 deposition.\textsuperscript{32} Conversely, reduced C3 deposition predicts less generation of proinflammatory anaphylotoxins and cytotoxic complement components, which may contribute to smaller infarct size and reduced neutrophil trafficking in Cr mice. These data suggest that TLR4 activation may be linked and proximal to complement activation in MIR.

Reduced myocardial inflammation in Cr mice prompted us to consider that defective TLR4 signaling may nonspecifically blunt all pathways of inflammation. However, no difference in the number of thioglycollate (TG)-elicited granulocytes was observed between the Sn and Cr mice. This result suggests that neutrophil trafficking is not inherently defective in Cr mice and that TLR4 is selectively involved in inflammatory pathways. A key difference in TG versus MIR-induced inflammation is the presence of tissue damage in MIR. This is consistent with the hypothesis that endogenous TLR4 ligands are present at sites of cellular injury or necrosis. This is supported by recent studies identifying HSP60 as a putative ligand for TLR4 and other endogenous host-derived molecules that appear to require TLR4 for activity.\textsuperscript{7} Therefore, TLR4, and possibly other TLRs, would be activated in circumstances involving tissue injury and necrosis in addition to activation caused by recognized microbial ligands.

Conclusions

In summary, 2 distinct TLR4-deficient murine strains were protected from myocardial infarction caused by ischemia-reperfusion injury. Inflammatory pathways linked to MIR-induced infarction were reduced in TLR4-deficient Cr mice, including PMN infiltration, oxidative stress, and complement activation. This study demonstrates that in addition to its role in innate immune responses, TLR4 serves a proinflammatory function in a murine model of MIR injury.

Acknowledgments

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References