Role of Cardiac- and Myeloid-MyD88 Signaling in Endotoxin Shock

A Study with Tissue-specific Deletion Models

Yan Feng, M.D., Ph.D., Lin Zou, M.D., Ph.D., Chan Chen, M.D., Ph.D., Dan Li, M.D., Wei Chao, M.D., Ph.D.

ABSTRACT

Background: Myeloid differentiation factor 88 (MyD88) is an adaptor molecule critical for host innate immunity. Studies have shown that signaling via MyD88 contributes to cytokine storm, cardiac dysfunction, and high mortality during endotoxin shock. However, the specific contribution of MyD88 signaling of immune and cardiac origins to endotoxin shock remains unknown.

Methods: Tissue-specific MyD88 deletion models: Cre-recombinase transgenic mice with α-myosin heavy chain (α-MHC) or lysozyme M promoters were cross-bred with MyD88-loxP (MyD88fl/fl) mice, respectively, to generate cardiomyocyte- (α-MHC-MyD88−/−) or myeloid-specific (Lyz-MyD88−/−) MyD88 deletion models and their respective MyD88fl/fl littermates. Endotoxin shock model: Mice were subjected to 15 mg/kg lipopolysaccharide (intraperitoneal injection). Cardiac function was measured by echocardiography and cytokines by multiplex assay and quantitative reverse transcription-polymerase chain reaction.

Results: α-MHC-MyD88−/− mice had 61 and 87% reduction in MyD88 gene and protein expression in cardiomyocytes, respectively, whereas Lyz-MyD88−/− had 73 and 67% decrease, respectively, in macrophages (n = 3 per group). After lipopolysaccharide treatment, the two groups of MyD88fl/fl littermates had 46% (n = 10) and 60% (n = 15) of mortality, respectively. Both α-MHC-MyD88−/− and Lyz-MyD88−/− mice had markedly improved survival. Compared with the MyD88fl/fl littermates, Lyz-MyD88−/− mice had warmer body temperature, attenuated systemic and cardiac inflammatory cytokine production, and significantly improved cardiac function, whereas α-MHC-MyD88−/− mice had decreased myocardial inducible nitric oxide synthase induction and modestly preserved cardiac function.

Conclusions: Both cardiomyocyte- and myeloid-MyD88 signaling play a role in cardiac dysfunction and mortality during endotoxin shock. Myeloid-MyD88 signaling plays a predominant role in systemic and cardiac inflammation after endotoxin challenge. (Anesthesiology 2014; 121:1258-69)

What We Already Know about This Topic

• Previous studies have shown that after a lethal dose of lipopolysaccharide, MyD88−/− mice exhibit no sign of cardiac dysfunction, much attenuated cytokines production, and markedly improved survival, demonstrating the essential role of MyD88 signaling in the pathogenesis of endotoxin shock.

• This study used cardiomyocyte- and myeloid-specific MyD88 deletion models to evaluate the contribution of cardiac versus circulating MyD88 signaling to the pathogenesis of endotoxin shock.

What This Article Tells Us That Is New

• Both cardiac- and myeloid-MyD88 signaling play an important role in the mortality and cardiomyopathy after a lethal dose of lipopolysaccharide. Moreover, myeloid-MyD88 signaling plays a predominant role in mediating systemic and cardiac cytokine responses, whereas cardiomyocyte-MyD88 signaling is mainly responsible for mediating myocardial inducible nitric oxide synthase induction during endotoxin shock.

Sepsis is the systemic inflammatory syndrome in response to invading pathogens and their components. It is the 10th leading cause of death in the United States.1,2 Despite advances in antibiotic therapy and supportive care, sepsis is still among the major causes of death in the noncardiac intensive care units.3 Multiorgan dysfunction, in particular cardiovascular collapse, dramatically increases sepsis mortality.4 Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize various pathogen-associated molecular patterns molecules derived from various pathogens and activate host innate immune defense against pathogens invasion.5–7 To date, 13 mouse TLRs have been reported.8 All TLRs, except TLR3, signal through myeloid differentiation factor 88 (MyD88)–dependent pathway and activate the transcript factor nuclear factor-KB, which in turn leads to the production of multiple inflammatory mediators.

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including cytokines, chemokines, and antimicrobial peptides. However, inappropriate and uncontrolled production of proinflammatory cytokines and mediators results in profound drop in blood pressure, impaired microcirculation, attenuated cardiac output, and ultimate cardiovascular collapse and death.

Lipopolysaccharide, also termed endotoxin, is a major component of the outer membranes of Gram-negative bacteria and is responsible for systemic cytokine storm and organ dysfunction during endotoxin shock and severe sepsis. Lipopolysaccharide is recognized by TLR4 and activates TLR4 signaling through both MyD88-dependent and MyD88-independent (Toll/interleukin [IL]-1 receptor domain-containing adaptor inducing interferon-β [Trif]-dependent) pathways. Mice with TLR4 gene site mutation or deletion are completely unresponsive to lipopolysaccharide, and TLR4 antagonists were found to markedly attenuate endotoxin shock in animals. Given its critical role in TLR signaling, it is not surprising that systemic deletion of MyD88 or Trif confers a powerful protection against lipopolysaccharide-induced cardiac dysfunction and high mortality. Interestingly, our previous studies using bone marrow chimeric models demonstrate that nonhematopoietic (parenchymal), instead of hematopoietic, TLR2, which signals through MyD88-dependent pathway, plays a predominant role in the development of cardiac dysfunction during polymicrobial sepsis. However, the specific contribution of cardiac versus circulating MyD88 signaling to the pathogenesis of endotoxin shock remains unclear.

To dissect the complex role of cardiac and extra-cardiac MyD88 signaling in cardiac dysfunction and mortality during endotoxin shock, we generated cardiomyocyte- and myeloid-specific MyD88 knockout mice using Cre-loxP system and subjected the tissue-specific MyD88 knockout mice and the littermate control mice to lethal dose of lipopolysaccharide. Our data suggest that both cardiomyocytes- and myeloid-MyD88 signaling play a role in cardiac dysfunction and mortality during endotoxin shock and that myeloid-MyD88 signaling plays a predominant role in systemic and cardiac inflammation.

Materials and Methods

Animals

Cre-recombinase transgenic mice with α-myosin heavy chain (α-MHC) or lysozyme M promoters and MyD88-loxP (MyD88fl/fl) mice, all in C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88−/− mice were generated by Kawai et al. and had been backcrossed more than 10 generations into the C57BL/6 strain. Unless stated otherwise, all mice used in the study were 8- to 12-weeks old and weighed 20 to 30 g. Mice were fed the same bacteria-free diet (Prolabo Isopro RMH 3000; LabDiet, Brentwood, MO) and water. The animal protocols used in the study were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital (Boston, Massachusetts). The experiments were performed in compliance with the guideline from the National Institutes of Health (Bethesda, Maryland). Simple randomization method was used to assign animals to various experimental conditions. All mice used were sex and age matched.

Generation of Cardiac- and Myeloid-MyD88 Knockout Mice

Transgenic mice expressing Cre-recombinase under the control of α-MHC promoter or lysozyme M promoter were cross-bred with mice with loxP sites flanking exon 3 of MyD88 gene (MyD88fl/fl) to generate cardiomyocyte-(α-MHC-MyD88−/−) or myeloid-specific (Lyz-MyD88−/−) MyD88 knockout mice, respectively (fig. 1). The MyD88fl/fl littermates were used as control. Breeding colonies were maintained by mating MyD88fl/fl with α-MHC-MyD88−/− or Lyz-MyD88−/− mice. Mice were genotyped by polymerase chain reaction using genomic DNA isolated from tail tips. The primers used for genotyping and gene deletion were summarized in table 1.

Adult Cardiomyocyte and Macrophage Isolation

Adult ventricular cardiomyocytes were prepared using Tyrode buffer (sodium chloride 137 mM, potassium chloride 5.4 mM, Hepes 0.5 mM, magnesium chloride 5.4 mM, calcium chloride 5.4 mM, HEPES 0.5 mM, magnesium chloride 5.4 mM). Mice were killed by cervical dislocation and hearts were removed. Hearts were flushed of blood with cold Tyrode buffer and minced in Tyrode buffer containing collagenase (Calbiochem, La Jolla, CA). Cardiomyocytes were isolated using a collagenase digestion followed by low gravity sedimentation. Macrophages were isolated by flushing the peritoneal cavity of mice with cold Tyrode buffer and allowing cells to sediment for 30 minutes.
Table 1. Primer Sequences for Genotyping and qRT-PCR

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<tr>
<th>Strain</th>
<th>Primer sequences for genotyping</th>
</tr>
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<tbody>
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<td>α-MHC-Cre</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Control Reverse: 5'-GTCAGGATGTCAGTGTTTCTC-3’</td>
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<tr>
<td>Lyz-Cre</td>
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<td>Wildtype: 5'-TTACAGTGCAAGGCTGACGC-3’</td>
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<th>Strain</th>
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<td>MyD88 deletion</td>
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<td></td>
<td>Reverse: 5'-AGGCTGAGTGCAAACCTTTG-3’</td>
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<td>Mouse IL-1β</td>
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<tr>
<td></td>
<td>Reverse: 5'-AGGCCACAGGATTTTTGTC-3’</td>
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<tr>
<td>Mouse TNFα</td>
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</tr>
<tr>
<td></td>
<td>Reverse: 5'-GACACTCTCGGAGAATCTG-3’</td>
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<td>Mouse IL-6</td>
<td>Forward: 5'-AGTTGCTCTTCTGAGGACTA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCCAGGATTTCCGACAGAGA-3’</td>
</tr>
<tr>
<td>Mouse iNOS</td>
<td>Forward: 5'-CTCCTCTTGAGGAAACTG-3’</td>
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<td></td>
<td>Reverse: 5'-GGTGCAAACCTTCGAGGT-3’</td>
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<tr>
<td>Mouse MyD88</td>
<td>Forward: 5'-CCACAAACAAAGGACTTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TACCTCTCTAGCAGACCT-3’</td>
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IL = interleukin; iNOS = inducible nitric oxide synthase; Lyz-Cre = Cre-recombinase transgenic mice under the control of lysozyme M promoter; α-MHC-Cre = Cre-recombinase transgenic mice under the control of α-myosin heavy chain promoter; MyD88 = myeloid differentiation factor 88; MyD88loxP = MyD88loxP control mice with loxP sites flanking exon 3 of MyD88 gene; qRT-PCR = Quantitative reverse transcription-polymerase chain reaction; TNFα = tumor necrosis factor α.

0.5 mM) containing 0.3 mg/g collagenase D, 0.4 mg/g collagenase B (Roche, Indianapolis, IN), and 0.05 mg/g protease XIV (Sigma, St. Louis, MO) of body weight. The cell suspension was filtered through 250-μm mesh to remove undigested tissues and sediment for 15 min. The supernatant, containing small cells, was discarded and the sedimentation was repeated twice. Finally, the adult cardiomyocytes were spun down at 1,000 rpm for 1 min. Bone marrow cells were flushed from mouse femur and tibia. After spun down, the cells were resuspended in Roswell Park Memorial Institute medium supplied with 10% fetal bovine serum, 5% horse serum, and 1 ng/ml macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN), and 4 × 10⁶ bone marrow cells were seeded into a well of six-well plate. Three days later, the culture medium was changed and cells were treated with 10 ng/ml Pam3Cys or 25 μg/ml poly(I:C) (Enzo Life, Farmingdale, NY) for 2 h.

**Endotoxin Shock Model**

To induce endotoxin shock, lipopolysaccharide (Escherichia coli 0111:B4; Sigma) was administered at the dose of 15 mg/kg body weight by intraperitoneal injection followed by administration of 1 ml of prewarmed normal saline. The same volume of normal saline was administered to the control mice.

**Mouse Echocardiography**

Mice were lightly anesthetized with ketamine (20 mg/kg). Transthoracic echocardiographic images were obtained 1
day before and again 6 h after saline or lipopolysaccharide administration. Thirty minutes before echocardiographic measurements, 1 ml of prewarmed saline was injected intraperitoneally into each mouse, and the mouse cages were warmed to 30°C under light for 30 min. All images were collected 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.

Mortality Study

After lipopolysaccharide administration, mice were monitored every 4 h during the day time and every 8 h at night and for total up to 96 h.

Measurement of Body Temperature

Body temperature was measured rectally before and again 6 h after saline or lipopolysaccharide administration using a digital thermometer probe (DC Temperature Controller; FHC Inc., Bowdoin, ME).

Multiplex Cytokine Immunoassays

Plasma were prepared at 4°C and stored at −80°C. Cytokine/chemokine concentrations were determined using a fluorescent bead-based multiplex immunoassay (Luminex kine/chemokine concentrations were determined using a Placenta were prepared at 4°C and stored at −80°C. Cyto-

Multiplex Cytokine Immunoassays

Plasma were prepared at 4°C and stored at −80°C. Cytokine/chemokine concentrations were determined using a fluorescent bead-based multiplex immunoassay (Luminex Corporation, Austin, TX). In brief, antibody for each cytokine was covalently immobilized to a fluorescent microsphere by a manufacturer (Millipore, Billerica, MA). Several fluorescent microspheres immobilized with different target cytokines were mixed together and incubated with samples. After overnight incubation, cytokines bound on the surface of microspheres were detected using a cocktail of biotinylated antibodies. After binding of streptavidin–phycocerythrin conjugates, the reporter fluorescent signal was measured with a Luminex 200® reader (Luminex Corporation). Final cytokine concentrations were calculated based on a standard curve constructed in each experiment.

Quantitative Reverse Transcription-polymerase Chain Reaction

Quantitative reverse transcription-polymerase chain reaction was performed as described previously. Changes in relative gene expression normalized to 18S ribosomal RNA were determined using the relative $C_T$ method (where $C_T$ is the threshold cycle number). The sequences of the oligonucleotide primers are listed in table 1.

Western Blotting

Homogenates of cell pellets were centrifuged at 12,000g at 4°C for 30 min. Proteins were separated in 4 to 20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with 1:1,000 diluted MyD88 antibody (Cell Signaling Tech, Danvers, MA) as described previously.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (Graphpad, La Jolla, CA). Unless stated otherwise, the distributions of the continuous variables were expressed as the mean ± SD. The survival rate was expressed as the percentage of live animals, and Mantel–Cox log-rank test was used to determine survival differences between the groups. The P values of echocardiographic measurements were based on the two-tailed unpaired Student t test. For those cytokine levels below detection limit, values input at the detection limit were used. For cytokine production, the statistical significance of the difference between groups (e.g., normal saline vs. lipopolysaccharide, wild type vs. knockout mice) was measured by two-way ANOVA with Bonferroni correction posttests. Of note, the sample sizes were based on our prior experiences rather than a formal statistical power calculation. The null hypothesis was rejected for $P$ value less than 0.05 with the two-tailed test.

Results

Generation of Cardiomyocyte- and Myeloid-specific MyD88 Knockout Mice

As shown in table 2, there was no difference in the body and heart weights among the three strains of mice, namely MyD88Δϕ−/−, α-MHC-MyD88−/−, and Lyz-MyD88−/−. To determine the tissue-specific deletion of MyD88, we isolated adult cardiomyocytes and bone marrow–derived macrophages (Mϕ) from the three strains of mice. As shown in figure 2A, the genotyping experiments indicate that in α-MHC-MyD88−/− mice, there was specific MyD88 gene deletion only in cardiomyocytes, whereas normal MyD88 gene expression was seen in all other cells/tissues examined, such as Mϕ, lung, spleen, kidney, and skeletal muscle. In Lyz-MyD88−/− mice, there was targeted MyD88 gene deletion only in Mϕ. Of note, the low levels of MyD88 gene deletion observed in the lung and liver were likely from residual Mϕ of the myeloid lineage. Quantitative reverse transcription-polymerase chain reaction and Western blot confirmed the tissue-specific MyD88 gene deletion and protein down-regulation (fig. 2, B and C) in cardiomyocytes and in Mϕ. Quantitatively, compared with MyD88Δϕ−/− control mice, α-MHC-MyD88−/− mice had 61 and 87% reduction in MyD88 messenger RNA and protein expression levels, respectively, in cardiomyocytes, whereas Lyz-MyD88−/− mice had 73 and 67% decrease in Mϕ, respectively. Functionally (fig. 2D), Mϕ isolated from Lyz-MyD88−/− mice had an attenuated tumor necrosis factor-α (TNFα) production in
response to Pam3cys (a TLR2 ligand) but a normal response to poly(I:C) (a TLR3 ligand). These data suggest that the specific MyD88 gene deletion in myeloid lineage specifically attenuates TLR2-MyD88 signaling and has no impact on TLR3-Trif pathway in Mφ.

To test whether or not MyD88 deletion alters TLR4 expression, TLR4 protein expression in Mφ was detected by Western blot. The data suggested that TLR4 expression was modestly increased in Mφ isolated from systemic MyD88−/− mice, but not in Mφ isolated from Lyz-MyD88−/− and α-MHC-MyD88−/− mice (fig. 1 in Supplemental Digital Content 1, http://links.lww.com/ALN/B69).

We also generated an inducible cardiomyocytes-targeted MyD88 deletion model. As detailed in figure 2 in Supplemental Digital Content 1, http://links.lww.com/ALN/B69, there was significant, but incomplete, reduction of cardiac-specific MyD88 gene transcript (46%) and subsequent protein (36%) expression after systemic tamoxifen administration. In this model, constitutive cardiac expression of Cre with or without loxP sites resulted in transient dilated cardiomyopathy within a week of tamoxifen injection but completely recovered by 22 days as reported.27 Given the incomplete deletion and the observed dilated cardiomyopathy, we decided not to use the system for the current study.

Cardiomyocyte- or Myeloid-specific MyD88 Deletion Improves Survival during Endotoxin Shock

Our previous data have shown that MyD88−/− mice were totally resistant to lethal dose of lipopolysaccharide (15 mg/kg) with no mortality, demonstrating the essential role of MyD88 signaling in endotoxin shock.19 To delineate the specific role of cardiac versus Myeloid-MyD88 in endotoxin-induced mortality, we subjected MyD88−/− control mice and the two strains of MyD88 knockout mice to the

Table 2. Body and Heart Weight of α-MHC-MyD88−/−, Lyz-MyD88−/−, MyD88fl/fl Mice

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body Weight (g)</td>
<td>Heart Weight (mg)</td>
</tr>
<tr>
<td>MyD88fl/fl</td>
<td>28.0 ± 0.7</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>α-MHC-MyD88−/−</td>
<td>27.5 ± 0.9</td>
<td>122 ± 7</td>
</tr>
<tr>
<td>Lyz-MyD88−/−</td>
<td>28.4 ± 0.5</td>
<td>119 ± 5</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

The three strains of mice were all in C57BL/6 background and between 16–24 weeks of age. Lyz-MyD88−/− = myeloid-specific MyD88 knockout mice; α-MHC-MyD88−/− = cardiomyocyte-specific MyD88 knockout mice; MyD88 = myeloid differentiation factor 88; MyD88fl/fl = MyD88-loxP control mice.
same lethal dose of lipopolysaccharide (fig. 3A). Similar to the littermate controls and different from total MyD88−/− mice, both α-MHC-MyD88−/− and Lyz-MyD88−/− mice showed the signs of endotoxin shock, such as a crouched position, shivering, and ruffled fur. However, despite of the signs of sickness, both α-MHC-MyD88−/− and Lyz-MyD88−/− mice had markedly improved survival with 0 and 13% mortality, respectively, compared with their littermate controls, who had 60 and 46% mortality, respectively, at 96 h after lipopolysaccharide administration (fig. 3, B and C). These data suggest that both cardiac and myeloid-MyD88 signaling play a role in the mortality during endotoxin shock.

**Effect of MyD88 Deletion on Body Temperature during Endotoxin Shock**

Body temperature has been shown to be an independent predictor of severity and survival at the onset of sepsis.28,29 After lipopolysaccharide challenge, mouse rectal temperature decreased gradually. In the littermate controls (MyD88fl/fl), the core temperature decreased from 37° ± 0°C to 33° ± 3°C at 6 h and 29° ± 3°C at 24 h after lipopolysaccharide injection (fig. 3, D and E). As shown in figure 3D, α-MHC-MyD88−/− mice exhibited a similar decrease in body temperature (34° ± 2°C at 6 h and 30° ± 3°C at 24 h). However, Lyz-MyD88−/− mice had higher body temperature compared with the litters (35° ± 2°C vs. 33° ± 2°C at 6 h; 32° ± 3°C vs. 29° ± 4°C at 24 h, P < 0.01) (fig. 3E). These data suggest that bone marrow–derived MyD88 signaling probably plays a more prominent role in the development of hypothermia during endotoxin shock.

Tissue hypoperfusion during sepsis may contribute to the hypothermia. We measured blood lactate level, an indicator of tissue hypoperfusion, in the MyD88fl/fl mice subjected to saline or lipopolysaccharide. As shown in figure 3A in Supplemental Digital Content 1, http://links.lww.com/ALN/B69, 18 h after treatment, the blood lactate level in lipopolysaccharide-treated MyD88fl/fl mice was modest but insignificantly increased compared with that in saline-injected control mice (1.5 ± 0.4 mM vs. 1.2 ± 0.4 mM, P > 0.05) (fig. 3A in Supplemental Digital Content 1, http://links.lww.com/ALN/B69) and the body temperature of the endotoxemic mice was dramatically dropped (from 36.1° ± 0.4°C to 26.4° ± 0.4°C, P < 0.001) (fig. 3B in Supplemental Digital Content 1, http://links.lww.com/ALN/B69). The data suggested that the body temperature is not correlated to tissue hypoperfusion as indicated by blood lactate level.

**Fig. 3.** Effect of cardiomyocyte- and myeloid-specific MyD88 deletion on survival rate and body temperature during endotoxin shock. Both cardiac- (α-MHC-MyD88−/−) and myeloid- (Lyz-MyD88−/−) MyD88 deletion mice were subjected to lipopolysaccharide (LPS) (15 mg/kg, intraperitoneal injection) together with the littersmate control (MyD88fl/fl). (A) The diagram of time line of the experiments. The mortality was monitored for up to 96 h (B and C) and body temperature was measured at 6 and 24 h after LPS injection (D and E). Each error bar represents mean ± SD. ** **P < 0.01; n = 10 in each group in B and D, n = 15 mice in each group in C and E. Echo. = echocardiography; Lyz-MyD88−/− = myeloid-specific MyD88 knockout mouse; α-MHC = α-myosin heavy chain; α-MHC-MyD88−/− = cardiomyocyte-specific MyD88 knockout mouse; MyD88 = myeloid differentiation factor 88; MyD88fl/fl = MyD88-loxP control mouse; temp. = temperature.
The Impact of Cardiac- or Myeloid-specific MyD88 Deletion on the Cardiac Function during Endotoxin Shock

The baseline cardiac functions, as measured by echocardiography, between the age-sex-matched cardiac- or myeloid-MyD88 knockout mice and their MyD88fl/fl littermates were the same (table 3). Six hours after lipopolysaccharide, both MyD88fl/fl littermate controls for the two knockout strains developed a marked left ventricle dysfunction with 33 and 51% reduction in FS, respectively (38 ± 13% vs. 57 ± 3%, P < 0.001, and 31 ± 5% vs. 63 ± 4%, P < 0.001). Compared with their septic littermate controls, septic Lyz-MyD88−/− mice had significantly improved cardiac function with 39% increase in FS (FS: 43 ± 6% vs. 31 ± 5%, P < 0.001). In comparison, the septic α-MHC-MyD88−/− mice had similar values of FS as that of septic Lyz-MyD88−/− mice (41 ± 9% vs. 43 ± 6%). However, compared with their septic littermate controls (38 ± 13%), the improvement in the septic α-MHC-MyD88−/− was modest and did not reach statistical significance.

MyD88 Deletion in Myeloid Cells Attenuates Systemic Cytokine Production during Endotoxin Shock

Lipopolysaccharide induced a marked increase in plasma IL-1β, IL-6, and TNFα in the MyD88fl/fl littermates. Although systemic MyD88-deficiency (MyD88−/−) almost completely blocked lipopolysaccharide-induced cytokines production (fig. 4), α-MHC-MyD88−/− mice had similar levels of the plasma cytokines compared with the MyD88fl/fl littermates after lipopolysaccharide injection. In contrast, myeloid-specific MyD88 deletion (Lyz-MyD88−/−) significantly attenuated the plasma levels of IL-1β, IL-6, and TNFα at 6 h and IL-1β and TNFα at 18 h after lipopolysaccharide injection, although the cytokine levels were still substantially higher than systemic MyD88-deficient mice (fig. 4).

Myeloid-MyD88 Signaling Contributes to Cardiac Cytokines Production during Endotoxin Shock

To explore the contribution of MyD88 signaling to the cardiac cytokines production, we measured cardiac proinflammatory cytokine production by quantitative reverse transcription-polymerase chain reaction. Eighteen hours after lipopolysaccharide treatment, MyD88−/− littermates had robust IL-6 and TNFα expression, but not IL-1β, in the myocardium compared with mice injected with saline (fig. 5). Systemic MyD88-deficiency almost completely blocked cardiac IL-6 and TNFα production after lipopolysaccharide administration. In contrast, α-MHC-MyD88−/− mice produced similar levels of cardiac IL-6 and TNFα compared with the control mice after lipopolysaccharide treatment. Importantly, myeloid-specific MyD88 deletion (Lyz-MyD88−/−) significantly reduced cardiac TNFα and IL-6 production compared with the MyD88fl/fl littermates (fig. 5).

Cardiomyocyte-specific MyD88 Deletion Attenuates Myocardial Inducible Nitric Oxide Synthase Production during Endotoxin Shock

Lipopolysaccharide is known to induce inducible nitric oxide synthase (iNOS) expression in various tissues in vivo and in cardiomyocytes in vitro. Cardiac iNOS production has been linked to cardiac dysfunction during endotoxia. We found that lipopolysaccharide administration induced a significant increase in myocardial iNOS messenger RNA expression in the MyD88fl/fl littermates. Systemic MyD88-deficiency (MyD88−/−) completely blocked lipopolysaccharide-induced iNOS production (fig. 6). Although Lyz-MyD88−/− mice had similar levels of myocardial iNOS production compared with the MyD88fl/fl littermates, α-MHC-MyD88−/− mice had significantly lower myocardial iNOS level (fig. 6). These data indicate that cardiomyocyte- (but not myeloid-) specific MyD88 signaling plays a predominant role in mediating iNOS production in the heart during endotoxia.

Discussion

Earlier studies have shown that after a lethal dose of lipopolysaccharide, MyD88−/− mice exhibit no sign of cardiac dysfunction, much attenuated cytokines production, and markedly improved survival, demonstrating the essential role of MyD88 signaling in the pathogenesis of endotoxin shock. To delineate the specific contribution of cardiac versus myeloid-MyD88 in endotoxin shock.
circulating MyD88 signaling to the pathogenesis of cardiac dysfunction and cytokine production during endotoxin shock, we generated cardiomyocyte- and myeloid-specific MyD88 deletion models and made several observations in the current study. First, when subjected to a lethal dose of endotoxin, deletion of either cardiac or myeloid-MyD88 conferred a remarkable survival benefit, suggesting an important role of both cardiomyocyte and circulating MyD88 signaling in the endotoxin-induced mortality. Second, both myeloid- and cardiomyocytes-MyD88-deficient mice showed improvement in cardiac function compared with the littermates subjected to lipopolysaccharide. Finally, myeloid-MyD88-deficient mice exhibited warmer body temperature and significantly attenuated systemic and cardiac inflammatory cytokine responses, demonstrating the critical role of the circulating MyD88 signaling of myeloid lineage in systemic and local inflammation during endotoxin shock. In contrast, cardiomyocyte-specific MyD88 deficient mice showed attenuated iNOS production in the heart after lipopolysaccharide administration.

In our study, recombination of α-MHC-Cre or Lyz-Cre with loxP system resulted in 87 and 67% reduction of MyD88 gene expression in cardiomyocytes and macrophages, respectively. There are a few explanations for this incomplete deletion observed in these cells. First, cardiomyocyte samples could potentially contain noncardiomyocytes such as resident macrophages, which maintain MyD88 expression. In our study, we isolated adult cardiomyocytes after enzymatic...
digestion of the heart and a series of sedimentation procedures. Any contamination by noncardiomyocytes could have contributed to the observed MyD88 gene expression in the α-MHC-Cre-MyD88−/− cardiomyocyte samples. Second, there is indeed an incomplete deletion in the α-MHC-Cre-MyD88−/− cardiomyocytes. This incomplete gene deletion was reported in the original work by Agah et al. when they first constructed α-MHC-Cre transgenic mice.21 They found that, in their α-MHC-Cre/CAG-CATZ+ mice, the chloramphenicol acetyltransferase (CAT) gene deletion led to 90% reduction of the CAT activity in myocardium compared with that in α-MHC-Cre/CAG-CATZ−/− mice.22 Also, they found that different α-MHC-Cre strain showed different degree of gene deletion. Similarly, others report that in cells of the myeloid lineage, there is approximately 80% deletion of the loxP-flanked target genes, such as β-polymerase gene and RFX5 gene, in developing macrophages (e.g., bone marrow–derived macrophages), whereas the deletion efficiency is more complete in fully differentiated peritoneal macrophages and neutrophils (83 to 99%).22 Most likely, the observed incomplete deletion reflects the efficacy of Cre-recombinase in target cells. Our data of MyD88 expression in bone marrow–derived macrophages were consistent with the above reports. Moreover, functionally, the bone marrow–derived macrophages isolated from the Lys-MyD88−/− mice had markedly attenuated, but not completely abolished, TNFα production in response to TLR2 activation by Pam3csys. These suggest that the myeloid-MyD88 signaling was significantly attenuated in Lys-MyD88−/− mice. Taken together, these data clearly demonstrate that using the Cre-loxP system, we have successfully generated cardiomyocyte- and myeloid-specific MyD88 gene deletion models.

Cre-recombinase overexpression may cause dose-dependent cytotoxicity.27,34 Antje et al. generated the mice expressing Cre-recombinase under the control of α-MHC promoter and found that the strains with high-expression of Cre-recombinase in the heart developed dilated cardiomyopathy and most of them died prematurely, whereas the transgenic lines with low level of Cre-recombinase were healthy.35 Mice with low-level cardiac Cre expression did not have any sign of heart failure at the age of 8- to 10-months old and survived up to 18 months.36 In our studies, we found that α-MHC-MyD88−/− mice were healthy and there was no difference in heart weight and baseline cardiac function between α-MHC-MyD88−/− mice (with Cre expression in the heart) and the MyD88fl/fl control mice (without Cre expression). The limitation of the Cre-loxP approach for tissue-specific deletion is the inability to control the timing of Cre-mediated recombination. That is, the tissue-specific gene disruption could occur within the developing embryo, fetus, or neonates. An inducible Cre system, such as inducible MerCreMer under the control of α-MHC promoter, could be used to achieve temporal and tissue-specific deletion in adult heart.37 However, the inducible Cre system has its own share of problems, including transient dilated cardiomyopathy and partial gene deletion.27,37,38 We have tested the tamoxifen-induced MerCreMer system and encountered the same issues. We found that the α-MHC-MerCreMer-MyD88−/− mice had incomplete (only 40%) MyD88 gene deletion and dilated cardiomyopathy, which peaked at 8 days and recovered at 22 days after first dose of tamoxifen (fig. 2 in Supplemental Digital Content 1, http://links.lww.com/ALN/B69).

Both cardiomyocyte- and myeloid-specific MyD88−/− mice exhibit markedly improved survival in response to endotoxin shock. However, unlike the global MyD88-deficient mice that were unresponsive to and totally protected from lethal dose of endotoxin, both cardiomyocyte- and myeloid-specific MyD88 deficient mice exhibited the signs of endotoxemia, including hypothermia and cardiac dysfunction. These data suggest that MyD88 signaling of both origins plays a role in the pathogenesis of lethal endotoxin shock. Deletion of either cardiomyocyte or myeloid-MyD88 alone will only achieve partial protection.

We found that myeloid-specific MyD88 deletion significantly reduced both systemic and cardiac proinflammatory cytokines responses, such as TNFα and IL-6. These data strongly suggests that MyD88 signaling in bone marrow–derived immune cells such as tissue macrophages and circulating neutrophils/monocytes are mainly responsible for mediating systemic as well as local cardiac cytokine production during endotoxin shock. In consistent with our observation, previous studies have demonstrated that MyD88 signaling in myeloid cells is responsible for acute inflammatory-driven anorexia and local adrenal inflammation in lipopolysaccharide-treated animals. Overproduction of multiple inflammatory mediators, cytokines in particular,
has been closely associated with the symptomatology and pathogenesis of sepsis in both experimental models\textsuperscript{43–45} and human clinical studies.\textsuperscript{43–45} Cytokines can cause profound vasodilation and compromise tissue perfusion.\textsuperscript{56,47} Systemic microcirculation dysfunction is considered to be a fundamental cause of multiple organ dysfunction in sepsis.\textsuperscript{38} Excessive amount of cytokines (e.g., TNFα) increases endothelial permeability,\textsuperscript{49,50} tissue edema, and in turn organ dysfunction. Therefore, animals lacking MyD88 signaling in the myocardial lineage cells (Lyz-MyD88\textsuperscript{−/−}) exhibit reduced systemic cytokine levels, warmer body temperature, improved cardiac function, and better survival compared with their littermates during endotoxin shock.

We found that α-MHC-MyD88\textsuperscript{−/−} mice had improved survival, but yet modest improvement in cardiac dysfunction compared with control mice after the lethal dose of lipopolysaccharide. It is worth noting although α-MHC-MyD88\textsuperscript{−/−} mice had nearly the same cardiac contractile function as that of Lyz-MyD88\textsuperscript{−/−} mice (FS: 41 ± 9\% vs. 43 ± 6\%), the difference in cardiac function between α-MHC-MyD88\textsuperscript{−/−} and MyD88\textsuperscript{−/−} littermates did not reach statistical significance. This is likely due to different FS values in their respective littermate controls (FS: 38 ± 13\% vs. 31 ± 5\%). Nevertheless, a few previous studies suggest that lipopolysaccharide-induced cardiac dysfunction may be an indirect effect secondary to immune cell TLR4 activation. For example, Tavener et al.\textsuperscript{31} found that cardiomyocytes isolated from lipopolysaccharide-treated mice exhibited reduced sarcomere shortening and Ca\textsuperscript{2+} transients, whereas in vitro treatment with lipopolysaccharide failed to inhibit cardiomyocyte function. Further studies in chimeric mice suggest that TLR4 in bone marrow–derived hematopoietic cells is probably responsible for cardiac dysfunction during endotoxin shock.

Accumulating data demonstrated that nitric oxide plays a critical role in the pathogenesis of myocardial dysfunction in sepsis.\textsuperscript{54} Nitric oxide is synthesized by NOS from l-arginine. There are three total NOS isoforms exist in the heart: neuronal (NOS1), inducible (iNOS or NOS2), and endothelial (NOS3). NOS1 and NOS3 are constitutively expressed in many cells, such as neuronal cells, cardiomyocytes, and endothelial cells, whereas iNOS is robustly induced in response to lipopolysaccharide administration \textit{in vivo} and \textit{in vitro}.\textsuperscript{30,31} iNOS-deficient mice or mice-treated iNOS inhibitor are protected from endotoxin-induced cardiac dysfunction, demonstrating the critical role of iNOS.\textsuperscript{32,33} iNOS inhibition also protects adult cardiomyocytes against declined contractility induced by lipopolysaccharide \textit{in vitro}.\textsuperscript{55} Thus, taken together, the decreased cardiac iNOS production in α-MHC-MyD88\textsuperscript{−/−} mice might serve as a potential mechanism for the observed cardiac functional improvement in these mice during endotoxin shock.

In summary, we generated cardiomyocyte- and myeloid-specific MyD88 deletion models to evaluate the contribution of cardiac \textit{versus} circulating MyD88 signaling to the pathogenesis of endotoxin shock. We found that both cardiac- and myeloid-MyD88 signaling play an important role in the mortality and cardiomyopathy after a lethal dose of lipopolysaccharide. Moreover, myeloid-MyD88 signaling plays a predominant role in mediating systemic and cardiac cytokine responses, whereas cardiomyocyte-MyD88 signaling is mainly responsible for mediating myocardial iNOS induction during endotoxin shock.

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**Competing Interests**

The authors declare no competing interests.

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