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 (MyD88$^{fl/fl}$) mice, respectively, to generate cardiomyocyte- (α-MHC-MyD88$^{-/-}$) or myeloid-specific (Lyz-MyD88$^{-/-}$) MyD88 deletion models and their respective MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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)**

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-κB, which in turn leads to the production of multiple inflammatory mediators

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.

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication February 17, 2014. Accepted for publication July 9, 2014. From the Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

including cytokines, chemokines, and antimicrobial peptides.[9] 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.[10–13]

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[14] or deletion[15] are completely unresponsive to lipopolysaccharide, and TLR4 antagonists were found to markedly attenuate endotoxin shock in animals.[16,17] 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.[18,19] 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.[20] 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 (MyD88$^{fl/fl}$) mice, all in C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$ mice were generated by Kawai *et al.*[18] 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 (Prolab 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[21] or lysozyme M promoter[22] were cross-bred with mice with loxP sites flanking exon 3 of *MyD88* gene (MyD88$^{fl/fl}$)[23] to generate cardiomyocyte- (α-MHC-MyD88$^{-/-}$) or myeloid-specific (Lyz-MyD88$^{-/-}$) MyD88 knockout mice, respectively (fig. 1). The MyD88$^{fl/fl}$ littermates were used as control. Breeding colonies were maintained by mating MyD88$^{fl/fl}$ 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

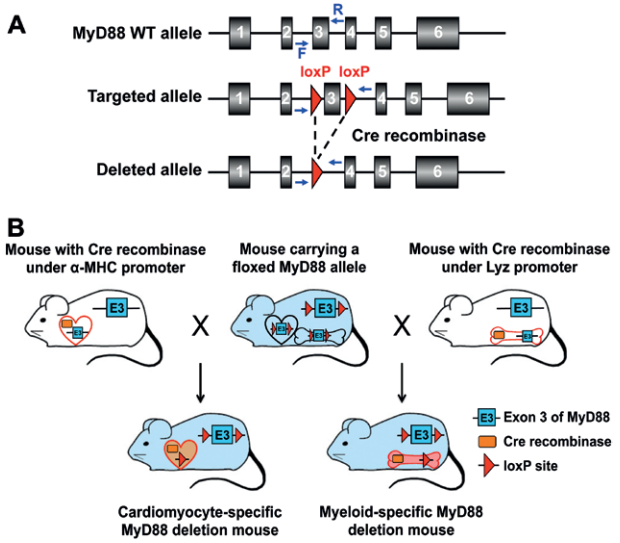

Fig. 1. Generation of cardiomyocyte- and myeloid-specific MyD88 deletion models using the Cre-loxP system. (*A*) Diagram indicating the locations of MyD88 exons (E) and loxP sites flanking E3, and the deletion of the E3 by Cre-recombinase. (*B*) Schematic view of cross-breeding between α-MHC-Cre (*left*) or Lyz-Cre (*right*) transgenic mice, mice carrying loxP sites flanking the E3 (*middle*) and the generation of cardiomyocyte- or myeloid-specific MyD88 deletion mice. E = exon; F = forward primer; Lyz = lysozyme M; α-MHC = α-myosin heavy chain; MyD88 = myeloid differentiation factor 88; R = reverse primer; WT = wild type.

Table 1. Primer Sequences for Genotyping and qRT-PCR

Strain	Primer sequences for genotyping
α-MHC-Cre	Tg Forward: 5'-ATGACAGACAGATCCCTCCTATCTCC-3' Tg Reverse: 5'-CTCATCACTCGTTGCATCATCGAC-3' Control Forward: 5'-CAAATGTTGCTTGTCTGGTG-3' Control Reverse: 5'-GTCAGTCGAGTGCACAGTTT-3'
Lyz-Cre	Mutant: 5'-CCCAGAAATGCCAGATTACG-3' Wildtype: 5'-TTACAGTCGGCCAGGCTGAC-3' Common: 5'-CTTGGGCTGCCAGAATTTCTC-3'
MyD88$^{fl/fl}$	Forward: 5'-GTTGTGTGTGTCCGACCGT-3' Reverse: 5'-GTCAGAAACAACCACCACCATGC-3'
Gene	**Primer sequences for detecting gene deletion**
MyD88 deletion	Forward: 5'-ATACCGGAAGCAGATGGATG-3' Reverse: 5'-AGGCTGAGTGCAAACTTGGT-3'
Gene	**Primer sequences for qRT-PCR**
Mouse 18S	Forward: 5'-CGGCTACCACATCCAAGGAA-3' Reverse: 5'-GCTGGAATTACCGCGGCT-3'
Mouse IL-1β[56]	Forward: 5'-GCCCATCCTCTGTGACTCAT-3' Reverse: 5'-AGGCCACAGGTATTTTGTCG-3'
Mouse TNFα[56]	Forward: 5'-CTGGGACAGTGACCTGGACT-3' Reverse: 5'-GCACCTCAGGGAAGAGTCTG-3'
Mouse IL-6[56]	Forward: 5'-AGTTGCCTTCTTGGGACTGA-3' Reverse: 5'-TCCACGATTTCCCAGAGAAC-3'
Mouse iNOS	Forward: 5'-CTCACTGGGACAGCACAGAA-3' Reverse: 5'-GGTCAAACTCTTGGGGTTCA-3'
Mouse MyD88	Forward: 5'-CCCACAAACAAAGGAACTGG-3' Reverse: 5'-TCATCTCCTGCACAAACTCG-3'

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; MyD88$^{fl/fl}$ = MyD88-loxP 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 4×10^6 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.[19,24] M-mode images were obtained from a parasternal short-axis view at the mid-ventricular level with a clear view of papillary muscle. Left ventricle internal diameters at end-diastole and end-systole (LVIDd and LVIDs, respectively) were measured. The fractional shortening (FS) was defined as (LVIDd – LVIDs) / LVIDd × 100%. The values of three consecutive cardiac cycles were averaged.

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 Corporation, Austin, TX).[19,24] 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–phycoerythrin 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.[25] 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 12,000*g* 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.[26]

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 $MyD88^{fl/fl}$, 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 residential 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 $MyD88^{fl/fl}$ 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

Table 2. Body and Heart Weight of α-MHC-MyD88$^{-/-}$, Lyz-MyD88$^{-/-}$, MyD88$^{fl/fl}$ Mice

	Male		Female	
	Body Weight (g)	Heart Weight (mg)	Body Weight (g)	Heart Weight (mg)
MyD88$^{fl/fl}$	28.0 ± 0.7	120 ± 4	21.7 ± 0.3	110 ± 10
α-MHC-MyD88$^{-/-}$	27.5 ± 0.9	122 ± 7	21.7 ± 1.3	111 ± 8
Lyz-MyD88$^{-/-}$	28.4 ± 0.5	119 ± 5	22.6 ± 1.5	114 ± 5
N	4	4	3	3

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; MyD88$^{fl/fl}$ = MyD88-loxP control mice.

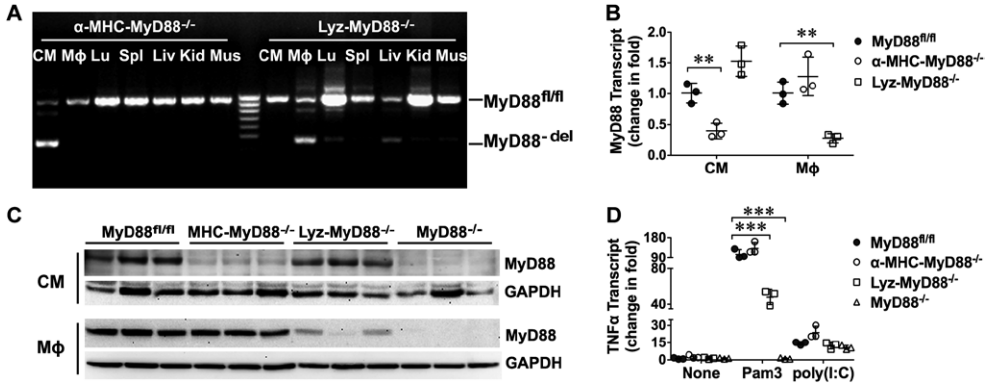

Fig. 2. Characterization of cardiomyocyte- and myeloid-specific MyD88 deletion mice. (*A*) Exon 3 of *MyD88* gene in various tissues DNA from α-MHC-MyD88$^{-/-}$ or Lyz-MyD88$^{-/-}$ mice was amplified by polymerase chain reaction using specific primers. Upper band (950 base pairs) indicated the intact loxP-flanked *MyD88* gene. Lower band (500 base pairs) indicated the deletion of exon 3 of *MyD88* gene. (*B*) Transcript of MyD88 gene in cardiomyocyte (CM) or bone marrow–derived macrophage (Mϕ) isolated from α-MHC-MyD88$^{-/-}$ or Lyz-MyD88$^{-/-}$ mice was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Each *error bar* represents mean ± SD. ** $P < 0.01$, n = 3 in each group. (*C*) Western blot was used to detect MyD88 protein expression in CM or Mϕ isolated from α-MHC-MyD88$^{-/-}$ or Lyz-MyD88$^{-/-}$ mice. (*D*) Mϕ isolated from α-MHC-MyD88$^{-/-}$, Lyz-MyD88$^{-/-}$, MyD88$^{fl/fl}$, and MyD88$^{-/-}$ mice were treated with 10 ng/ml of Pam3Cys (a TLR2 ligand activating TLR2 → MyD88 signaling) or 25 μg/ml of Poly(I:C) (a TLR3 ligand activating TLR3 → Trif signaling) (Enzo Life, Farmingdale, NY). Two hours later, the cells were harvested for tumor necrosis factor-α (TNFα) production as measured by qRT-PCR. Each *error bar* represents mean ± SD. *** $P < 0.001$, n = 3 in each group. del = deletion; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Kid = kidney; Liv = liver; Lu = lung; Lyz-MyD88$^{-/-}$ = myeloid-specific MyD88 knockout mice; Mϕ = bone marrow–derived macrophage; (α-) MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; α-MHC = α-myosin heavy chain; Mus = skeletal muscle; MyD88 = myeloid differentiation factor 88; MyD88$^{-/-}$ = MyD88 knockout mice; MyD88$^{fl/fl}$ = MyD88-loxP control mice; Pam3 = Pam3Cys; Spl = spleen; TLR = Toll-like receptor.

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$^{fl/fl}$ control mice and the two strains of MyD88 knockout mice to the

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 (MyD88$^{fl/fl}$), 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 littermates (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 MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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.

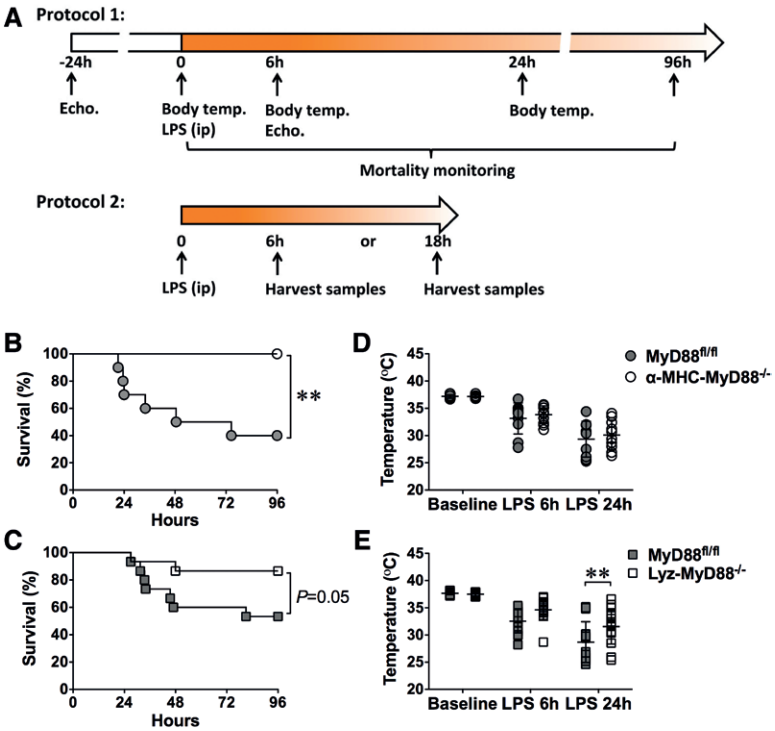

Fig. 3. Effect of cardiomyocyte- and myeloid-specific MyD88 deletion on survival rate and body temperature during endotoxin shock. Both cardiac- (α-MHC-MyD88$^{-/-}$) and myeloid- (Lyz-MyD88$^{-/-}$) MyD88 deletion mice were subjected to lipopolysaccharide (LPS) (15 mg/kg, intraperitoneal injection) together with the littermates control (MyD88$^{fl/fl}$). (*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 mice; α-MHC = α-myosin heavy chain; α-MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; MyD88 = myeloid differentiation factor 88; MyD88$^{fl/fl}$ = MyD88-loxP control mice; 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 MyD88$^{fl/fl}$ littermates were the same (table 3). Six hours after lipopolysaccharide, both MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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*[30,31] and in cardiomyocytes *in vitro*.[31] Cardiac iNOS production has been linked to cardiac dysfunction during endotoxemia.[32,33] We found that lipopolysaccharide administration induced a significant increase in myocardial iNOS messenger RNA expression in the MyD88$^{fl/fl}$ 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 MyD88$^{fl/fl}$ 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 endotoxemia.

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,[18,19] demonstrating the essential role of MyD88 signaling in the pathogenesis of endotoxin shock. To delineate the specific contribution of cardiac *versus*

Table 3. Serial Echocardiographic Measurements of Left Ventricle Function before and 6 h after Lipopolysaccharide Treatment

	Baseline		Lipopolysaccharide 6 h		Baseline		Lipopolysaccharide 6 h	
	MyD88$^{fl/fl}$	α-MHC-MyD88$^{-/-}$	MyD88$^{fl/fl}$	α-MHC-MyD88$^{-/-}$	MyD88$^{fl/fl}$	Lyz-MyD88$^{-/-}$	MyD88$^{fl/fl}$	Lyz-MyD88$^{-/-}$
HR, beats/m	723 ± 31	707 ± 23	584 ± 33†	637 ± 36†#	717 ± 30	696 ± 29	595 ± 20†	617 ± 39†
LVIDd, mm	2.7 ± 0.1	2.8 ± 0.1	3.0 ± 0.2*	2.8 ± 0.2‖	2.8 ± 0.2	3.0 ± 0.1§	3.0 ± 0.2*	3.1 ± 0.2
LVIDs, mm	1.2 ± 0.1	1.1 ± 0.1	1.9 ± 0.5†	1.7 ± 0.3†	1.0 ± 0.1	1.2 ± 0.1§	2.1 ± 0.3†	1.8 ± 0.2†#
FS, %	57 ± 3	59 ± 4	38 ± 13†	41 ± 9†	63 ± 4	60 ± 3‡	31 ± 5†	43 ± 6†**
N	10	10	10	10	10	10	10	10

The three strains of mice were subjected to 15 mg/kg of lipopolysaccharide treatment. Six hours later, the mice were anesthetized and the cardiac function was measured by echocardiography as detailed in Materials and Methods.

* P < 0.01, † P < 0.001 *vs.* Baseline; ‡ P < 0.05, § P < 0.01 *vs.* MyD88$^{fl/fl}$-Baseline; ‖ P < 0.05, # P < 0.01, ** P < 0.001 *vs.* MyD88$^{fl/fl}$-lipopolysaccharide 6 h.

FS = fractional shortening; HR = heart rate; LVIDd = left ventricular internal diameter at end-diastole; LVIDs = left ventricular internal diameter at end-systole; Lyz-MyD88$^{-/-}$ = myeloid-specific MyD88 knockout mice; α-MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; MyD88$^{fl/fl}$ = MyD88-loxP control mice.

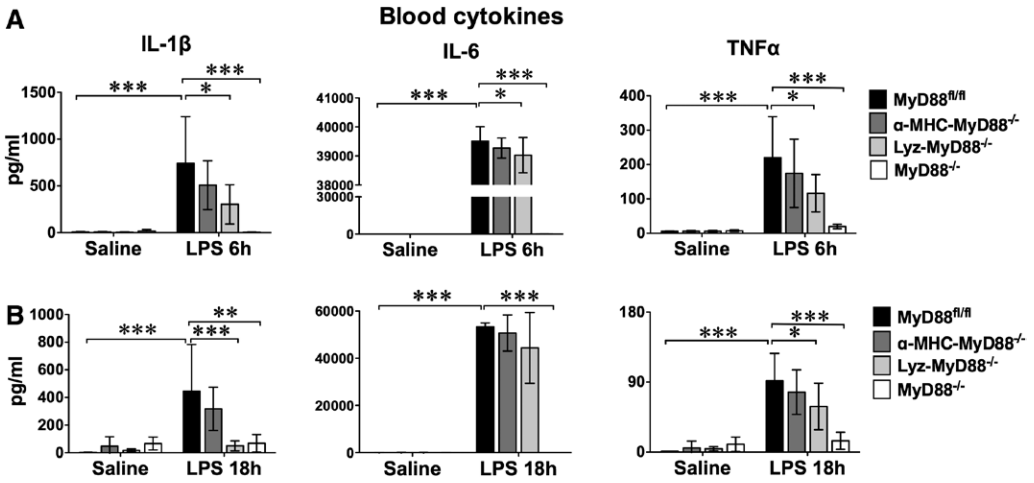

Fig. 4. Effect of cardiomyocyte- and myeloid-specific MyD88 deletion on cytokine production in plasma at 6 h (*A*) and 18 h (*B*) after lipopolysaccharide (LPS) administration. Cardiac- (α-MHC-MyD88$^{-/-}$), myeloid- (Lyz-MyD88$^{-/-}$) MyD88 deletion mice, control (MyD88$^{fl/fl}$) mice, and systemic MyD88 knockout (MyD88$^{-/-}$) mice were treated with LPS (15 mg/kg, intraperitoneal injection) or saline. Six or 18 h later, blood was collected. Plasma interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNFα) were measured with a multiplex fluorescent bead-based immunoassay. Each *error bar* represents mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 3 mice in MyD88$^{-/-}$ Saline group, n = 4 mice in other Saline groups and MyD88$^{-/-}$ LPS group, n = 7 mice in other LPS groups. Lyz-MyD88$^{-/-}$ = myeloid-specific MyD88 knockout mice; α-MHC = α-myosin heavy chain; α-MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; MyD88 = myeloid differentiation factor 88; MyD88$^{-/-}$ = MyD88 knockout mice; MyD88$^{fl/fl}$ = MyD88-loxP control mice.

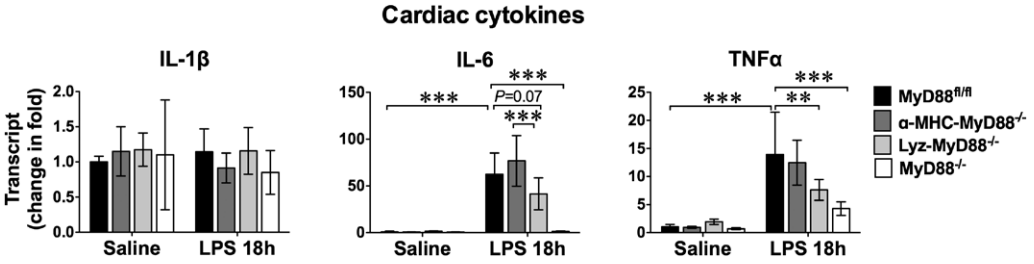

Fig. 5. Effect of cardiomyocyte- and myeloid-specific MyD88 deletion on cardiac cytokine production during endotoxin shock. Cardiac- (α-MHC-MyD88$^{-/-}$), myeloid- (Lyz-MyD88$^{-/-}$) MyD88 deletion mice, control (MyD88$^{fl/fl}$) mice, and systemic MyD88 knockout (MyD88$^{-/-}$) mice were subjected to lipopolysaccharide (LPS) (15 mg/kg, intraperitoneal injection) or saline. Eighteen hours later, heart was harvested and myocardial interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNFα) were measured by quantitative reverse transcription-polymerase chain reaction. Each *error bar* represents mean ± SD. ** $P < 0.01$, *** $P < 0.001$, n = 3 mice in MyD88$^{-/-}$ Saline group, n = 4 mice in other Saline groups and MyD88$^{-/-}$ LPS group, n = 7 mice in other LPS groups. Lyz-MyD88$^{-/-}$ = myeloidspecific MyD88 knockout mice; α-MHC = α-myosin heavy chain; α-MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; MyD88 = myeloid differentiation factor 88; MyD88$^{-/-}$ = MyD88 knockout mice; MyD88$^{fl/fl}$ = MyD88-loxP control mice.

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 linage 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 residential macrophages, which maintain MyD88 expression. In our study, we isolated adult cardiomyocytes after enzymatic

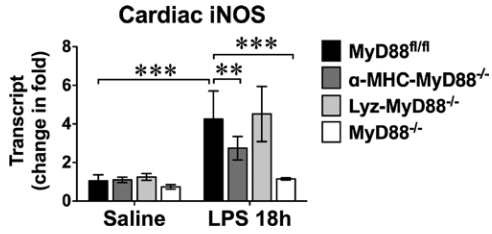

Fig. 6. Effect of cardiomyocyte- and myeloid-specific MyD88 deletion on myocardial inducible nitric oxide synthase (iNOS) production during endotoxin shock. Cardiac- (α-MHC-MyD88$^{-/-}$), myeloid- (Lyz-MyD88$^{-/-}$) MyD88 deletion mice, control (MyD88$^{fl/fl}$) mice, and systemic MyD88 knockout (MyD88$^{-/-}$) mice were subjected to lipopolysaccharide (LPS) (15 mg/kg, intraperitoneal injection) or saline. Eighteen hours later, heart was harvested and myocardial iNOS was measured by quantitative reverse transcription-polymerase chain reaction. Each *error bar* represents mean ± SD. ** $P < 0.01$, *** $P < 0.001$, n = 3 mice in MyD88$^{-/-}$ Saline group, n = 4 mice in other Saline groups and MyD88$^{-/-}$ LPS group, n = 7 mice in other LPS groups. Lyz-MyD88$^{-/-}$ = myeloid-specific MyD88 knockout mice; α-MHC = α-myosin heavy chain; α-MHC-MyD88$^{-/-}$ = cardiomyocyte-specific MyD88 knockout mice; MyD88 = myeloid differentiation factor 88; MyD88$^{-/-}$ = MyD88 knockout mice; MyD88$^{fl/fl}$ = MyD88-loxP control mice.

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.[21] Also, they found that different α-MHC-Cre strain showed different degree of gene deletion. Similarly, others report that in cells of the myeloid linage, 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 Lyz-MyD88$^{-/-}$ mice had markedly attenuated, but not completely abolished, TNFα production in response to TLR2 activation by Pam-3cys. These suggest that the myeloid-MyD88 signaling was significantly attenuated in Lyz-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 MyD88$^{fl/fl}$ 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[27] 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[39] and local adrenal inflammation[40] 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[41,42] and human clinical studies.[43–45] Cytokines can cause profound vasodilation and compromise tissue perfusion.[46,47] Systemic microcirculation dysfunction is considered to be a fundamental cause of multiple organ dysfunction in sepsis.[48] Excessive amount of cytokines (*e.g.*, TNFα) increases endothelial permeability,[49,50] tissue edema, and in turn organ dysfunction. Therefore, animals lacking MyD88 signaling in the myeloid linage cells (Lyz-MyD88$^{-/-}$) 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$^{-/-}$ 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$^{-/-}$ mice had nearly the same cardiac contractile function as that of Lyz-MyD88$^{-/-}$ mice (FS: 41 ± 9% *vs.* 43 ± 6%), the difference in cardiac function between α-MHC-MyD88$^{-/-}$ and MyD88$^{fl/fl}$ 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.*[51] found that cardiomyocytes isolated from lipopolysaccharide-treated mice exhibited reduced sarcomere shortening and Ca^{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 endotoxic shock.[51–53] Based on our current study, we propose that both myeloid and cardiac MyD88 signaling plays a role in cardiac dysfunction during endotoxin shock.

Accumulating data demonstrated that nitric oxide plays a critical role in the pathogenesis of myocardial dysfunction in sepsis.[54] Nitric oxide is synthesized by NOS from L-arginine. There are total three 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 *in vivo* and *in vitro*.[30,31] iNOS-deficient mice or mice-treated iNOS inhibitor are protected from endotoxin-induced cardiac dysfunction, demonstrating the critical role of iNOS.[32,33] iNOS inhibition also protects adult cardiomyocytes against declined contractility induced by lipopolysaccharide *in vitro*.[55] Thus, taken together, the decreased cardiac iNOS production in α-MHC-MyD88$^{-/-}$ 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 *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.

Acknowledgments

The authors sincerely thank Yuntai Yao, M.D., Ph.D., Yu Gong, Ph.D., and Jiayan Cai, B.S. (Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts), for their assistance.

This work was supported in part by the grants R01-GM080906 and R01-GM097259 (to Dr. Chao) from the National Institutes of Health, Bethesda, Maryland.

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Feng: Massachusetts General Hospital, 149 13th Street, Room 4423, Charlestown, Massachusetts 02129. yfeng@partners.org. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Marik PE: Surviving sepsis: Going beyond the guidelines. Ann Intensive Care 2011; 1:17
2. Miniño AM, Heron MP, Murphy SL, Kochanek KD; Centers for Disease Control and Prevention National Center for Health Statistics National Vital Statistics System: Deaths: Final data for 2004. Natl Vital Stat Rep 2007; 55:1–119
3. Levy MM, Dellinger RP, Townsend SR, Linde-Zwirble WT, Marshall JC, Bion J, Schorr C, Artigas A, Ramsay G, Beale R, Parker MM, Gerlach H, Reinhart K, Silva E, Harvey M, Regan S, Angus DC: The Surviving Sepsis Campaign: Results of an international guideline-based performance improvement program targeting severe sepsis. Intensive Care Med 2010; 36:222–31
4. Zanotti-Cavazzoni SL, Hollenberg SM: Cardiac dysfunction in severe sepsis and septic shock. Curr Opin Crit Care 2009; 15:392–7
5. Kawai T, Akira S: The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat Immunol 2010; 11:373–84
6. Feng Y, Chao W: Toll-like receptors and myocardial inflammation. Int J Inflam 2011; 2011:170352
7. Chao W: Toll-like receptor signaling: A critical modulator of cell survival and ischemic injury in the heart. Am J Physiol Heart Circ Physiol 2009; 296:H1–12
8. Miggin SM, O'Neill LA: New insights into the regulation of TLR signaling. J Leukoc Biol 2006; 80:220–6
9. Kawai T, Akira S: Signaling to NF-κB by Toll-like receptors. Trends Mol Med 2007; 13:460–9
10. Parrillo JE: Pathogenetic mechanisms of septic shock. N Engl J Med 1993; 328:1471–7

11. Parrillo JE: Septic shock—Vasopressin, norepinephrine, and urgency. N Engl J Med 2008; 358:954–6
12. Rittirsch D, Flierl MA, Ward PA: Harmful molecular mechanisms in sepsis. Nat Rev Immunol 2008; 8:776–87
13. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. N Engl J Med 2003; 348:138–50
14. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. Science 1998; 282:2085–8
15. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the Lps gene product. J Immunol 1999; 162:3749–52
16. Sha T, Sunamoto M, Kitazaki T, Sato J, Ii M, Iizawa Y: Therapeutic effects of TAK-242, a novel selective Toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model. Eur J Pharmacol 2007; 571:231–9
17. Ehrentraut S, Lohner R, Schwederski M, Ehrentraut H, Boehm O, Noga S, Langhoff P, Baumgarten G, Meyer R, Knuefermann P: *In vivo* Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. Shock 2011; 36:613–20
18. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S: Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999; 11:115–22
19. Feng Y, Zou L, Zhang M, Li Y, Chen C, Chao W: MyD88 and Trif signaling play distinct roles in cardiac dysfunction and mortality during endotoxin shock and polymicrobial sepsis. Anesthesiology 2011; 115:555–67
20. Zou L, Feng Y, Zhang M, Li Y, Chao W: Nonhematopoietic toll-like receptor 2 contributes to neutrophil and cardiac function impairment during polymicrobial sepsis. Shock 2011; 36:370–80
21. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD: Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle *in vivo*. J Clin Invest 1997; 100:169–79
22. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I: Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999; 8:265–77
23. Hou B, Reizis B, DeFranco AL: Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity 2008; 29:272–82
24. Zou L, Feng Y, Chen YJ, Si R, Shen S, Zhou Q, Ichinose F, Scherrer-Crosbie M, Chao W: Toll-like receptor 2 plays a critical role in cardiac dysfunction during polymicrobial sepsis. Crit Care Med 2010; 38:1335–42
25. Feng Y, Zhao H, Xu X, Buys ES, Raher MJ, Bopassa JC, Thibault H, Scherrer-Crosbie M, Schmidt U, Chao W: Innate immune adaptor MyD88 mediates neutrophil recruitment and myocardial injury after ischemia-reperfusion in mice. Am J Physiol Heart Circ Physiol 2008; 295:H1311–8
26. Feng Y, Zou L, Si R, Nagasaka Y, Chao W: Bone marrow MyD88 signaling modulates neutrophil function and ischemic myocardial injury. Am J Physiol Cell Physiol 2010; 299:C760–9
27. Koitabashi N, Bedja D, Zaiman AL, Pinto YM, Zhang M, Gabrielson KL, Takimoto E, Kass DA: Avoidance of transient cardiomyopathy in cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. Circ Res 2009; 105:12–5
28. Blanqué R, Meakin C, Millet S, Gardner CR: Hypothermia as an indicator of the acute effects of lipopolysaccharides: Comparison with serum levels of IL1β, IL6 and TNFα. Gen Pharmacol 1996; 27:973–7
29. Pittet D, Thiévent B, Wenzel RP, Li N, Auckenthaler R, Suter PM: Bedside prediction of mortality from bacteremic sepsis. A dynamic analysis of ICU patients. Am J Respir Crit Care Med 1996; 153:684–93
30. Liu S, Adcock IM, Old RW, Barnes PJ, Evans TW: Lipopolysaccharide treatment *in vivo* induces widespread tissue expression of inducible nitric oxide synthase mRNA. Biochem Biophys Res Commun 1993; 196:1208–13
31. Zhu X, Zhao H, Graveline AR, Buys ES, Schmidt U, Bloch KD, Rosenzweig A, Chao W: MyD88 and NOS2 are essential for toll-like receptor 4-mediated survival effect in cardiomyocytes. Am J Physiol Heart Circ Physiol 2006; 291:H1900–9
32. Ullrich R, Scherrer-Crosbie M, Bloch KD, Ichinose F, Nakajima H, Picard MH, Zapol WM, Quezado ZM: Congenital deficiency of nitric oxide synthase 2 protects against endotoxin-induced myocardial dysfunction in mice. Circulation 2000; 102:1440–6
33. Ichinose F, Hataishi R, Wu JC, Kawai N, Rodrigues AC, Mallari C, Post JM, Parkinson JF, Picard MH, Bloch KD, Zapol WM: A selective inducible NOS dimerization inhibitor prevents systemic, cardiac, and pulmonary hemodynamic dysfunction in endotoxemic mice. Am J Physiol Heart Circ Physiol 2003; 285:H2524–30
34. Molkentin JD, Robbins J: With great power comes great responsibility: Using mouse genetics to study cardiac hypertrophy and failure. J Mol Cell Cardiol 2009; 46:130–6
35. Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, Jay PY: Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. J Card Fail 2006; 12:392–8
36. Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB: Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. J Clin Invest 1999; 104:1703–14
37. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD: Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. Circ Res 2001; 89:20–5
38. Andersson KB, Winer LH, Mørk HK, Molkentin JD, Jaisser F: Tamoxifen administration routes and dosage for inducible Cre-mediated gene disruption in mouse hearts. Transgenic Res 2010; 19:715–25
39. Ruud J, Wilhelms DB, Nilsson A, Eskilsson A, Tang YJ, Ströhle P, Caesar R, Schwaninger M, Wunderlich T, Bäckhed F, Engblom D, Blomqvist A: Inflammation- and tumor-induced anorexia and weight loss require MyD88 in hematopoietic/myeloid cells but not in brain endothelial or neural cells. FASEB J 2013; 27:1973–80
40. Kanczkowski W, Alexaki VI, Tran N, Großklaus S, Zacharowski K, Martinez A, Popovics P, Block NL, Chavakis T, Schally AV, Bornstein SR: Hypothalamo-pituitary and immune-dependent adrenal regulation during systemic inflammation. Proc Natl Acad Sci U S A 2013; 110:14801–6
41. Johnson J, Meyrick B, Jesmok G, Brigham KL: Human recombinant tumor necrosis factor α infusion mimics endotoxemia in awake sheep. J Appl Physiol (1985) 1989; 66:1448–54
42. Mayoral JL, Schweich CJ, Dunn DL: Decreased tumor necrosis factor production during the initial stages of infection correlates with survival during murine gram-negative sepsis. Arch Surg 1990; 125:24–7; discussion 28
43. Casey LC, Balk RA, Bone RC: Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. Ann Intern Med 1993; 119:771–8
44. Blackwell TS, Christman JW: Sepsis and cytokines: Current status. Br J Anaesth 1996; 77:110–7
45. Gårdlund B, Sjölin J, Nilsson A, Roll M, Wickerts CJ, Wretlind B: Plasma levels of cytokines in primary septic shock in humans: Correlation with disease severity. J Infect Dis 1995; 172:296–301

46. Vicaut E, Hou X, Payen D, Bousseau A, Tedgui A: Acute effects of tumor necrosis factor on the microcirculation in rat cremaster muscle. J Clin Invest 1991; 87:1537–40
47. Takahashi K, Ando K, Ono A, Shimosawa T, Ogata E, Fujita T: Tumor necrosis factor-α induces vascular hyporesponsiveness in Sprague-Dawley rats. Life Sci 1992; 50:1437–44
48. Lehr HA, Bittinger F, Kirkpatrick CJ: Microcirculatory dysfunction in sepsis: A pathogenetic basis for therapy? J Pathol 2000; 190:373–86
49. Jahr J, Grände PO: *In vivo* effects of tumor necrosis factor-α on capillary permeability and vascular tone in a skeletal muscle. Acta Anaesthesiol Scand 1996; 40:256–61
50. Koga S, Morris S, Ogawa S, Liao H, Bilezikian JP, Chen G, Thompson WJ, Ashikaga T, Brett J, Stern DM, Pinsky DJ: TNF modulates endothelial properties by decreasing cAMP. Am J Physiol 1995; 268: C1104–13
51. Tavener SA, Long EM, Robbins SM, McRae KM, Van Remmen H, Kubes P: Immune cell Toll-like receptor 4 is required for cardiac myocyte impairment during endotoxemia. Circ Res 2004; 95:700–7
52. Binck BW, Tsen MF, Islas M, White DJ, Schultz RA, Willis MS, Garcia JV, Horton JW, Thomas JA: Bone marrow-derived cells contribute to contractile dysfunction in endotoxic shock. Am J Physiol Heart Circ Physiol 2005; 288:H577–83
53. Tavener SA, Kubes P: Cellular and molecular mechanisms underlying LPS-associated myocyte impairment. Am J Physiol Heart Circ Physiol 2006; 290:H800–6
54. Flynn A, Chokkalingam Mani B, Mather PJ: Sepsis-induced cardiomyopathy: A review of pathophysiologic mechanisms. Heart Fail Rev 2010; 15:605–11
55. Baumgarten G, Knuefermann P, Schuhmacher G, Vervölgyi V, von Rappard J, Dreiner U, Fink K, Djoufack C, Hoeft A, Grohé C, Knowlton AA, Meyer R: Toll-like receptor 4, nitric oxide, and myocardial depression in endotoxemia. Shock 2006; 25:43–9
56. Favre J, Musette P, Douin-Echinard V, Laude K, Henry JP, Arnal JF, Thuillez C, Richard V: Toll-like receptors 2-deficient mice are protected against postischemic coronary endothelial dysfunction. Arterioscler Thromb Vasc Biol 2007; 27: 1064–71