MyD88 and Trif Signaling Play Distinct Roles in Cardiac Dysfunction and Mortality during Endotoxin Shock and Polymicrobial Sepsis

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ABSTRACT

Background: Toll-like receptors (TLRs) such as TLR2, TLR4, and TLR9 contribute to the pathogenesis of polymicrobial sepsis. These TLRs signal via the common myeloid differentiation factor 88 (MyD88)-dependent pathways. TLR4 also signals through MyD88-independent but TIR domain-containing adaptor inducing interferon-β-mediated transcription factor (Trif)-dependent pathway. The role of the two signaling pathways in cardiac dysfunction during polymicrobial sepsis and endotoxin shock is unknown.

Methods: Sepsis was generated by cecum ligation and puncture. Mice were divided into sham and cecum ligation and puncture groups or subjected to saline or endotoxin. Left ventricular function was assessed in a Langendorff apparatus or by echocardiography. Cytokines were examined using a multiplex immunoassay. Neutrophil migratory and phagocytic functions were assessed using flow cytometry.

Results: In comparison with wild-type mice, MyD88−/− but not Trif−/− mice had markedly improved cardiac function and survival after cecum ligation and puncture. In comparison, both MyD88−/− and Trif−/− mice were protected from cardiac depression and mortality during endotoxin shock. Septic MyD88−/− but not Trif−/− mice had diminished cytokine production in serum and in peritoneal space in comparison with wild-type mice after cecum ligation and puncture. In contrast, both MyD88−/− and Trif−/− mice had attenuated serum cytokines in comparison with wild-type mice after endotoxin challenge. Neither MyD88 nor Trif signaling had any effect on neutrophil phagocytic function or bacterial clearance at 24 h of polymicrobial sepsis.

Conclusions: These studies establish that MyD88 but not Trif signaling plays a critical role in mediating cardiac dysfunction, systemic inflammation, and mortality during polymicrobial sepsis. Both MyD88 and Trif are essential for cardiac depression and mortality during endotoxin shock.

Sepsis is the systemic inflammatory response syndrome that occurs during infection. It has an estimated prevalence of 751,000 cases each year; more than 210,000 of these patients die.1 Sepsis is the 10th leading cause of death in the United States.2 Cardiovascular collapse induced by cardiac dysfunction and profound vasodilatation represents a main feature of septic shock and contributes to its high mortality.

Germline-encoded innate immune receptors such as Toll-like receptors (TLRs) represent the first line of host defense against pathogen invasion. TLRs play an important role in infectious and noninfectious tissue injury.3–5 All TLRs, with the exception of TLR3, signal through the common MyD88-dependent pathway.6 TLR3 exclusively and TLR4 partially signal via MyD88-independent but Trif-dependent pathway.7,8 These innate immune receptors play a pivotal role in host innate immune response and modulate adaptive immunity against foreign pathogens.9 On the other hand, studies have established that inappropriate and imbalanced host inflammatory response via TLR-dependent mechanisms may also contribute to the
pathogenesis of sepsis. For example, TLR2, TLR4, and TLR9 are found to mediate host inflammatory response and immune cell dysfunction and contribute to the high mortality in lethal models of polymicrobial sepsis. 10–14 Deficiency of MyD88, the adapter for the three TLRs, leads to attenuated systemic inflammation and reduced lymphocyte apoptosis and affects the survival in mouse models of polymicrobial peritonitis. 15,16 Trif is a key adaptor for TLR3 and TLR4 signaling and is primarily responsible for viral (and bacterial) stimulation via the induction of type I interferons (IFNs) including IFN-α and IFN-β. 8 Reports on the role of type I IFN signaling in polymicrobial sepsis are controversial, and the effect of the pathway seems to depend on both the severity and type of animal models of bacterial sepsis. 17,18 In animal models of endotoxin shock, a TLR4-mediated event, both MyD88 and Trif deficiency lead to a survival benefit. 6,7

The heart expresses at least two receptors involved in TLR signaling, i.e., TLR2 and TLR4. 19–22 Studies have suggested that these receptors are in part responsible for cardiac dysfunction in endotoxic shock (by TLR4) and in bacterial sepsis. For example, TLR4 is essential for endotoxin-induced left ventricular (LV) dysfunction, 21 whereas TLR2 is involved in cardiac dysfunction induced by bacterial infection. 14,20 However, the relative contributions of MyD88 versus Trif signaling to the development of cardiac dysfunction in these two models of sepsis are unknown.

Here, we sought to test the role of MyD88 and Trif signaling pathways in the pathogenesis of polymicrobial peritonitis sepsis and endotoxin shock. We found that in comparison with wild-type (WT) mice, mice deficient in MyD88 but not Trif had markedly improved cardiac function, significant reduction in systemic and peritoneal cytokine concentrations, and markedly improved survival after polymicrobial sepsis. Importantly, both MyD88 and Trif are essential for endotoxin-induced cardiac dysfunction. Thus, the current study establishes that MyD88 signaling is the dominant pathway mediating cardiac dysfunction and contributes to the high mortality during severe polymicrobial sepsis and that both MyD88 and Trif pathways play an equally important role in myocardial depression during endotoxin shock.

**Materials and Methods**

**Animals**

WT mice (C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88−/− mice were generated by Kawai et al. 6 and had been backcrossed more than 10 generations into the C57BL/6 strain. 16 Trif−/− mice were generated by Yamamoto et al. 8 All mice used in the study were 8 to 12 weeks old and weighed 20–30 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 the Massachusetts General Hospital (Boston, MA). The experiments were performed in compliance with the guideline from the National Institutes of Health (Bethesda, MD).

**Mouse Model of Polymicrobial Sepsis**

Polymicrobial peritonitis was generated by cecum ligation and puncture (CLP) as described previously. 14,23 Briefly, after general anesthesia, the cecum was ligated 1.0 cm from the tip of the cecum, punctured twice with an 18-gauge needle, and squeezed gently to expel a small amount of fecal materials before being returned to the abdominal cavity. The sham-operated mice underwent laparotomy but without CLP. After surgery, prewarmed normal saline (0.1 ml/g body weight) was administered subcutaneously.

**Endotoxin Shock Model**

To induce endotoxin shock in mice, lipopolysaccharide (Escherichia coli 0111:B4, Sigma Chemical Company, St Louis, MO) was administered at the dose of 15 mg/kg body weight by intraperitoneal injection followed by administration of 1 ml prewarmed saline. The same volume of normal saline was administered to the control mice. Thirty min before echocardiographic measurements, 1 ml prewarmed saline was injected into each mouse and the mouse cages were warmed to 30°C under light for 15–20 min.

**Langendorff Perfusion of Isolated Heart**

LV functions of the hearts isolated from septic or sham mice were measured in a Langendorff perfusion apparatus as described previously with some modifications. 14,24,25 Briefly, 24 hours after sham or CLP procedures, mice were heparinized (1,000 IU/kg) subcutaneously and euthanized. The hearts were excised and aortas were cannulated. The coronary arteries were perfused in a Langendorff apparatus at a constant pressure of 90 mmHg with modified Krebs-Henseleit buffer containing 118 mM NaCl, 24 mM NaHCO3, 10 mM glucose, 4.7 mM KCl, 4.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, and 2 mM pyruvate, pH 7.4, at 37°C. The perfusate was saturated with continuous gas flow consisting of 95% O2 and 5% CO2. A balloon was made with wrapped saran film connected to a PE-50 polyethylene tube (Becton Dickinson and Company, Sparks, MD). The neck-to-tip distance of the balloon was approximately 6–7 mm. The balloon was inserted into the LV chamber through the mitral valve with an incision in the left atrium and was connected to a pressure transducer (APT300, Harvard Apparatus, Holliston, MA) for continuous measurement of LV pressure. The balloon was inflated with deionized water (15–20 μl) to adjust left ventricular end-diastolic pressure to 6–10 mmHg. The perfused hearts were paced at 420 beats/min with pacing electrodes placed on the right atrium. After 10 min of perfusion, LV function data (LV systolic pressure, left ventricular end-diastolic pressure, and heart rate) were recorded continuously on a data acquisition system (PowerLab, AD Instruments, Colorado Springs, CO). Left ventricular developed pressure (LVDP) was calculated as the difference between peak systolic pressure and left ventricular end-diastolic pressure. dP/dtmax and...
dP/dt_{max} were calculated as the maximum rate of increase and decrease of LVDP, respectively.

**Echocardiography in Mice**

Mice were lightly anesthetized with ketamine (20 mg/kg). Transthoracic echocardiographic images were obtained 1 day before saline or lipopolysaccharide administration (baseline), so animals had sufficient time to recover from light anesthesia, and again 6 h after saline or lipopolysaccharide administration. 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. M-mode images were obtained from a parasternal short-axis view at the midventricular level with a clear view of papillary muscle. LV internal diameters at end-diatole and end-systole (LVIDd and LVIDs, respectively) were measured. The fractional shortening was defined as (LVIDd-LVIDs)/LVIDd × 100%. The values of three consecutive cardiac cycles were averaged.

**Mortality Study**

After sham or CLP procedure, mice were observed every 12 h for up to 14 days. Mice in whom saline or lipopolysaccharide was administered were closely monitored for up to 96 h.

**Multiplex Cytokine/Chemokine Immunoassays**

Twenty-four hours after sham or CLP procedures, blood and peritoneal lavage fluid were collected. Cell-free supernatants were saved for cytokine measurements and the cell pellets were discarded. Twenty-four hours after sham or CLP procedures, 5 ml of normal saline was injected into the peritoneal space and mixed thoroughly by injection of microspheres were detected by a cocktail of biotinylated antibodies. After binding of streptavidin-phycoerythrin (BD Company, Sparks, MD), and incubated at 37°C for 30 min. Cells were then washed, stained with Gr-1 antibody, and analyzed with flow cytometry for Gr-1+ (allophycocyanin) and phagocytic neutrophils (fluorescein isothiocyanate−), which were expressed as a percentage of Gr-1+ neutrophils.

**Bacterial Counts**

Twenty-four hours after sham or CLP procedures, 5 ml of sterile normal saline were injected into the peritoneal space and mixed thoroughly by gentle massage of the abdomen. Three ml of the peritoneal lavage were collected. Blood samples were collected and anticoagulated. Samples were serial diluted, plated on Trypticase soy agar with 5% sheep blood (BD Company, Sparks, MD), and incubated at 37°C for 14–16 h. Colony-forming units were counted and expressed as log_{10} of colony-forming unit/ml of blood or lavage fluid.

**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 ± SE. The P values of cytokine production analysis were applied on the log_{10} scale and based on the two-tailed independent Student t test. For those cytokine production values below detection limit, values input at the detection limit were used in a nonparametric test and the P values were based on Mann–Whitney U test results. For echocardiographic measurement, LVDP and dP/dt data, the statistical significance of the difference between two groups (e.g., sham vs. CLP, normal saline vs. lipopolysaccharide, WT vs. knockout mice) at the different time points was measured by two-way ANOVA with Bonferroni correction posttests and repeated measurements. Of note, these specific comparisons were made based on a priori hypotheses rather than pure statistical considerations. Neutrophil migration and bacterial loading data were analyzed with one-way ANOVA with Tukey posttest. 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 null hypothesis was rejected for P < 0.05.

**Results**

**MyD88−/− but Not Trif−/− Mice have Improved Cardiac Function during Polymicrobial Sepsis**

To test cardiac dysfunction in polymicrobial sepsis, we isolated the hearts from sham-operated or septic mice 24 h after surgeries and perfused them in a Langendorff apparatus at a constant pressure. The ex vivo system measures LV contrac-
tile function with a constant preload that may not be achievable in an in vivo septic condition. Under these conditions, septic WT mice exhibited LVDP (67.5 ± 6.5 mmHg at 40 min of perfusion time), dP/dt_max (3127 ± 304 mmHg/s), and dP/dt_min (1884 ± 223 mmHg/s), which were significantly lower than in sham-operated mice (fig. 1A). Importantly, compared with septic WT mice, septic MyD88−/− mice had markedly improved LVDP (96.0 ± 1.4 mmHg), dP/dt_max (4374 ± 193 mmHg/s) and dP/dt_min (2783 ± 108 mmHg/s) at 40 min of perfusion time (fig. 1A). In contrast, Trif−/− mice subjected to CLP did not exhibit any improvement in the cardiac function in comparison with septic WT mice (Fig. 1B). Of note, consistent with our previous work,25 the current data indicate that C57BL/6 WT, MyD88−/−, and Trif−/− mice have similar baseline cardiac functions after sham operation (fig. 1A and B).

MyD88−/− but Not Trif−/− Mice have a Higher Survival Rate during Polymicrobial Sepsis

To assess the overall effect of MyD88 and Trif signaling pathways during bacterial sepsis, we examined the survival rates of WT, MyD88−/−, and Trif−/− mice after CLP and sham procedures. As illustrated in figure 2, polymicrobial peritonitis after CLP induced severe sepsis that resulted in a mortality rate of up to 80% within 3 days in WT mice (20% on day 2; 80% on day 3 and day 14). Trif−/− mice followed a mortality course similar to that of WT mice, reaching 70% mortality on day 2 and 80% on day 11 after CLP procedure. There was no significant difference in mortality between WT and Trif−/− mice after CLP. In comparison, MyD88−/− mice with polymicrobial sepsis had a marked reduction in the mortality rate (0% on day 2, 20% day 3, and 40% at day 14). There was no mortality in the three groups of mice subjected to sham operation. These data suggest that the MyD88-dependent pathway plays a predominant role in mediating cardiac dysfunction during polymicrobial sepsis and contributes to its high mortality.

Both MyD88−/− and Trif−/− Mice are Protected from Cardiac Dysfunction during Endotoxin Shock

TLR4 mediates cardiac dysfunction during endotoxin shock.21 TLR4 signals via both MyD88- and Trif-dependent pathways.6,7 However, the relative contribution of the
two pathways to cardiac dysfunction during endotoxin shock is unknown. We first measured LV function \textit{ex vivo} in the hearts isolated from mice treated with saline or lipopolysaccharide for 6 h. As indicated in figure 3, in comparison with saline-treated animals, there was a marked decrease in LV contractile function 6 h after lipopolysaccharide administration as demonstrated by reduced LVDP (70.6 ± 6.7 mmHg at 40 min of perfusion time vs. 94.4 ± 2.9 mmHg), dP/dt max (3,022 ± 581 vs. 3,842 ± 694 mmHg/s), and dP/dt min (1,664 ± 297 vs. 2,283 ± 463 mmHg/s). In contrast, MyD88^{-/-} mice were protected with normal LV function after lipopolysaccharide treatment with normal LVDP (105 ± 6 mmHg at 40 min of perfusion), dP/dt max (4,234 ± 333 mmHg/s), and dP/dt min (2,439 ± 166 mmHg/s) (fig. 3A). Compared with saline-treated Trif^{-/-} mice, lipopolysaccharide-treated Trif^{-/-} mice had a trend of somewhat decreased LV function although the difference was statistically insignificant (fig. 3B). We also measured cardiac function \textit{in vivo} using echocardiography 1 day before and 6 h after saline or lipopolysaccharide or saline injection as illustrated in figure 4A and summarized in table 1. Six hours after lipopolysaccharide but not saline administration, there was a marked depression in LV contractile function as demonstrated by a 64% reduction in fraction shortening (56% vs. 20%) and a 92% increase in LV internal diameter at end-systole (LVIDs: 1.3 vs. 2.5 mm). In contrast, MyD88^{-/-} and Trif^{-/-} mice had only slightly depressed LV contractile function and fraction shortening was slightly decreased by 11% and 13%, respectively, in MyD88^{-/-} and Trif^{-/-} mice compared with WT mice after lipopolysaccharide administration (table 1). LVIDs and LVIDd were only mildly affected in septic MyD88^{-/-} and Trif^{-/-} mice by lipopolysaccharide administration. Associated with the improved cardiac function in MyD88^{-/-} and Trif^{-/-} mice was their marked prolonged survival (Fig. 4B). At the dose of 15 mg/kg, lipopolysaccharide administration resulted in profound shock and a high mortality within 24 h in WT mice, whereas saline-treated mice had zero mortality (N = 4, data not shown). Both MyD88^{-/-} and Trif^{-/-} mice were largely protected from endotoxin shock-induced death. Thus, in contrast with that in polymicrobial sepsis, cardiac dysfunction and high mortality induced by endotoxin shock critically depend on both MyD88 and Trif signaling.

**MyD88 but Not Trif Signaling Plays a Predominant Role in Local and Systemic Cytokine Response during Polymeric Bacterial Sepsis**

Given the distinct role of MyD88 versus Trif signaling in cardiac dysfunction and mortality during polymicrobial sepsis, we next examined how MyD88 or Trif would affect cytokine and chemokine production during polymicrobial peritonitis infection. Cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) are potent proinflammatory mediators that are known for contributing to septic mortality whereas IL-10 is antiinflammatory. Keratinocyte chemoattractant and macrophage inflammatory protein-2 are potent chemoattractants for neutrophils.27,28 As illustrated in Fig. 5, in comparison with sham-operated mice that had minimal concentrations of cytokines/chemokines, septic WT mice had a marked increase in IL-6, IL-10, TNFα, keratinocyte chemoattractant, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2 concentrations in the peritoneal lavage (fig. 5A) as well as serum (fig. 5B). MyD88 deficiency led to a significant decrease in keratinocyte chemoattractant and IL-10 concentrations in the peritoneum (fig. 5A) and remarkably, almost abolished all six cytokines production in the serum (fig. 5B). Of note, the peritoneal concentrations of the cytokines represent the diluted values as the peritoneal fluid was mixed with 5 ml normal saline before it was collected. In contrast, septic Trif^{-/-} mice had similar concentrations of the cytokines compared with the septic WT littermates, both in the peritoneum (fig. 6A) and in the serum (fig. 6B). Taken together, these data clearly suggest that MyD88 but not Trif signaling plays a predominant role in mediating both local and systemic cytokine response during peritoneal polymicrobial sepsis.

**MyD88 and Trif Signaling Play an Important Role in Systemic Cytokine Response during Endotoxin Shock**

Similar to the CLP model, the endotoxin shock model induced a marked increase in serum cytokine production including IL-6, TNFα, and IL-1β in WT mice. In comparison, MyD88^{-/-} mice had much lower concentrations of IL-6, TNFα, and IL-1β (fig. 7). Similarly, Trif^{-/-} mice had much lower concentrations of TNF-α and IL-1β and significantly decreased IL-6 concentration. These data suggest that both MyD88 and Trif signaling play an important role in systemic inflammation during endotoxin shock.

![Fig. 2. Mouse mortality during polymicrobial sepsis. Agematched WT, MyD88^{-/-}, and Trif^{-/-} mice were subjected to CLP or sham procedures and the mortality of the septic mice were followed daily for up to 14 days. CLP = cecum ligation and puncture; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type. The number of animals in each group: WT-Sham = 5, WT-CLP = 5, MyD88^{-/-}-Sham = 5, MyD88^{-/-}-CLP = 6, Trif^{-/-}-Sham = 5, Trif^{-/-}-CLP = 6. * P < 0.05 versus WT-CLP.](image-url)
MyD88 and Trif Have Distinct Roles in Neutrophil Functions during Polymicrobial Peritonitis

To assess the effect of MyD88 and Trif signaling on neutrophil function during sepsis, we first examined neutrophil recruitment into the peritoneal space after CLP in WT, MyD88−/−, and Trif−/− mice. We have previously shown that only a small number of Gr-1+ neutrophils (2 × 10⁶ cells/cavity), which represents approximately 30% of the peritoneal cell population, are present in the peritoneum of the sham-operated mice. The percentage of the peritoneal Gr-1+ neutrophils increases to 85% in CLP mice. Similarly, the current study indicates that 24 h after CLP, a large number of neutrophils migrated into the peritoneal space of WT CLP mice (32 × 10⁶ cells/cavity) (fig. 8A). In comparison with septic WT mice, MyD88−/− mice had a 41% reduction in the number of recruited peritoneal Gr-1+ neutrophils (P < 0.05). In contrast, Trif−/− mice had 59% of increase in neutrophil migration compared with septic WT mice (P < 0.05) (fig. 8A). Because phagocytosis is an important function of neutrophils, we next examined the effect of MyD88 and Trif signaling on the phagocytic function of neutrophils by measuring their ability to engulf fluorescent beads ex vivo. As indicated in fig. 8B, there was no difference in the percentage of phagocytic peritoneal neutrophils among MyD88−/−, Trif−/−, and WT mice. These studies suggest that MyD88 or Trif signaling affects neutrophil migratory function in distinct ways but played a minimal role in the phagocytic function of neutrophils during severe polymicrobial peritonitis.

MyD88 or Trif Deficiency Has No Effect on Bacterial Clearance during Polymicrobial Sepsis

We next examined the bacterial loading in the blood and the peritoneal lavage from WT, MyD88−/−, and Trif−/− mice 24 h after sham or CLP surgery. No bacteria grew in any of the sham samples (data not shown). All of the mice subjected to CLP surgery exhibited significant bacterial counts in the plasma as well as in the peritoneal lavage (fig. 9). However, there was no difference among the three strains of mice, indicating that MyD88 or Trif signaling plays a minimal role in the host bacterial clearance during severe polymicrobial sepsis.

Fig. 3. Langendorff measurements of LV function in WT, MyD88−/−, and Trif−/− mice during endotoxin shock. Age-matched WT, MyD88−/−, and Trif−/− mice were administered LPS (15 mg/kg, intraperitoneal injection) or saline. Six hours later, the hearts were isolated and perfused in a Langendorff apparatus. Left ventricular (LV) contractile function was measured as described in Materials and Methods. (A) MyD88−/− versus WT; (B) Trif−/− versus WT. dP/dt max = the maximum first derivative of LVDP; dP/dt min = the minimum first derivative of LVDP; LPS = lipopolysaccharide; LVDP = left ventricular developed pressure; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type. Each data point and error bar represents the mean ± SE. The number of animals in each group: WT-Saline = 6, WT-LPS = 7, MyD88−/−-Saline = 5, MyD88−/−-LPS = 5, Trif−/−-Saline = 4, Trif−/−-LPS = 4. ** P < 0.01; *** P < 0.001, MyD88−/−-LPS or WT-Saline versus WT-LPS; # P < 0.05; ## P < 0.01; ### P < 0.001, WT-LPS versus WT-Saline.
Fig. 4. Both MyD88−/− and Trif−/− mice are resistant to endotoxin shock. Age-matched WT, MyD88−/−, and Trif−/− mice were administered with LPS (15 mg/kg, intraperitoneal injection) or saline (data not shown). The mice were subsequently examined for left ventricular function by serial echocardiography and mortality. (A) Representative M-mode echocardiograms were presented at the baseline and 6 h after LPS administration. (B) Survival rate after LPS administration. LPS = lipopolysaccharide; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type. N = 5 in all 3 LPS groups; * P < 0.05 versus WT.

Discussion

The current study demonstrates that signaling via MyD88 and Trif, the two adaptors critical for the innate immune function, plays distinct roles in cardiac dysfunction and mortality in two different models of sepsis, namely endotoxin shock and polymicrobial peritoneal sepsis. We found that in comparison with WT mice, MyD88−/− mice had markedly improved cardiac function and prolonged survival with both

Table 1. Serial Echocardiographic Measurements of Left Ventricular Function 24 h before and 6 h after Lipopolysaccharide Administration

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<th>Baseline 6 h</th>
<th>Lipopolysaccharide 6 h</th>
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<td></td>
<td>Baseline 6 h</td>
<td>Lipopolysaccharide 6 h</td>
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<tr>
<td>Heart rate, beats/min</td>
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<td>WT</td>
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<td>Trif−/−</td>
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<tr>
<td>Trif−/−</td>
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<td>Fractional shortening, %</td>
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<td>Trif−/−</td>
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Baseline echocardiography was measured 1 day before saline or lipopolysaccharide administration (baseline) and again 6 h after saline or lipopolysaccharide administration (6 h). Values are mean ± SE. The number of animals in each group: saline groups = 4; lipopolysaccharide = 5.

* P < 0.05; ** P < 0.01; *** P < 0.001 versus lipopolysaccharide baseline; # P < 0.05; ## P < 0.01; ### P < 0.001 versus WT-lipopolysaccharide-6 h; † P < 0.05 versus MyD88−/−-lipopolysaccharide-6 h; § P < 0.05; §§ P < 0.01; §§§ P < 0.001 versus saline-6 h.

LVIDd = left ventricular internal diameter at end-diastole; LVIDs = left ventricular internal diameter at end-systole; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type.
endotoxin shock and polymicrobial sepsis. Trif−/− mice, on the other hand, were only protected in endotoxin shock with improved cardiac function and survival, but were not protected during polymicrobial sepsis. The improved heart function and survival in MyD88−/− mice were associated with markedly attenuated systemic and local inflammation during bacterial sepsis. Finally, neither MyD88 nor Trif signaling had any major effect on neutrophil phagocytic function and bacterial clearance in severe polymicrobial peritonitis sepsis.

Numerous animal models of sepsis have been used that replicate either signs and symptoms or laboratory findings observed in human sepsis. Chaudry et al.29 introduced a widely used classification of three septic models: bacterial infusion models, endotoxin models, and polymicrobial peritonitis models. Infusion models use bolus or short-term infusion of bacteria.30 These models have several limitations31 and do not correlate well with the clinical situations whereas in most instances, there is a focus of infection providing continuous dissemination of bacteria. Endotoxin models in-
volve administration of lipopolysaccharide and simulates the clinical situation of septic shock. Endotoxin models are highly reproducible and can provide great insight into inflammatory processes. However, these models lack an infectious focus and do not closely mimic the pathophysiology observed in patients with sepsis. Bacterial peritonitis models closely resemble the clinical scenario of sepsis after bowel perforation. The most widely used peritonitis model is CLP. Similar to many clinical cases of sepsis, the CLP model induces polymicrobial sepsis, but the model has wide variability in terms of the host inflammatory and physiologic responses and the degree of bacteremia and mortality rates. Nevertheless, the CLP model used in the current study represents a clinically relevant model of sepsis and has been validated in several laboratories. Hotchkiss et al. have demonstrated that both gram-positive and gram-negative organisms are present in blood cultures in CLP mice but not in sham-operated mice.

Previous studies have established that TLRs such as TLR2, TLR4, and TLR9 all contribute to the pathogenesis of polymicrobial sepsis. TLR2-deficient mice have a decreased inflammatory response, improved neutrophil migration, and improved hemodynamics and LV contractile function during sepsis. Interestingly, TLR2 deficiency seems to offer no protection against a lethal gram-negative (E. coli) bacterial sepsis. In contrast, either TLR4 deletion or inhibition seems to confer protection in lethal E. coli or polymicrobial sepsis. Similarly, mice deficient in TLR9, a sensor for unmethylated CpG motifs present in bacterial DNA, are resistant to lethal polymicrobial sepsis and have improved survival. It should be pointed out that in most of

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**Fig. 6.** Trif deficiency has no effect on peritoneal lavage and serum cytokine levels during polymicrobial sepsis. WT and Trif−/− mice underwent sham or CLP procedures. Twenty-four hours later, peritoneal lavage and blood were collected. Peritoneal lavage and serum IL-6, IL-10, TNFα, KC, MCP-1, and MIP-2 were measured with a multiplex fluorescent bead-based immunoassay. (A) Peritoneal lavage cytokines. (B) Serum cytokines. Each error bar represents mean ± SE. The number of animals in each group: WT-Sham = 3, WT-CLP = 4, Trif−/−-Sham = 3, Trif−/−-CLP = 6. CLP = cecum ligation and puncture; IL = interleukin; KC = keratinocyte chemoattractant; MCP-1 = monocyte chemoattractant protein-1; MIP-2 = macrophage inflammatory protein-2; MyD88 = myeloid differentiation factor 88; TNF = tumor necrosis factor; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type.
MyD88 and Trif signaling in septic cardiac dysfunction

In septiسمic peritonitis, a highly inflammatory model with extracellular bacterial infection, the presence of the TLR signaling is often protective and plays a critical role in host immune response against invading pathogen.11,16,36–38

Using the CLP model16 or a similar model,15 studies have established the critical role of MyD88 signaling in the pathogenesis of polymicrobial sepsis. In a colon ascendent stent peritonitis model, a highly inflammatory model with extremely high mortality, MyD88−/− mice were found to be protected with improved survival and attenuated systemic inflammation within the first 48 h.15 However, in a CLP model with a slower onset of peritoneal polymicrobial sepsis, MyD88−/− mice had worse survival compared with WT mice despite significantly attenuated systemic inflammation and reduced lymphocyte apoptosis in these mice.16 In comparison, the role of Trif signaling in polymicrobial sepsis is not well understood. Again, in a less severe sepsis model, Trif−/− mice have reduced cytokine production including TNFα, IL-6, and IL-10, suggesting Trif signaling may contribute to systemic inflammation in the mild form of animal sepsis.16 Trif signaling is primarily responsible for host antiviral responses and activation of type I IFN.7,8,39 Deficiency in TLR340 or Trif41 leads to increased viral load, decreased antiviral cytokines, and increased mortality. One of the main downstream effectors of the Trif pathway is type I IFNs, which can also be induced via MyD88-dependent mechanisms.8 Type I IFN signaling is implicated in the inflammatory response and mortality in a lethal form of polymicrobial sepsis.17 However, in a milder form of polymicrobial sepsis, type I IFN signaling is protective.18 The current study extends the previous studies and demonstrates that MyD88 signaling is the dominant determinant in mediating cardiac dysfunction and mortality, whereas Trif signaling plays no major role in the development of cardiac dysfunction and mortality in severe polymicrobial sepsis. But in endotoxin shock, MyD88 and Trif play an equally important role in mediating cardiac depression and high mortality. Therefore, given its critical role in the endotoxin-elicited cardiac depression, the lack of effect by Trif signaling on cardiac function during polymicrobial sepsis noted in the current study suggests that endotoxin is probably not the cause of cardiac dysfunction during polymicrobial peritonitis sepsis. This is consistent with a previous study that demonstrates that loss of the host ability to respond to bacterial lipopolysaccharide, i.e., in TLR4def, has no effect on survival of mice in a lethal model of polymicrobial peritonitis sepsis,42 suggesting that host mobilizes different innate defense mechanisms in endotoxemia and polymicrobial septic peritonitis.

![Fig. 7. Effect of MyD88 and Trif deficiency on endotoxin-induced cytokine production in serum. WT, MyD88+/−, and Trif−/− mice were administered LPS (15 mg/kg, intraperitoneal injection) or saline. Six hours later, blood was collected. Serum IL-6, TNFα, and IL-1β were measured with a multiplex fluorescent bead-based immunoassay. Each error bar represents mean ± SE. The number of animals in each group: WT-Saline = 5, WT-LPS = 7, MyD88−/−-Saline = 5, MyD88−/−-LPS = 5, Trif−/−-Saline = 3, Trif−/−-LPS = 6. * P < 0.05; ** P < 0.01; *** P < 0.001. IL = interleukin; LPS = lipopolysaccharide; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type.](image1)

![Fig. 8. Effect of MyD88 and Trif deficiency on neutrophil migration and phagocytic functions during polymicrobial sepsis. Twenty-four hours after CLP procedures, peritoneal cells were harvested from lavage fluid and manually counted. 5 × 10⁶ cells from each mouse were loaded for flow cytometry analysis of Gr-1− neutrophils or for phagocytosis analysis. (A) Total Gr-1− neutrophils in the peritoneum. Neutrophil numbers were calculated based on the total cells numbers manually counted multiplied by the percentage of neutrophils as measured by flow cytometry gated on Gr-1+. The horizontal bars represent the mean values in each mouse group. The number of animals in each group: WT-CLP = 18, MyD88+/−-CLP = 18, Trif−/−-CLP = 7. (B) Percentage of phagocytic neutrophils in the peritoneal space. Peritoneal cells were incubated with opsonized fluorescent microspheres at 37°C for 30 min and analyzed with flow cytometry. Percentages of phagocytic (FITC-stained) neutrophils (Gr-1−) were presented. The number of animals in each group: WT-CLP = 17; MyD88+/−-CLP = 15; Trif−/−-CLP = 6. * P < 0.05; ** P < 0.001. CLP = cecum ligation and puncture; FITC = fluorescein isothiocyanate; MyD88 = myeloid differentiation factor 88; Trif = TIR-domain-containing adaptor protein inducing interferon-β mediated transcription-factor; WT = wild-type.](image2)
cytokine production may have contributed to the improved signaling in the infectious peritoneum. The attenuated systemic production of IL-6, IL-10, TNFα, keratinocyte chemoattractant, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2 seems independent of MyD88, and Trif, strongly suggesting a predominant role of MyD88 signaling in systemic inflammation during peritoneal polymicrobial sepsis. It is noteworthy that MyD88 deficiency has more selective effect on the peritoneal neutrophil migration. A recent report by Kelly-Scumpia et al. has demonstrated that mice deficient for type I IFN receptors (IFNAR−/−) have impaired peritoneal neutrophil migration in a much less severe form of polymicrobial peritonitis during the early stage and impaired bacterial clearance at a later stage. In contrast, Trif−/− mice seem to have an enhanced neutrophil migration. Some limitations of the current study should be noted. First, the mouse model of polymicrobial sepsis induces a profound systemic inflammatory response that is often observed in the early stage of sepsis. It does not create immunosuppression that often is the feature of patients who survive the initial hyperinflammatory injury but then succumb to secondary infection and organ failure later on. Therefore, the altered neutrophil migratory function in MyD88−/− and Trif−/− mice did not affect the overall bacterial clearance because both MyD88−/− and Trif−/− mice had the same levels of bacterial load at 24 h of polymicrobial sepsis.

Some limitations of the current study should be noted. The plasma concentrations of cytokines such as TNFα, IL-6, and IL-1β correlate with patient survival during sepsis. In the current study, MyD88 deletion almost abolished systemic production of IL-6, IL-10, TNFα, keratinocyte chemoattractant, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2, only impairing keratinocyte chemoattractant and IL-10 production, whereas IL-6, TNFα, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2 production seems independent of MyD88 signaling in the infectious peritoneum. The attenuated systemic cytokine production may have contributed to the improved cardiac function and better survival in these septic MyD88−/− mice. However, elimination of deleterious cardiac MyD88 signaling, independent of systemic cytokines, could also contribute to the improved cardiac function. Our previous study has demonstrated that direct activation of TLR2 signaling is capable of inhibiting sarcomere shortening and intracellular Ca2+ transients in isolated cardiomyocyte.

Neutrophils play a critical role in sepsis pathogenesis. To delineate the mechanisms by which MyD88 signaling contributes to septic pathogenesis, we investigated the role of MyD88 signaling in neutrophil functions such as migration, phagocytosis, and bacterial clearance. We have previously shown that polymicrobial peritoneal infection induces a large number of neutrophil influx and dramatic increase in the percentage of neutrophils in the peritoneum 24 h after CLP. Importantly, systemic deficiency of MyD88 attenuates peritoneal neutrophil migration. This is somewhat different from what Weighardt et al. reported. In that study, the investigators used the colon ascendens stent peritonitis model and found no difference in peritoneal neutrophil migration and bacterial clearance between WT mice and MyD88−/− mice 12 h after colon ascendens stent peritonitis. However, the colon ascendens stent peritonitis model creates such acute and severe sepsis pathology that nearly 80% of the septic mice die within 24 h. In our study, the CLP model confers a less acute sepsis course with approximately 20% mortality at 24 h and thus permits us to examine neutrophil migration at a later time. Our data suggest that the peritoneal neutrophil migration in response to polymicrobial infection is at least partially MyD88-dependent. Moreover, such MyD88 dependency for a robust neutrophil migration to the sites of inflammation appears independent of pathogens or pathogenic components because neutrophil migration in the thioglycolate-elicited peritonitis, a noninfectious model of tissue inflammation, is also dependent on MyD88 signaling. Nevertheless, the altered neutrophil migratory function in MyD88−/− and Trif−/− mice did not affect the overall bacterial clearance because both MyD88−/− and Trif−/− mice had the same levels of bacterial load at 24 h of polymicrobial sepsis.

In summary, the current study has demonstrated the distinct roles of the two key innate immune signaling pathways, namely MyD88 and Trif, in mediating cardiac dysfunction and mortality during endotoxin shock and polymicrobial peritonitis sepsis. Both MyD88 and Trif signaling are important for endotoxin shock-elicited cardiac dysfunction, an exclusive TLR4-mediated event. In contrast, MyD88 signaling appears to be the predominant pathway controlling system inflammatory re-
sponse and cardiac dysfunction during polymicrobial sepsis, a pathologic process known to involve multiple TLRs. Trif signaling plays no major role in this process. This study indicates that specifically targeting MyD88 signaling may represent a novel strategy in managing patients with cardiovascular dysfunction during bacterial sepsis.

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References


36. Albigera B, Dahlberg S, Henriques-Normark B, Normark S: Role of the innate immune system in host defence against

**ANESTHESIOLOGY REFLECTIONS**

**A Rural Scene by Dennis Jackson**

While corresponding with Librarian Walter Necker in 1964, pharmacologist and anesthesiologist Dennis E. Jackson suggested the possibility of painting a work of art for the Wood Library-Museum. The following year, Dr. William Dornette discussed delivering that painting to the WLM. A Rural Scene (left) is likely that very painting. Featuring two people aboard a small boat on a lake in the countryside, A Rural Scene is signed in the lower right hand corner by its artist. Jackson’s signature (right) reflects the artistic side of this pioneer, who brought carbon dioxide absorption into mainstream anesthetic practice. (Copyright © the American Society of Anesthesiologists, Inc. This image also appears in the Anesthesiology Reflections online collection available at www.anesthesiology.org.)

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