Toll-like Receptor 4 Is Essential to Preserving Cardiac Function and Survival in Low-grade Polymicrobial Sepsis

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ABSTRACT

Background: Toll-like receptor 4 (TLR4), the receptor for endotoxin, mediates hyperinflammatory response and contributes to high mortality during both endotoxin shock and severe sepsis. However, little is known about the role of TLR4 in the pathogenesis of low-grade polymicrobial sepsis, which is often associated with immunosuppression.

Methods: Low-grade polymicrobial sepsis was generated by cecum ligation and puncture. Mortality was monitored in wild-type (C57BL/10ScSn) and TLR4^{def} (C57BL/10ScCr) mice. *Ex vivo* heart and individual cardiomyocyte function were assessed in Langendorff (Hugo Sachs Elektronik; Harvard Apparatus, Holliston, MA) and IonOptix systems (IonOptix, Milton, MA), respectively. Serum chemistry was tested for liver and kidney injury. Cytokines were examined using a multiplex immunoassay. Neutrophil migratory and phagocytic functions were assessed using flow cytometry. Reactive oxygen species were measured using redox-sensitive dichlorodihydrofluorescein dye.

Results: Following cecum ligation and puncture, wild-type mice developed bacterial peritonitis with mild cardiac dysfunction (n = 3 in sham and n = 8 in cecum ligation and puncture) and a mortality of 23% within 14 days (n = 22). In comparison, septic TLR4^{def} mice had deleterious cardiac dysfunction (n = 6 in sham and n = 10 in cecum ligation and puncture), kidney and liver injury (n = 7), and much higher mortality at 81% (n = 21). The deleterious effects observed in septic TLR4^{def} mice were associated with increased local and systemic cytokine response, reduced neutrophil migratory and phagocytic function, increased reactive oxygen species generation in leukocytes, and impaired bacterial clearance.

Conclusion: TLR4 plays an essential role in host defense against low-grade polymicrobial sepsis by mediating neutrophil migratory/phagocytic functions, attenuating inflammation, reducing reactive oxygen species generation, and enhanced bacterial clearance. (ANESTHESIOLOGY 2014; 121:1270-80)

S EPSIS has an estimated prevalence of 751,000 cases each year.[1] Between 1979 and 2000, there was a steady increase in the incidence of sepsis.[2] Even though the total in-hospital mortality rate fell to 17.9% during the period from 1995 through 2000, the total number of sepsis-related deaths continued to rise.[2] Myocardial depression and associated hemodynamic collapse are among the major causes of death in severe sepsis.[3]

Toll-like receptors (TLRs) are an important member of the innate immunity and represent the first line of host defense against pathogen invasion.[4] As illustrated in figure 1, various TLRs detect different pathogens through the pathogen-associated molecular patterns recognition. All TLRs with exception of TLR3 signal through MyD88.[4] TLR4 also signals *via* Trif.[4] TLRs such as TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 have been identified in cardiomyocytes.[5] Natural deletion of TLR4, a receptor for lipopolysaccharide (endotoxin),[6] protects against lipopolysaccharide-induced cardiac dysfunction.[7,8] We have demonstrated that genetic

> **What We Already Know about This Topic**
>
> - Toll-like receptors are an important member of the innate immunity and represent the first line of host defense against pathogen invasion
> - While the role of toll-like receptor 4 signaling in endotoxin shock is well defined, its role in bacterial sepsis is less clear
> - This study investigated the role of toll-like receptor 4 in cardiac dysfunction, neutrophil impairment, and bacterial clearance in a low-grade bacterial sepsis model
>
> **What This Article Tells Us That Is New**
>
> - Deletion of toll-like receptor 4 in mice results in attenuated neutrophil function, decreased bacterial clearance, deleterious cardiac dysfunction and kidney/liver injury, and markedly increased mortality during low-grade polymicrobial sepsis

deletion of MyD88 or Trif, two adaptors downstream of TLR4, confers a profound protection with markedly improved cardiac function and survival in an endotoxin shock model.[9] These findings establish that TLR4 signaling

is responsible for myocardial depression and mortality during endotoxin shock.

The pathogenesis of bacterial sepsis has been described as immunological imbalance characterized by early hyperinflammatory response featured by proinflammatory cytokine storm and late immunosupressive phase characterized by a shift to antiinflammatory cytokines, T-cell anergy, and immune cell death.[10] While hyperinflammatory response associated with endotoxin shock or severe sepsis could be lethal, immunosupression is believed to be the predominant cause for morbidity and mortality of many intensive care unit septic patients who have survived the initial hyperinflammatory attack.[11] Death in the immunosuppressed septic patients is typically due to failure to control the primary infection and the acquisition of secondary hospital-acquired infections.[11–13] Therefore, an effective host defense is crucial for the survival of septic patients, particularly for those immunosuppressed patients. In an animal model of low-grade polymicrobial

sepsis, as defined by relatively low mortality, followed by a second hit of bacterial challenge, Muenzer *et al.*[14] demonstrate that immunosuppresion created by the low-grade sepsis model increases susceptibility to the secondary bacterial infection.

While the role of TLR4 signaling in endotoxin shock is well defined, its role in bacterial sepsis is less clear. The reports on the role of TLR4 in severe bacterial sepsis have been somewhat conflicting. Both protective and contributory roles have been proposed for TLR4 in severe lethal bacterial sepsis.[15,16] Nevertheless, the role of TLR4 signaling in low-grade polymicrobial sepsis is unclear. Our overall hypothesis was that intact TLR4 signaling is essential for host immune defense against polymicrobial infection during low-grade sepsis. Specifically, we hypothesized that mice lacking TLR4 would have impaired neutrophil function and higher bacterial load as compared with mice possessing TLR4. Consequently, we anticipated that these TLR4-deficient mice would have higher mortality,
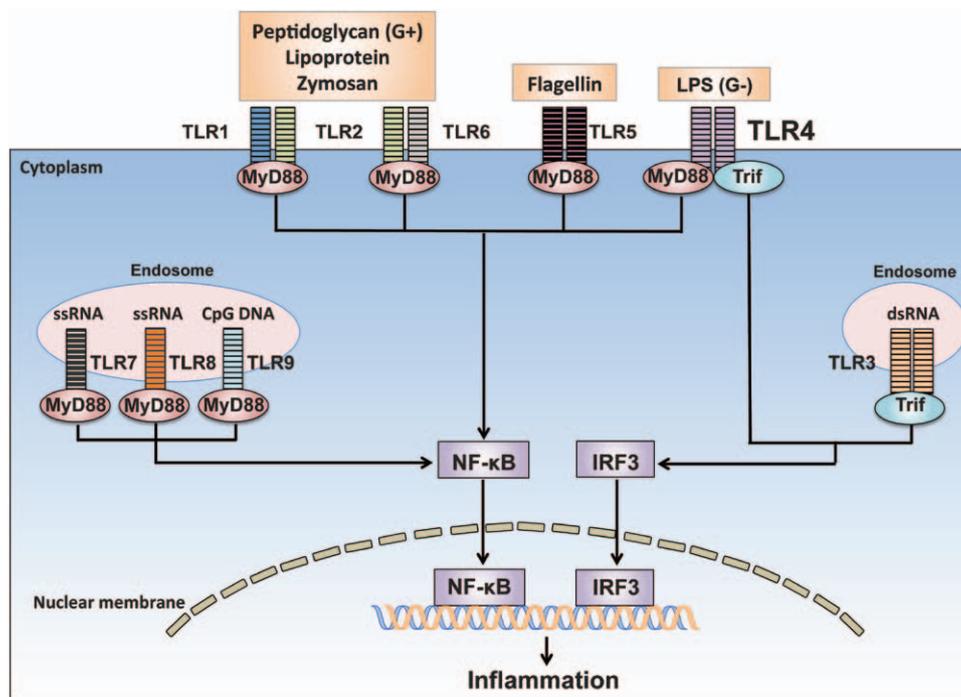

Fig. 1. Pathogen sensing by toll-like receptors (TLRs). TLRs are pattern-recognition receptors. All TLRs are transmembrane proteins. Some TLRs such as TLR1, 2, 4, 5, and 6 are expressed on the cell surface, whereas others such as TLR3, 7, 8, and 9 are located almost exclusively in intracellular compartments such as endosomes. Different TLRs recognize different microbial components.[49] For example, TLR4 senses lipopolysaccharide (LPS), a wall component of Gram-negative (G–) bacteria such as *Escherichia coli*. TLR2 recognizes lipoprotein, a wall component of Gram-positive (G+) bacteria such as *Staphylococcus aureus* or *Streptococcus pneumoniate.* TLR2 heterodimerizes either with TLR1 to recognize triacylated lipopeptide or with TLR6 to recognize diacylated lipopeptides. TLR5 senses bacterial flagellin, a protein component of flagella. TLR3 recognizes viral double-stranded RNA (dsRNA), whereas TLR7 and 8 are the sensors for single-stranded RNA (ssRNA). Finally, TLR9 senses bacterial CpG-rich hypomethylated DNA (CpG DNA) motifs. With the exception of TLR3, all TLR members signal through the adaptor myeloid differentiation primary-response gene 88 (MyD88) to recruit the downstream kinases. TLR3 signals through the adaptor TIR domain containing adaptor protein inducing interferon-β (Trif)-mediated transcription factor. TLR4 signals through both MyD88- and Trif-dependent pathways. Activation of these signaling pathways ultimately activates the transcription factors such as nuclear factor-κB (NF-κB) and IFN regulatory factor 3 (IRF3), which leads to production of diverse proinflammatory cytokines.[50]

worsening cardiac function, and deleterious kidney and hepatic injury.

Materials and Methods

Animals

Eight- to 12 week-old, age- and sex-matched mice were used for the studies. Wild-type (WT) (C57BL/10ScSn) and TLR4[def] mice (C57BL/10ScCr) were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/10ScCr is also referred to as C57BL/10ScNJ (stock no. 003752) with WT IL-12Rβ2 allele. C57BL/10ScCr mice have a deletion of the tlr4 gene, which results in the absence of both TLR4 messenger RNA and protein and, thus, a defective response to lipopolysaccharide. C57BL/10ScCr mice differs from C3H/HeJ mice with a point tlr4 mutation that causes an amino acid substitution.[6] C57BL/10ScSn mice were used as the appropriate WT controls for the TLR4[def] mice. All mice were housed and maintained in temperature-controlled, air-conditioned facilities with 12h/12h light–dark cycles and fed with the same bacteria-free diet (Prolab Isopro RMH 3000; LabDiet, Brentwood, MO). All animal experiments were performed with the approval of the Subcommittee on Research Animal Care of Massachusetts General Hospital (Charlestown, Massachusetts). Simple randomization method was used to assign animals to various experimental conditions. Different groups were processed identically throughout the whole experiment. For example, (1) all mice used were sex and age matched, (2) all mice were of the same inbred strains (except for the sex difference, mice of an inbred strain were genetically alike), and (3) mice were housed on the same shelves in the same rooms before and after surgery.

Mouse Model of Low-grade Polymicrobial Sepsis

In brief, cecum was ligated 1.0 cm from the tip and punctured through to through with an 18-gauge needle. A small amount of fecal materials was squeezed gently to expel before the ligated cecum was returned to the abdominal cavity. The sham-operated mice underwent laparotomy but without cecum ligation and puncture (CLP). After surgery, prewarmed normal saline (0.05 ml/g body weight) was administered subcutaneously. The low grade of sepsis in C57BL/10ScSn mice was defined by low mortality rate (–20%),[17] modest cytokine responses, and mild organ dysfunction. Of note, all surgeries were performed by operators blinded to the strain information.

Endotoxin Shock Model

Endotoxin shock model was induced as previously described.[7,9] Mice were administered with lipopolysaccharide (15 mg/kg body weight) (*Escherichia coli* 0111:B4; Sigma, St. Louis, MO) by intraperitoneal injection followed by administration of 1 ml of prewarmed normal saline.

Langendorff System

Left ventricular (LV) function was assessed in a Langendorff perfusion system (Hugo Sachs Elektronik—Harvard Apparatus, March-Hugstetten, Germany) as described previously.[18,19] Briefly, mice were heparinized (1,000 IU/kg, subcutaneously) and euthanized. After the heart was excised, the aorta was quickly cannulated and retrograde-perfused at a constant flow rate (3 ml/min) at 37°C with modified Krebs–Henseleit buffer (NaCl, 118.5 mM; NaHCO$_3$, 25 mM; D-Glucose, 11.2 mM; KCl, 4.7 mM; MgSO$_4$, 1.2 mM; KH$_2$PO$_4$, 1.2 mM; sodium pyruvate, 2 mM; and CaCl$_2$, 2 mM). The heart was paced at 7 Hz (420 beats/min). After 20 min of coronary perfusion, LV end-systolic pressure, LV end-diastolic pressure, and dP/dt$_{max}$ (maximal first derivative of LV developed pressure [LVDP]) were contiuously recorded for up to 30 min. LVDP was calculated as follows: LVDP = LV end-systolic pressure – LV end-diastolic pressure.

Cardiomyocyte Sarcomere Shortening and Intracellular Calcium Measurement

Sarcomere shortening and Ca^{2+} transients were recorded simultaneously on an IonOptix system (IonOptix, Milton, MA) as described previously.[18,20,21] Adult cardiomyocytes were incubated with membrane-permeable fluorescent indicator fura-2 AM (1 μM) (Invitrogen, Carlsbad, CA) and probenecid (0.5 mM), placed in a flow chamber that was perfused with 1.2 mM Ca^{2+} Tyrode solution (NaCl 137 mM, KCl 5.4 mM, HEPES 0.5 mM, MgCl$_2$ 0.5 mM, Glucose 5.5 mM), and electrically paced at 1, 2, 4, and 6 Hz *via* platinum wires. The Ca^{2+} transients and sarcomere shortening were analyzed based on single-cell-averaged tracing. The final values were derived from 14 to 15 individual cells in each group and calculated for statistical analysis. Four mice from each group were used to prepare cardiomyocytes for the functional studies.

Echocardiographic Assessment of Cardiac Function

Transthoracic echocardiographic images were obtained 1 day before (baseline) and again at 6h after lipopolysaccharide administration. In brief, 30 mins prior to echocardiographic measurements, 1 ml of prewarmed normal saline was injected to each mouse and mouse cages were warmed to 30°C under light for 15 to 20 min. Mice were lightly anesthetized with ketamine (20 mg/kg). All images were collected using a 13.0-MHz linear probe (Vivid 7; GE Medical System, Milwaukee, WI) as described previously.[9] M-mode images were obtained from a parasternal short-axis view at the mid-ventricular level with a clear view of papillary muscle. LV internal diameters at end-diastole and end-systole were measured. The fractional shortening was defined as (LV internal diameters at end-diastole – LV internal diameters at end-systole)/(LV internal diameters at end-diastole) × 100%. The values of three consecutive cardiac cycles were averaged. Of note, echocardiographic measurements were performed and analyzed by an operator blinded to strain information.

Flow Cytometry Analysis of Peritoneal Neutrophils

Twenty-four hours after surgeries, 5 ml of normal saline was injected into the peritoneal cavity and mixed thoroughly by gentle massage to the abdomen. Three milliliters of the peritoneal lavage fluid was collected and centrifuged. The supernatants were saved for cytokine measurements, and the cell pellets were resuspended and manually counted. A fraction of cells (5×10^5) from the peritoneal lavage was labeled with Gr-1 (anti-Ly-6C/Ly-6G; BD Biosciences, San Jose, CA) and gated on Gr-1 for neutrophil percentage in the recruited peritoneal cells. Total neutrophil numbers in the peritoneum were calculated based on the total cell numbers and the percentage of Gr-1$^+$ neutrophils.

Phagocytosis Assay

Phagocytosis assay was performed as described previously.[22,23] In brief, 4×10^5 cells were incubated with serum-opsonized fluorescein isothiocyanate (FITC)-labeled yellow-green fluorescent polystyrene microspheres (FluoSpheres; Invitrogen) at 37°C for 30 min. Cells were then washed, stained with allophycocyanin-labeled Gr-1 antibody, and analyzed with flow cytometry for phagocytic neutrophils (FITC$^+$/ allophycocyanin$^+$), which were expressed as percentage of Gr-1$^+$ neutrophils.

Bacterial Counts in the Peritoneal Exudates and Blood

Twenty-four hours after sham or CLP procedures, 5 ml of sterile normal saline was injected into the peritoneal space and mixed thoroughly by gentle massage to the abdomen. Three milliliters of the peritoneal lavage was collected. Blood was collected through cardiac puncture and anticoagulated with lithium heparin. Samples were serially diluted, plated on Trypticase soy agar with 5% sheep blood (BD Company, Sparks, MD), and incubated at 37°C for 14 to 16 h. Colony-forming units were counted and expressed as log$_{10}$ of colony-forming units per milliliter of blood or lavage fluid as we described previously.[18,23]

Serum Chemistry

Serum blood urea nitrogen (BUN) and creatinine were examined for kidney injury and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for liver injury. Blood was collected 24 h after sham or CLP surgery. Serum was prepared and stored at –80°C until analysis. Serum chemistry was measured by DRI-CHEM 7000 Chemistry Analyzer (HESKA, Des Moines, IA) according to the manufacturer's instruction.

Multiplex Cytokine Immunoassays

Twenty-four hours after sham or CLP procedures, blood was collected through cardiac puncture and anticoagulated with lithium heparin. Plasma were harvested after they were centrifuged at 1,000g for 10 min at 4°C and stored at –80°C. Five milliliters of saline was administered intra-peritonealy, and peritonal lavage fluid was harvested after gentle abdominal massage. Cell-free supernatant of the peritoneal lavage fluid was collected and stored at –80°C. Cytokine concentrations were determined using a fluorescent bead–based multiplex immunoassay (Luminex Co., Austin, TX) as previously described.[18,23] In brief, antibody for each cytokine was covalently immobilized to a set of fluorescent microspheres by manufacturer (Millipore, Billerica, MA). After overnight incubation, cytokines bound on the surface of microspheres were detected by a cocktail of biotinylated antibodies. Following binding of streptavidin–phycoerythrin conjugates, the reporter fluorescent signal was measured using a Luminex 200 reader. Final cytokine concentrations were calculated based on a standard cytokine curve obtained in each experiment.

Detection of Intracellular Reactive Oxygen Species

Peritoneal cells were harvested 12 h after sham and CLP procedures and incubated with the redox-sensitive dye dichlorodihydrofluorescein diacetate (Molecular Probes, Grand Island, NY), at 37°C for 30 min. Dichlorodihydrofluorescein diacetate was measured in FITC channel of flow cytometry and quantified by mean fluorescence intensity.

Mortality Study

Following sham or CLP procedures, mice were observed every 24 h for up to 14 days. Mice administered with saline or lipopolysaccharide were monitored every 6 h for up to 48 h. Mouse mortality was monitored by operators blinded to strain information.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). The distributions of the continuous variables were expressed as the mean ± standard error. Isolated heart function assessed by Langendorff and cardiomyocytes function were analyzed by two-way analysis of variance (ANOVA). Serum chemistry data, neutrophil migration, and reactive oxygen species (ROS) production were analyzed by two-way ANOVA with Bonferroni-adjusted P value for *post hoc* analyses between groups. Analysis of echocardiographic measurements was performed by two-way ANOVA with repeated measurements with Bonferroni *post hoc* test. Colony-forming unit was applied on the log$_{10}$ scale and based on Student t test. The survival data were analyzed with a log-rank test. Student t test was used for statistical analysis between groups of all other data. Of note, these specific comparisons were made based on *a priori* hypotheses rather than pure statistic considerations. The null hypothesis was rejected for P value less than 0.05 with the two-tailed test.

Results

TLR4^{def} Mice Have Increased Mortality in Low-grade Polymicrobial Sepsis

We subjected WT (C57BL/10ScSn) and TLR4^{def} (C57BL/10ScCr) mice to a CLP model of low-grade polymicrobial sepsis. We found that WT mice developed

low-grade sepsis with an accumulated mortality of 14% on day 2 and 23% on day 14 (fig. 2A). TLR4[def] mice had a marked increase in the mortality (48% on day 2, and 81% on day 14) compared with the WT control ($P < 0.001$). In stark contrast, these TLR4[def] mice were totally resistant to endotoxin-induced shock (fig. 2B). After a lethal dose of lipopolysaccharide administration (15 mg/kg), WT mice had 100% mortality within 28 h whereas TLR4[def] mice were completely protected with no mortality for up to 48 h. These data suggest that TLR4 signaling confers a potent survival benefit in low-grade polymicrobial sepsis and absence of TLR4 signaling leads to increased mortality.

TLR4[def] Mice Have Worse LV Dysfunction and Cardiomyocyte Functional Impairement in Low-grade Polymicrobial Sepsis

The hearts isolated from both sham and CLP mice were perfused in a Langendorff system. There was no difference in LV contractile function between WT and TLR4[def] groups after the sham procedure (fig. 3A). Twenty-four hours after CLP, however, there was a reduction in LV contractile function in WT mice as demonstrated by moderate but significant decrease in LVDP (24% reduction, 109 ± 4 vs. 83 ± 2 mmHg) and dP/dt$_{max}$ (26% reduction, $4,991 \pm 140$ vs. $3,679 \pm 101$ mmHg/s). Compared to septic WT mice, septic TLR4[def] mice had significantly worse LV contractile dysfunctions (42% reduction in LVDP and 43% reduction in dP/dt$_{max}$ at 30 min of perfusion; fig. 3A). Consistent with the mortality data above, TLR4[def] mice were largely protected with near-normal LV function in response to endotoxin shock (table and figure in Supplemental Digital Content 1, http://links.lww.com/ALN/B59). To further test the impact of TLR4 deficiency on sepsis-induced cardiac dysfunction, we isolated adult cardiomyocytes 24 h after CLP surgery and examined cardiomyocyte function as measured by sarcomere shortening and Ca^{2+} transients. As shown in figure 3, B and C, compared with septic WT

cardiomyocytes, septic TLR4[def] cardiomyocytes had attenuated sarcomere shortening (43% reduction, 6.7 ± 0.4 vs. $3.8 \pm 0.5\%$) and peak change in [Ca^{2+}]$_i$ (48% reduction, 271 ± 31 vs. 140 ± 14 nM). These data suggest a critical role of TLR4 in preserving cardiomyocyte function during low-grade polymicrobial sepsis.

TLR4 Deficiency Deteriorates Organ Injury during Low-grade Sepsis

We measured the serum levels of BUN, creatinine, ALT, and AST. As shown in figure 4, in WT mice, the low-grade model of sepsis did not cause any detectable acute kidney injury at 24 h with normal BUN and creatinine levels, but induced liver injury as evidenced by an increase in both ALT and AST (ALT: 22 ± 1.4 vs. 162 ± 25 U/l, $P = 0.007$; AST: 96 ± 5.6 vs. 392.4 ± 41.4 U/l, $P = 0.002$). In comparison, septic TLR4[def] mice had significantly worse kidney and liver injury as demonstrated by markedly elevated BUN/creatinine and ALT/AST (BUN: 18 ± 3 vs. 59 ± 9 mg/dl, $P = 0.001$; creatinine: 0.3 ± 0.04 vs. 2.8 ± 1.2 mg/dl, $P = 0.057$; ALT: 162 ± 25 vs. 281 ± 44 U/l, $P = 0.036$; AST: 392 ± 41 vs. $1,715 \pm 320$ U/l, $P = 0.015$, WT vs. TLR4[def], respectively).

TLR4[def] Mice Have Higher Bacterial Loading during Low-grade Polymicrobial Sepsis

We examined the bacterial loading in the blood and the peritoneal lavage from the mice subjected to CLP surgery. As indicated in figure 5, 24 h after CLP, TLR4[def] mice displayed a significant increase in the bacterial counts both in the blood and the peritoneal fluid (Log$_{10}$ scale, 5.6 ± 0.2 and 7.9 ± 0.3, respectively) when compared with WT mice (2.2 ± 0.6 and 5.0 ± 0.3, respectively).

TLR4[def] Mice Have Increased Local and Systemic Cytokine Responses, Impaired Neutrophil Functions, and Increased Leukocyte ROS Generation

Interleukin (IL)-6 is a proinflammatory cytokine during sepsis,[24,25] whereas IL-10 is antiinflammatory.[25] To determine
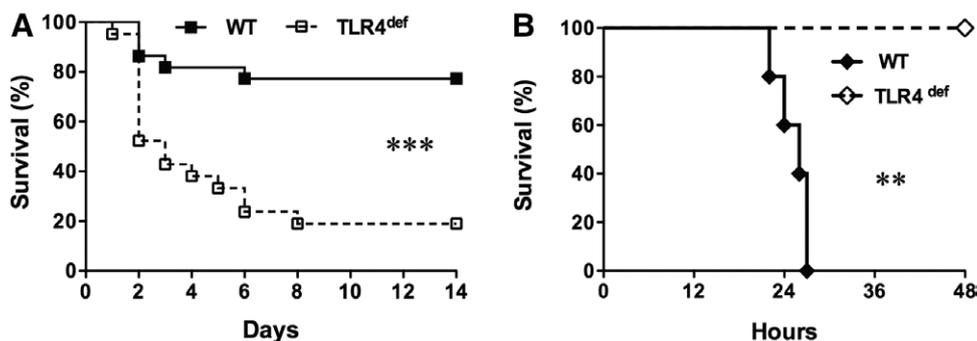
Fig. 2. Toll-like receptor 4 (TLR4) deficiency leads to improved survival in endotoxin shock but decreased survival in low-grade polymicrobial sepsis. Mice were subjected to cecum ligation and puncture or lipopolysaccharide administration and observed for mortality. (*A*) Survival curve in cecum ligation and puncture model. n = 22 in wild-type (WT) group, n = 21 in TLR4[def] group. ***$P = 0.0001$. (*B*) Survival curve in endotoxin shock model. n = 5 in each group. **$P = 0.003$. Log-rank test was used to analyze statistical difference in survival between the two groups.
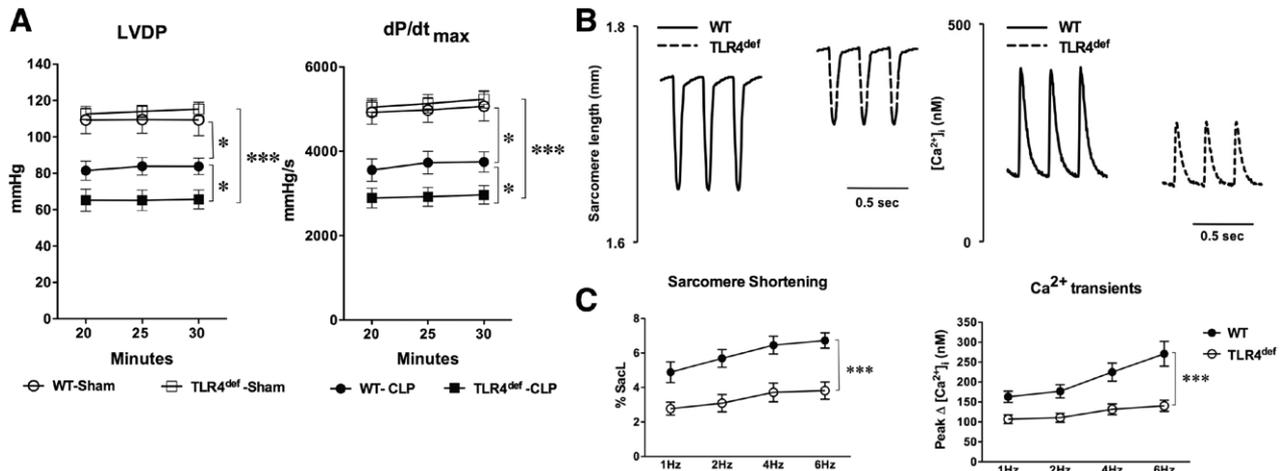
Fig. 3. Toll-like receptor 4 (TLR4)–deficient mice have deleterious left ventricle (LV) function compared with wild-type (WT) mice after cecum ligation and puncture (CLP) surgery. (*A*) LV function measured in a Langendorff perfusion system. Twenty-four hours after CLP or sham surgery, the heart was excised and perfused in a Langendorff system. LV function was measured. Each *error bar* represents the mean ± standard error, n = 4 in WT-sham group, n = 6 in TLR4^{def}-sham group, n = 8 in WT-CLP group, n = 10 in TLR4^{def}-CLP group. Two-way ANOVA was used for statistical analysis. *P < 0.05; ***P < 0.001. Detailed P values in left ventricle developed pressure (LVDP) assessment: WT-CLP *versus* WT-sham, P = 0.013; WT-CLP *versus* TLR4^{def}-CLP, P = 0.034. Detailed P values in dP/dt$_{max}$ assessment: WT-CLP *versus* WT-sham, P = 0.012; WT-CLP *versus* TLR4^{def}-CLP, P = 0.041. dP/dt$_{max}$ = the maximal rate of LV pressure development. (*B* and *C*) Sarcomere shortening and Ca^{2+} transients in isolated adult cardiomyocytes. (*B*) Representative tracing of sarcomere shortening and Ca^{2+} transients in isolated cardiomyocytes 24 h after CLP surgery. (*C*) Accumulated data of sarcomere shortening and Ca^{2+} transients. The data in each group were recorded from 14 to 15 single adult cardiomyocytes isolated from four mice. Two-way ANOVA was used for statistical analysis. ***P < 0.0001. SacL = sarcomere length.

the impact of TLR4 signaling on cytokine responses during low-grade polymicrobial sepsis, we harvested blood and peritoneal lavage 24 h after CLP. Compared with septic WT mice, TLR4^{def} mice exhibited markedly elevated levels of local and systemic IL-6 (28,040 pg/ml in the peritoneal lavage and 46,651 pg/ml in the plasma) and IL-10 (5,893 pg/ml in the lavage and 22,171 pg/ml in the plasma). In contrast, septic WT mice had significantly lower proinflammatory cytokines IL-6 (5,394 pg/ml in lavage, P = 0.0001 *vs.* TLR4^{def} group, and 1,192 pg/ml in plasma, P = 0.001 *vs.* TLR4^{def} group) and IL-10 (2,611 pg/ml in lavage, P = 0.022 *vs.* TLR4^{def} group, and 4,826 pg/ml in plasma, P = 0.001 *vs.* TLR4^{def} group; fig. 6A).

Innate immune cells such as neutrophils play a pivotal role in the host defense as well as the pathogenesis of sepsis.[26] To determine the impact of TLR4 signaling on neutrophil migratory function, we next examined neutrophil recruitment into the peritoneal space after CLP and the neutrophil phagocytic function *ex vivo* to engulf opsonized fluorescent beads. In the sham-operated mice, both WT and TLR4^{def}, there was a small number of leukocytes present in the peritoneal space and less than 33% of these leukocytes were Gr-1$^+$ neutrophils (fig. 6B). Twelve to 24 h after CLP, there was a marked and time-dependent increase in the number of neturophils recruited into the infectious peritioneal space of WT mice (37.8 ± 3.4 × 10^6 at 24 h). The percentage of neutrophils was also increased to nearly 70%. In comparison, there was significantly fewer number of neutrophils in the peritoneal space of the septic TLR4^{def}

mice (21.9 ± 2.9 × 10^6, P = 0.003 *vs.* WT-CLP) at 24 h (fig. 6B). Moreover, neutrophils isolated from TLR4^{def} mice had significantly lower phagocytic function compared with that of WT mice (64.0 ± 3.6% *vs.* 38.5 ± 7.1%, P = 0.012; fig. 6C). ROS, especially intracellular ROS, plays an important role in regulating cytokine production[27,28] and is associated with organ injury during sepsis.[28,29] ROS generation was assessed using redox-sensitive dye dichlorodihydrofluorescein by flow cytometry. As illustrated in figure 6D, there was a much higher level of the intracellular ROS production in the peritoneal cells of TLR4^{def} septic mice when compared with that of WT mice (P = 0.001). Together, these data suggest that TLR4 plays a vital role in maintaining normal neutrophil migratory and phagocytic function and in controlling ROS generation during low-grade polymicrobial sepsis.

Discussion

In the present study, we demonstrated an important role of TLR4 in the host defense mechanism in a clinically relevant mouse model of sepsis. In a low-grade model of polymicrobial sepsis, we found that TLR4 deficiency deteriorated cardiac function and induced hepatic and renal injury with markedly increased mortality. These deleterious effects of TLR4 deficiency were associated with grossly impaired neutrophil migratory and phagocytic functions and attenuated bacterial clearance. These data suggest that in low-grade polymicrobial sepsis, TLR4 signaling appears to be critical for maintaining normal host immune defense against
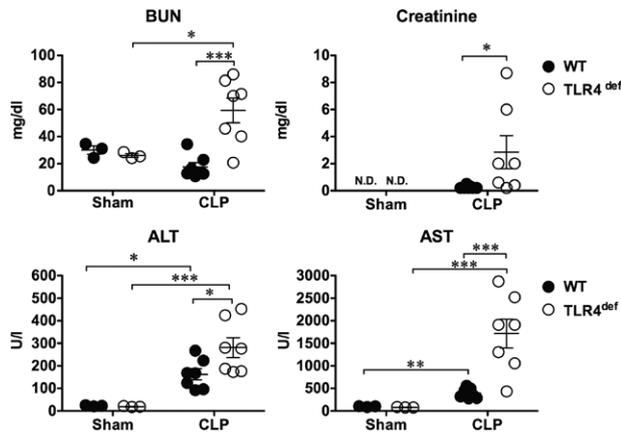
Fig. 4. Toll-like receptor 4 (TLR4)–deficient mice develop worse kidney and liver injury during low-grade sepsis. Mice were subjected to sham or cecum ligation and puncture (CLP) surgery. Serum was collected 24 h after surgery. Each *error bar* represents the mean ± standard error. Two-way ANOVA was used for statistical analysis and Bonferroni *post hoc* tests were applied for difference between groups. *$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$; n = 3 in sham group, n = 7 in CLP group. ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; N.D. = not detectable; WT = wild type.

Fig. 5. Toll-like receptor 4 (TLR4)–deficient mice have higher bacterial load in the blood and peritoneal space compared with wild-type (WT) mice following low-grade polymicrobial infection. Both the blood and peritoneal lavage were harvested 24 h after the cecum ligation and puncture surgery. After serial dilutions, the samples were incubated on agar plates at 37°C for 14–16 h. Bacterial colony-forming units (CFU) were counted. Each data point represents the CFU from one mouse and was plotted in a Log_{10} scale. *Horizontal bars* indicate the mean value of the CFU in each mouse group, and *t* test was applied for statistical difference analysis. ***$P < 0.001$. Detailed P value: $P = 0.0001$ in both blood and lavage CFU comparison between groups; n = 9.

bacterial invasion and protecting the vital organs from polymicrobial infection-induced injury.

Cardiac dysfunction represents a clinical feature of sepsis and contributes to its mortality. Several mechanisms have been proposed responsible for myocardial dysfunction during sepsis, including cardiosuppressive cytokines such as IL-6 and tumor necrosis factor-α, mitochondrial dysfunction, and alterations of myocardial calcium homeostasis.[30,31] It has been well documented that TLR4, in particular those of bone marrow–derived immune cells, mediates cardiac dysfunction induced by endotoxin,[7,32–34] a wall component of Gram-negative bacteria. Consistent with these previous findings, we found that systemic TLR4 deficiency in C57BL/10ScCr mice conferred a profound protection against endotoxin-induced cardiac dysfunction and prevented mortality. This is because during endotoxin shock, the key underlying pathology is systemic hyperinflammation featured by cytokine storm (*e.g.*, IL-6 and tumor necrosis factor-α), which leads to cardiovascular collapse and death. Lack of TLR4 would effectively block endotoxin-induced cytokine storm and, thus, confer survival benefit. In contrast, in the mouse model of low-grade peritonitis sepsis, which involves multiple live bacterial infection and only modest systemic cytokine response and low mortality, we found that TLR4 deficiency deteriorated cardiac function as demonstrated *ex vivo* in isolated heart and isolated adult cardiomyocytes following CLP procedure. There are several possible mechanisms that may explain the deleterious impact of TLR4 deficiency on cardiac function in low-grade bacterial sepsis observed in the current study. First,
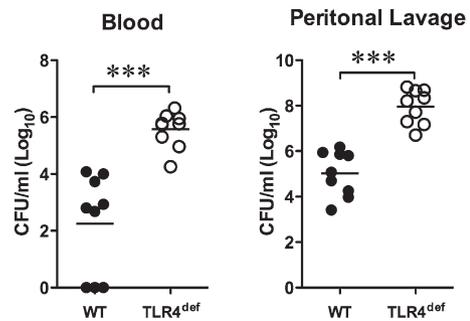
TLR4 is critically involved in the effective host immune defense against bacterial invasion. In our study, WT mice exhibit more robust neutrophil migratory and phagocytic functions and markedly reduced bacterial loading compared with mice lacking TLR4. We speculate that as a result of uncontrolled bacterial dissemination in the absence of TLR4, animals lacking TLR4 have more systemic cytokine production, including IL-6, a major cardiodepressant.[31,35] Moreover, higher bacterial load may lead to more ROS production in cardiac tissue and immune cells as demonstrated in the present study, which could induce tissue injury and adversely impact cardiomyocyte function. Second, the cardiac-protective benefit associated with TLR4 signaling might be attributed to a local and direct cardiac "preconditioning-like" effect of TLR4 activation during low-grade bacterial infection. Several studies have found that very small doses of lipopolysaccharide pretreatment confers a cardioprotective effect against hypoxic injury,[36–38] in part through interleukin-1 receptor-associated kinase 1, MyD88, and nitric oxide synthase 2 signaling pathway.[20,39] It is worth noting that the amount of lipopolysaccharide used in these models is extremely small (0.1 to 0.5 mg/kg) while the dose for endotoxin shock model is many times higher (between 10 mg/kg[32,33] and 15 mg/kg[9]). Therefore, it seems possible that a small amount of endotoxin released from bacterial peritonitis to the circulatory system in the early stage of the low-grade polymicrobial sepsis could activate TLR4 signaling and initiate a "pre-conditioning-like" cardiac protection against subsequent myocardial depression during polymicrobial sepsis. Supporting this notion are studies that demonstrate the preconditioning effect of low dose of endotoxin against both subsequent endotoxin
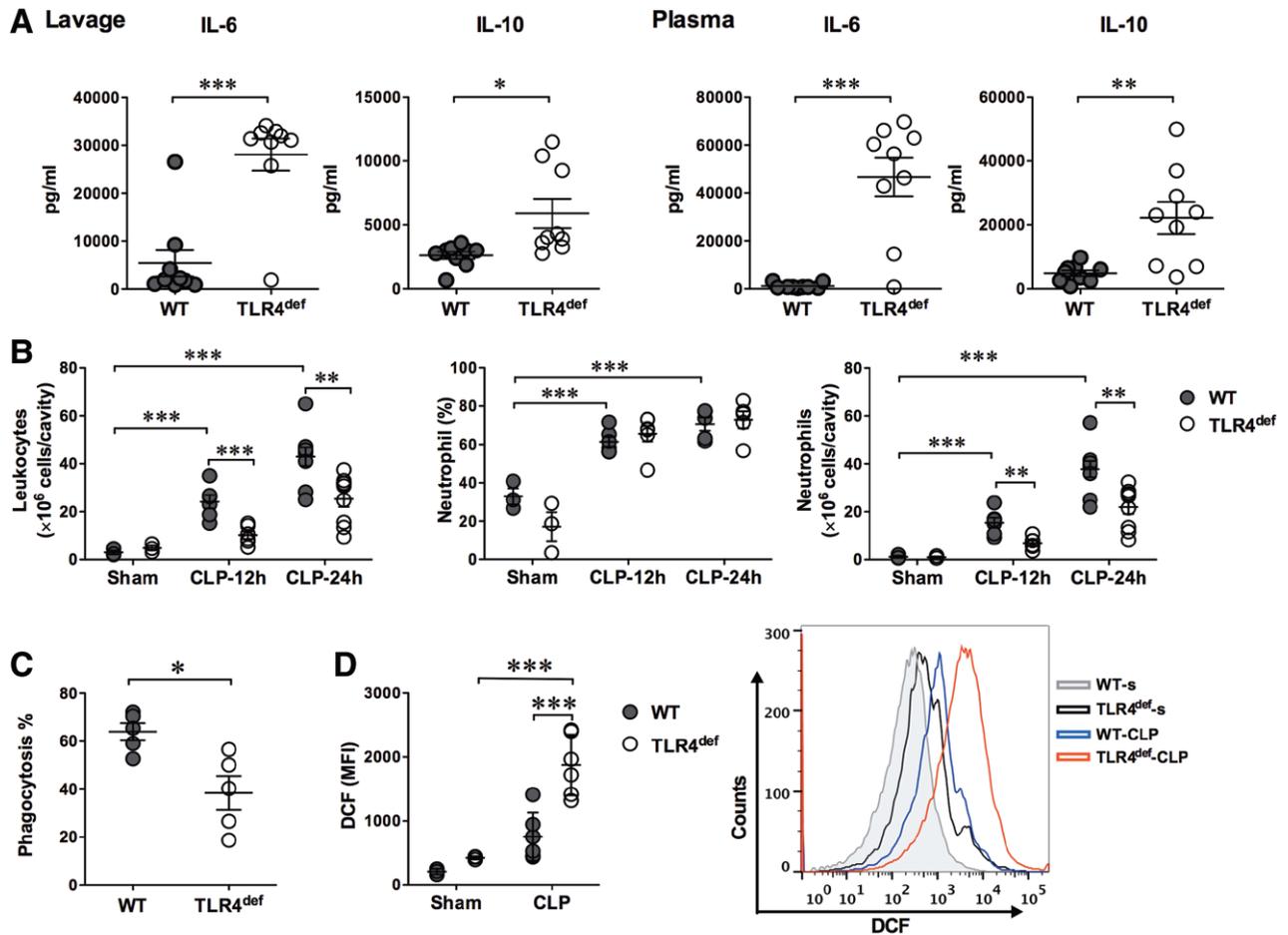
Fig. 6. Toll-like receptor 4 (TLR4) deficiency leads to increased inflammation, impaired neutrophil recruitment and phagocytic function, and increased leukocyte reactive oxygen species (ROS) production during low-grade polymicrobial sepsis. (*A*) Cytokines in the lavage and plasma of wild-type (WT) and TLR4def mice following cecum ligation and puncture (CLP). Twenty-four hours after CLP procedure, the peritoneal lavage and plasma were collected from the septic mice. Interleukin (IL)-6 and IL-10 were measured using a multiplex fluorescent bead–based immunoassay. *t* test was applied for difference between groups. N = 9 in each group. (*B*) Leukocytes in the peritoneal space following sham or CLP procedures. Total peritoneal cells were manually counted by hemacytometer. Neutrophils were calculated based on the total peritoneal cell numbers multiplied by the percentage of neutrophils as measured by flow cytometry. Two-way ANOVA was used for statistical analysis, and Bonferroni-adjusted *P* value was applied for *post hoc* analyses between groups. n = 3 in sham groups, n = 6 in CLP-12h groups, n = 9 in CLP-24h groups. (*C*) Percentages of phagocytic neutrophils. *t* test was applied for difference between the two groups; n = 5 in each group. (*D*) ROS production in peritoneal cells. Intracellular ROS of the peritoneal cells was measured by flow cytometry with dichlorodihydrofluorescein (DCF) stain 12 h after surgery. Two-way ANOVA was used for statistical analysis, and Bonferroni *post hoc* tests were applied for difference between groups. n = 3 in sham groups, n = 6 in CLP groups; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. MFI = mean fluorescence intensity; TLR4def-s = TLR4def-sham; WT-s = WT-sham.

challenges[40] and polymicrobial infection.[41] Meng *et al.*[40] demonstrate that animals pretreated with small dose of lipopolysaccharide (0.5 mg/kg) become resistant to subsequent lipopolysaccharide-induced cardiac dysfunction. Wheeler *et al.*[41] show that lipopolysaccharide pretreatment also attenuates systemic inflammatory response and improved bacterial clearance and survival in a CLP model of polymicrobial sepsis.

Successful bacterial clearance at the infection site and circulatory system is essential for survival in bacterial sepsis. In the present study, we demonstrated that compared with WT mice, TLR4-deficient mice had impaired bacterial

clearance in both peritoneal space and in the blood following CLP. There are several possible mechanisms that may be responsible for this. First, TLR4 signaling is important for neutrophil migratory function. The ability of neutrophils to migrate to the infection site is critical for a successful host defense. Our *in vivo* study demonstrates that compared with WT mice, TLR4-def mice have markedly attenuated neutrophil recruitment in the peritoneal space during both early (12 h) and late (24 h) polymicrobial infection. This is in consistent with a previous *in vitro* study demonstrating that TLR4 signaling promotes neutrophil migration by reducing chemokine receptor (chemokine (C-X-C motif) receptor 2

and macrophage inflammatory protein 2 receptors) internalization and desensitization.[42] Our own previous study[43] also demonstrates that signaling *via* MyD88, the downstream adaptor of TLRs including TLR4, is important for maintaining neutrophil chemokine receptor expression and migratory function. Thus, lack of TLR4-MyD88 signaling may impair neutrophil migratory function. In addition, an intact TLR4 signaling has an antiapoptotic survival effect in neutrophils. Thus, the relative longer survival life span of the peritoneal neutrophils in WT septic mice may contribute to better neutrophil phagocytic function compared with TLR4[def] mice.[44] Second, TLR4 is important for neutrophil phagocytic function. In our study, neutrophils isolated from septic TLR4[-def] mice exhibited significantly reduced phagocytic capability compared with those from WT septic mice. Consistent with this, Wheeler *et al.*[41] demonstrate that lipopolysaccharide preconditioning *via* TLR4 enhances macrophage phagocytosis of both Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*Staphylococcus aureus*), suggesting that activation of TLR4 signaling augments bacterial clearance through increased phagocytosis.

The present study establishes that TLR4 signaling is essential for overall survival and organ function in low-grade polymicrobial sepsis. However, in high-grade lethal models of sepsis, TLR4 signaling appears detrimental and absence of TLR4 actually improves survival[15,16] These observations suggest that the role of TLR4 signaling in the pathogenesis of polymicrobial sepsis is complex and may well depend on the severity of sepsis. Similar observations have been reported for the role of MyD88 and type I interferons, two downstream molecules of TLR4 signaling, in polymicrobial sepsis.[9,45–47] Collectively, these studies appear to suggest a common theme, that is, dual roles for TLR4 signaling in the pathogenesis of bacterial sepsis. In lethal and severe sepsis, where cytokine storm dominates the underlying pathology, TLR4 signaling mediates the harmful hyperinflammatory responses. In this case, absence of TLR4 signaling attenuates cytokine production, improves hemodynamics, reduces organ injury, and promotes survival. In low-grade or nonlethal sepsis, on the other hand, the main pathology is immunosuppression and bacterial invasion. TLR4 signaling provides an essential host immune defense mechanism such as neutrophil migration and phagocytosis as demonstrated in the present study. Absence of TLR4 signaling would weaken the host immune defense, compromise bacterial clearance, deteriorate organ injury and dysfunction, and reduce the survival.

The present and several previous studies have suggested a potential therapeutic value of TLR4 agonist in enhancing host immunity in bacterial sepsis. For example, Wheeler *et al.*[41] have reported that a low dose of lipopolysaccharide pretreatment attenuates inflammatory cytokine level in the plasma and peritoneal fluid in a CLP model of sepsis. In addition, lipopolysaccharide pretreatment improves bacterial clearance, phagocytosis, and survival during polymicrobial sepsis. Moreover, synthetic TLR agonists appear to stimulate innate resistance to infectious challenge.[48] Administration of the synthetic TLR4 agonists, namely, aminoalkyl gluocosaminide phosphates, provides strong protection against subsequent *Listeria* challenge in WT mice, but not in inactive TLR4 mutant mice.[48] These results support the role of TLR4 agonists as potential immunomodulatory agents in the setting of bacterial sepsis.

In summary, the current study demonstrates that natural deletion of TLR4 in C57BL/10ScCr mice results in attenuated neutrophil function, decreased bacterial clearance, deleterious cardiac function and kidney/liver injury, and markedly increased mortality during low-grade polymicrobial sepsis. These data suggest that TLR4 signaling is pivotal in the host defense by maintaining normal neutrophil functions and effective bacterial clearance and, thus, protecting the vital organs from sepsis-induced organ injury during the low-grade polymicrobial sepsis.

Acknowledgments

Competing Interests

The authors declare no competing interests.

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