ANESTHESIOLOGY

Toll-like Receptor 7 Contributes to Inflammation, Organ Injury, and Mortality in Murine Sepsis

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Toll-like receptor 7 responds to elevated single-stranded RNA by increasing cytokine production. Sepsis is characterized by elevated plasma levels of tissue damage (and pathogen)–associated molecular patterns, including RNA.

What This Article Tells Us That Is New

- Using murine models of bacterial sepsis, knockout of the Toll-like receptor 7 resulted in lower mortality and cytokine levels and less end-organ injury. Therefore, Toll-like receptor 7, which mediates innate immune response, contributes to harm in experimental sepsis.

ABSTRACT

Background: Sepsis remains a critical illness with high mortality. The authors have recently reported that mouse plasma RNA concentrations are markedly increased during sepsis and closely associated with its severity. Toll-like receptor 7, originally identified as the sensor for single-stranded RNA virus, also mediates host extracellular RNA-induced innate immune responses *in vitro* and *in vivo*. Here, the authors hypothesize that innate immune signaling *via* Toll-like receptor 7 contributes to inflammatory response, organ injury, and mortality during polymicrobial sepsis.

Methods: Sepsis was created by (1) cecal ligation and puncture or (2) stool slurry peritoneal injection. Wild-type and Toll-like receptor 7 knockout mice, both in C57BL/6J background, were used. The following endpoints were measured: mortality, acute kidney injury biomarkers, plasma and peritoneal cytokines, blood bacterial loading, peritoneal leukocyte counts, and neutrophil phagocytic function.

Results: The 11-day overall mortality was 81% in wild-type mice and 48% in Toll-like receptor 7 knockout mice after cecal ligation and puncture (N = 27 per group, $P = 0.0031$). Compared with wild-type septic mice, Toll-like receptor 7 knockout septic mice also had lower sepsis severity, attenuated plasma cytokine storm (wild-type *vs.* Toll-like receptor 7 knockout, interleukin-6: 43.2 [24.5, 162.7] *vs.* 4.4 [3.1, 12.0] ng/ml, $P = 0.003$) and peritoneal inflammation, alleviated acute kidney injury (wild-type *vs.* Toll-like receptor 7 knockout, neutrophil gelatinase-associated lipocalin: 307 ± 184 *vs.* 139 ± 41-fold, $P = 0.0364$; kidney injury molecule-1: 40 [16, 49] *vs.* 13 [4, 223]-fold, $P = 0.0704$), lower bacterial loading, and enhanced leukocyte peritoneal recruitment and phagocytic activities at 24 h. Moreover, stool slurry from wild-type and Toll-like receptor 7 knockout mice resulted in similar level of sepsis severity, peritoneal cytokines, and leukocyte recruitment in wild-type animals after peritoneal injection.

Conclusions: Toll-like receptor 7 plays an important role in the pathogenesis of polymicrobial sepsis by mediating host innate immune responses and contributes to acute kidney injury and mortality.

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Sepsis remains one of the intricate diseases with extremely high mortality. Between 2004 to 2009, sepsis incidence had a 22.3% annual increase in the United States and the hospital mortality of sepsis was 28.3%.[1,2] Although significant progress in our understanding of sepsis pathogenesis has been made during the past decades, the specific therapy for sepsis is still lacking and mortality remains unacceptably high. Therefore, further investigation into the complex molecular and cellular mechanisms of sepsis is warranted to identify new target for future therapeutic intervention.

Pathogen-associated molecular patterns and host damage–associated molecular patterns participate in innate immune activation during pathogen invasion and in sepsis. Toll-like receptors are a pivotal part of host innate immune defense and play a critical role in molecular pattern recognition. Among 10 Toll-like receptors, Toll-like receptor 7 and Toll-like receptor 8 were originally identified as the sensors for single-stranded RNA of viral origins.[3–5] Although their genes lie in close proximity on the X chromosome,[6] only Toll-like receptor 7

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is functional in mice.[7] Besides viral single-stranded RNAs, Toll-like receptor 7 can also recognize nucleic acids released from bacteria. For instance, human primary monocytes and macrophages sense *Staphylococcus aureus* single-stranded RNA *via* Toll-like receptor 8 and induce interferon-β production.[8] Moreover, vaccine composition formulated with a Toll-like receptor 7–dependent adjuvant induces high and broad protection against *S. aureus*.[9] These findings suggest that Toll-like receptor 7 signaling may play an important role in host innate immune response during bacterial infection. Our group has previously reported that host tissue RNAs including microRNAs were released into the blood circulation in a mouse model of polymicrobial sepsis and the plasma RNA concentrations are closely correlated with the severity of sepsis.[10] *In vitro* and *in vivo* studies show that extracellular RNAs and microRNA mimics can work as host damage–associated molecular patterns to induce a robust proinflammatory response such as cytokine production, immune cell activation, and complement activation.[10–12] Most importantly, the proinflammatory role of these extracellular RNAs and microRNAs proves to be dependent of Toll-like receptor 7–myeloid differentiation primary response 88 signaling both in cell cultures and in a peritonitis model *in vivo*.[10–12] However, the role of extracellular RNA and Toll-like receptor 7 signaling in sepsis pathogenesis remains unclear.

In the present study, we tested the hypothesis that Toll-like receptor 7 sensing plays an important role in mediating host innate immune responses, tissue injury, and mortality after bacterial sepsis. Taking a loss-of-function approach, we examined the impact of Toll-like receptor 7 deficiency to the host systemic and local cytokine responses, immune cell migration, leukocyte phagocytosis, bacterial clearance, acute kidney injury, and mortality during sepsis.

Materials and Methods

Animals

Male and age-matched wild-type (C57BL/6J) and Toll-like receptor $7^{-/-}$ mice ($Tlr7^{tm1Flv}$/J) were purchased from the Jackson Laboratories (USA). All mice were 8 to 16 weeks old and weighed between 22 and 30 g. Animals were housed in an animal facility of Massachusetts General Hospital (Boston, Massachusetts) or University of Maryland School of Medicine (Baltimore, Maryland) for at least one week before experiments under specific pathogen–free environment. They were fed with autoclaved bacteria-free diet, and the housing facilities were temperature-controlled and air-conditioned with 12-h/12-h light/dark cycles. All animal protocols were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital and Institutional Animal Care and Use Committee of University of Maryland School of Medicine and followed the guideline of the National Institutes of Health (Bethesda, Maryland). Simple randomization was used to assign animals, and the operators of the surgery (W.J. and L.G.) were blinded to the strain information.

Mouse Model of Polymicrobial Sepsis

Cecal ligation and puncture model was slightly modified based on what we described previously and was performed in the morning.[13] Briefly, after anesthetization (ketamine 100 mg/kg, xylazine 4 mg/kg), mice were subjected to laparotomy. The cecum was ligated 1.2 to 1.5 cm from the tip and punctured with an 18-gauge needle through-through. A small drop of feces was squeezed out gently. Sham-operated mice went through the same procedure but without cecal ligation and puncture. Postoperatively, mice were administered subcutaneously prewarmed saline (0.3 ml/10g). A dose of 3 mg/kg bupivacaine and 0.1 mg/kg buprenorphine was administered to treat postoperative pain. Rectal temperature was recorded at 24 h after surgery.

Mortality Study

After cecal ligation and puncture, mice (n = 27 per group) were observed every 4 h for the first 48 h and every 12 h for up to 11 days. Experimental operators and observers (W.J. and L.Z.) were blinded to the strain information. Because sham surgery resulted in no mortality,[14] no sham mice were included in the mortality study to minimize the unnecessary use of animals.

RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Kidney samples were collected at 24 h after surgery (n = 6 to 8 per group). RNA was extracted from the kidney with TRIzol (Sigma, USA) and cDNA synthesized with reverse transcriptase. Quantitative reverse-transcription polymerase chain reaction was performed as described previously.[13] The transcripts of glyceraldehyde 3-phosphate dehydrogenase, neutrophil gelatinase-associated lipocalin, and kidney injury molecule-1 were quantified using quantitative reverse-transcription polymerase chain reaction with glyceraldehyde 3-phosphate dehydrogenase as the internal control. The polymerase chain reaction primer sequences are listed below: glyceraldehyde 3-phosphate dehydrogenase (Forward: 5′-AACTTTGGCATTGTGGAAGG-3′, Reverse: 5′-GGATGCAGGGATGATGTTCT-3′); neutrophil gelatinase-associated lipocalin (Forward:5′-CTCAGAACTTGATCCCTGCC-3′, Reverse: 5′-TCCTTGAGGCCCAGAGACTT-3′); kidney injury molecule-1 (Forward: 5′-CATTTAGGCCTCATACTGC-3′, Reverse:5′-ACAAGCAGAAGATGGGCATT-3′). Transcript expression was calculated using the comparative Ct method normalized to glyceraldehyde 3-phosphate dehydrogenase ($2^{-\Delta\Delta}$Ct) and expressed as the fold difference in the cecal ligation and puncture or treatment group over the wild-type-sham group.

Bacterial Colony Formation and Quantification

Twenty-four hours after sham or cecal ligation and puncture procedures, 5 ml of sterile normal saline was injected into

the peritoneal cavities of wild-type and Toll-like receptor $7^{-/-}$ mice (n = 8 per group). After gently mixed, 4 ml of the peritoneal lavage was harvested. Blood was collected through inferior vena cava in EDTA-containing tubes. Bacterial counts of the peritoneal lavage and blood were determined by incubating 50 μl of the samples with serial dilutions on Trypticase soy agar plate containing 5% sheep blood (BD Company, USA) at 37°C for 36 h. Colonies were counted and expressed as log10 of units per milliliter of lavage fluid or blood. The supernatant of peritoneal lavage was stored at −80°C for enzyme-linked immunosorbent assay analysis.

Cytokine Enzyme-linked Immunosorbent Assay

Twenty-four hours after procedure, plasma and peritoneal lavages were collected and measured for interleukin-6, interleukin-1β, tumor necrosis factor-α, and chemokine ligand 2 using commercially available DuoSet or Quantikine HS enzyme-linked immunosorbent assay kits (R&D systems, USA) as described in the manufacturer's instructions.

Flow Cytometry Analysis of Peritoneal Cell Population and Phagocytic Function

Yellow-green fluorescent carboxylate-modified microspheres (Invitrogen/Thermo Fisher, USA) were opsonized by incubating with Roswell Park Memorial Institute-1640 cell culture medium containing 10% fetal bovine serum at 37°C for 30 mins. The opsonized microspheres were then injected intraperitoneally at 23 h after sham or cecal ligation and puncture surgery in a dose of 10^8 microspheres per 200 μl volume per mouse. After one hour, 5 ml of sterile normal saline was injected and mixed thoroughly by gentle massage. Four milliliters of peritoneal lavage fluid were collected and centrifuged. A faction of cells (4×10^5) were stained with the following surface markers: CD45-Phycoerythrin (clone 30-F11, 1:1,500 dilution, BD Biosciences, USA), Ly6G-Brillian Violet 421 (clone 1A8, 1:100 dilution, BD Biosciences), F4/80-Alexa Fluro 647 (clone T45-2342, 1:100 dilution, BD Biosciences) at 4°C for 30 min in the dark as described previously.[12] Cells were washed twice in 4 ml of ice-cold phosphate-buffered saline and resuspended in phosphate-buffered saline containing 5% fetal bovine serum, and then flow cytometry analysis was performed using a BD LSR II flow cytometer. The phagocytic function in single cell was expressed as the mean fluorescence intensity of microsphere within the chosen cell population.

Leukocytes Count in the Blood

Twenty-four hours after sham or cecal ligation and puncture surgery, mice (n = 9 to 12 per group) were anesthetized as described above. Blood was collected *via* cardiac puncture and transferred to blood collection tubes containing 3.2% sodium citrate as anticoagulant (Grenier, Fisher Scientific, USA). Leukocyte count was tested using an automated cell counter (Beckman COULTER AC-T diff™ Analyzer, USA).

Cecal Slurry Model of Polymicrobial Sepsis

Male and age-matched wild-type and Toll-like receptor $7^{-/-}$ mice were euthanized by anesthesia (ketamine 100 mg/kg, xylazine 4 mg/kg) followed by cervical dislocation. The whole cecum was dissected and the cecal contents were then collected using sterile forceps and spatula, weighed, and suspended in 5% dextrose (Hospira, USA) in water to make a cecal contents slurry at a concentration of 80 mg/ml. The cecal slurry was mixed well by vortex and filtered through a 100-μm sterile cell strainer. Mice were given the cecal slurry intraperitoneally in a dose of 1.3 mg per g body weight in 500 μl volume. The vehicle group received the same volume of 5% dextrose in water only. Twenty hours later, peritoneal lavage and plasma were collected. The cell migration and cytokine production were measured as mentioned above. The operator was blinded to the strain information.

Statistical Analysis

Mice were randomly allocated to treatment groups in balanced distribution. No statistical power calculation was conducted before the current study. Sample sizes were estimated based on previous similar studies and our preliminary data. Specific comparisons were made based on scientific hypotheses for the study. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., USA). Normality of all numerical data was assessed using D'Agostino-Pearson test. A parametric test was used if the hypothesis of normality was not rejected. Because of data variance observed in the study, unequal variance unpaired *t* test was assumed in all parametric comparisons with interval variables. Nonparametric Mann–Whitney *U* tests were applied to the data that did not pass normality test. Data are presented as mean ± SD when a parametric test was used or as median (interquartile range) otherwise. The survival distributions were presented as ratio variables and compared using log-rank (Mantel–Cox) test. There were no lost data. All data were included. The null hypothesis was rejected for $P < 0.05$ with two tails.

Results

Toll-like Receptor $7^{-/-}$ Mice Have Reduced Mortality and Attenuated Acute Kidney Injury in Polymicrobial Sepsis

As shown in figure 1A, wild-type cecal ligation and puncture mice showed an accumulated mortality of 48% on day 2 and 81% on day 11. In comparison, Toll-like receptor $7^{-/-}$ cecal ligation and puncture mice had a marked reduction in mortality (18% on day 2 and 48% on day 11, N = 27, $P = 0.0031$). Body temperature reportedly is a reliable surrogate marker of sepsis severity in mouse models.[15] Consistent with the mortality data, wild-type cecal ligation and puncture mice displayed a significantly lower rectal temperature compared with Toll-like receptor $7^{-/-}$ cecal ligation and puncture mice (28.3 ± 0.7 *vs.* 31.1 ± 1.1°C, $P = 0.0448$) (fig.1B) at 24 h after surgery, suggesting a less

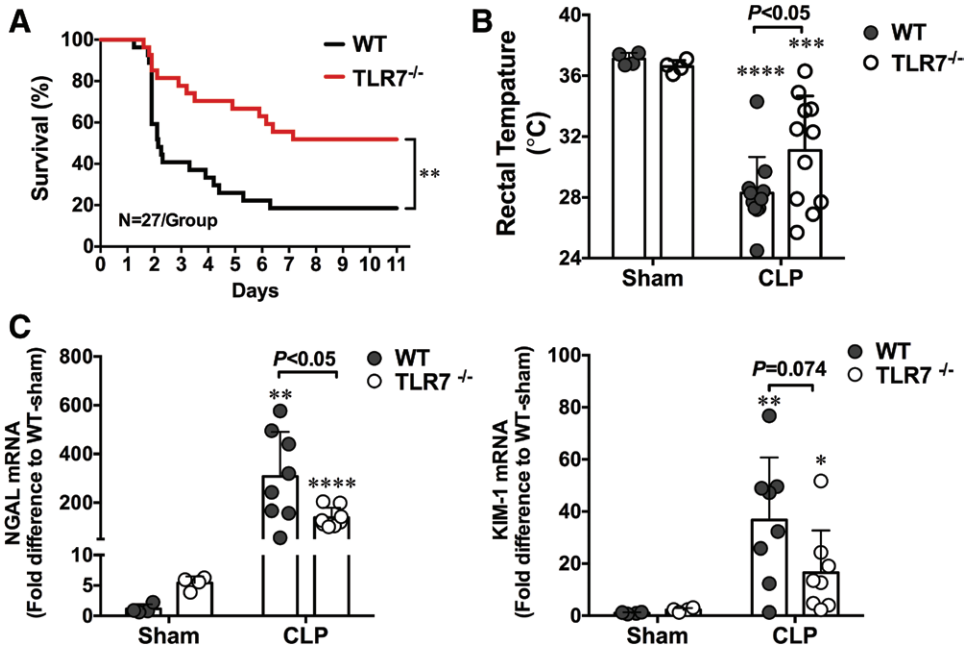

Fig. 1. Toll-like receptor 7 (TLR7)–deficient mice have improved survival and attenuated acute kidney injury after polymicrobial sepsis. (*A*) Survival rate of wild-type (WT) and TLR7$^{-/-}$ mice during sepsis. Mice were subjected to cecal ligation and puncture (CLP) surgery and observed for survival for up to 11 days. $^{**}P < 0.01$, n = 27 in WT and TLR7$^{-/-}$ group. (*B*) Rectal temperature at 24 h after CLP surgery. $^{***}P < 0.001$, $^{****}P < 0.0001$ *versus* sham group. Unequal variance *t* test, n = 11 per group. (*C*) Kidney neutrophil gelatinase–associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) mRNA expression in WT and TLR7$^{-/-}$ mice at 24 h postprocedure. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$ *versus* sham group. Unequal variance *t* test. N = 4 to 8 per group. Each bar represents mean ± SD.

severe septic condition in these Toll-like receptor 7$^{-/-}$ mice. Neutrophil gelatinase–associated lipocalin[16–18] and kidney injury molecule-1[19] are two highly sensitive biomarkers of acute kidney injury. As illustrated in figure 1C, wild-type cecal ligation and puncture mice quickly developed acute kidney injury as evidenced by markedly elevated kidney neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 gene expression. Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice had substantially lower neutrophil gelatinase–associated lipocalin (139 ± 41 *vs.* 307 ± 184 folds, $P = 0.0364$) and kidney injury molecule-1 (13[4, 223] *vs.* 40[16, 49]-fold, $P = 0.0704$) gene expression as compared with wild-type–cecal ligation and puncture. Together, these data suggest that lack of Toll-like receptor 7 signaling confers both survival and kidney-protecting benefits in the mouse model of polymicrobial sepsis.

Lack of Toll-like Receptor 7 Leads to Reduced Cytokine Production and Bacterial Loading in Sepsis

Early mortality and organ injury in sepsis is likely attributed to cytokine storm and other innate immune activities during the initial host response to invading pathogens.[20] To determine the role of Toll-like receptor 7 signaling in host inflammation after polymicrobial sepsis, we measured the proinflammatory cytokines interleukin-6, interleukin-1β, and tumor necrosis factor-α and chemokine ligand 2, known for their key role in sepsis pathogenesis. As illustrated in figure 2, we observed significant reduction in interleukin-6, tumor necrosis factor-α, and chemokine ligand 2, in the plasma and the peritoneal lavages of Toll-like receptor 7$^{-/-}$ mice as compared with that of wild-type mice after cecal ligation and puncture. However, interleukin-1β was only reduced in the peritoneal lavages, but not in the plasma, of Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice when compared with wild-type cecal ligation and puncture mice. To determine the role of Toll-like receptor 7 in bacterial clearance, we measured the bacterial loading both in the blood and the peritoneal lavage 24 h after sham and cecal ligation and puncture surgery. Almost no bacterial colonies were identified from sham samples. As shown in figure 3, there were marked bacterial loads in both blood and peritoneal lavage of cecal ligation and puncture mice. Compared with wild-type cecal ligation and puncture mice, Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice had a marked reduction in bacterial loading in both blood and the peritoneal lavage.

Toll-like Receptor 7$^{-/-}$ Mice Exhibited Enhanced Neutrophil and Small Peritoneal Macrophage Recruitment after Cecal Ligation and Puncture

The reduced bacterial loading in Toll-like receptor 7$^{-/-}$ mice as compared with wild-type mice suggested a possible role of Toll-like receptor 7 activation in functional impairment

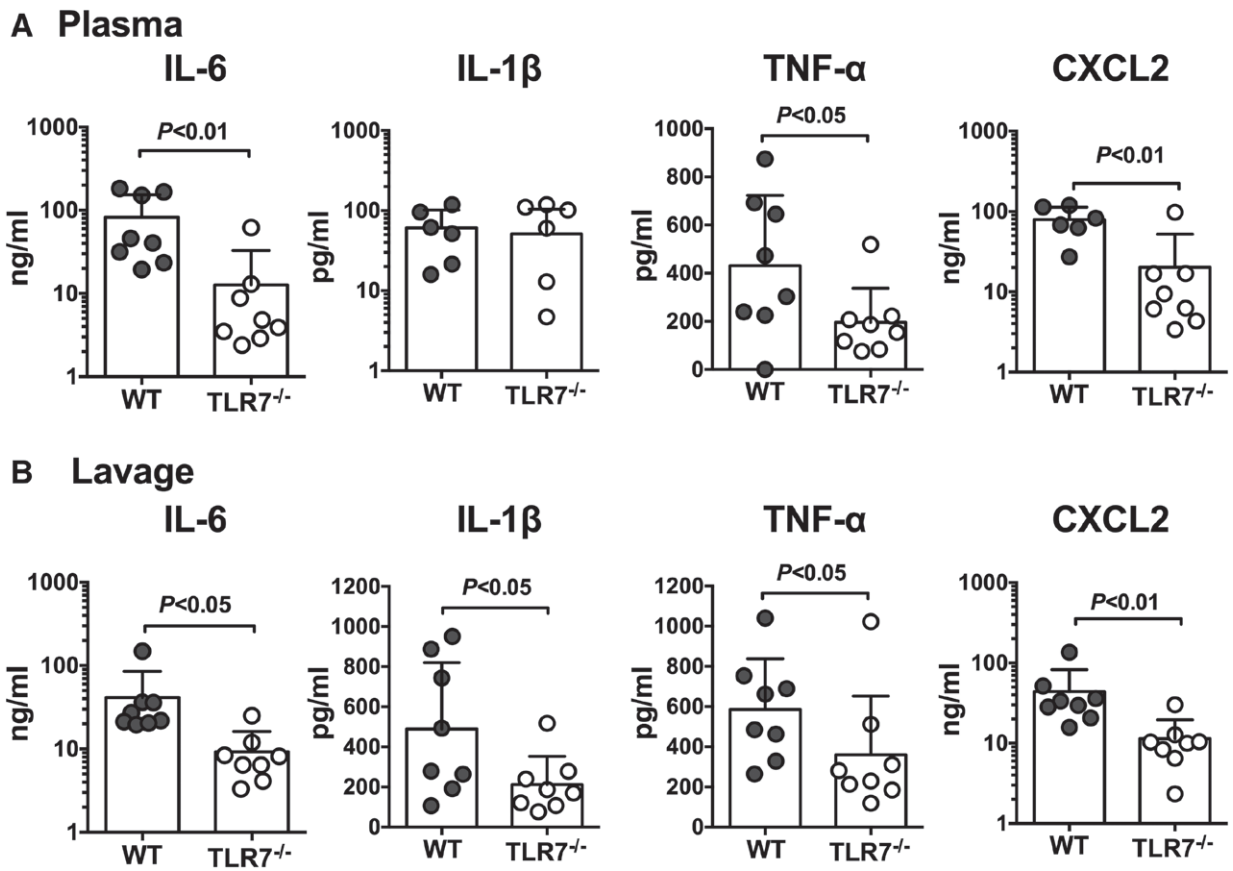

Fig. 2. Toll-like receptor 7–deficient (TLR7$^{-/-}$) mice display lower proinflammatory cytokine production in both blood circulation and peritoneal cavity after cecal ligation and puncture procedure. Twenty-four hours after cecal ligation and puncture procedure, cytokines in the plasma and peritoneal lavage were measured by enzyme-linked immunosorbent assay. (*A*) Plasma interleukin (IL) 6, IL-1β, tumor necrosis factor (TNF)-α, and chemokine ligand 2 (CXCL2) concentration. IL-6 and TNF-α are expressed as median with interquartile range and analyzed by Mann–Whitney *U* test. IL-1β and CXCL2 are expressed as mean ± SD and analyzed by unequal variance *t* test. (*B*) Peritoneal lavage IL-6, IL-1β, TNF-α, CXCL2. N = 6 to 8 per group. IL-6, IL-1β, and TNF-α are expressed as median with interquartile range and analyzed by Mann–Whitney *U* test. CXCL2 is expressed as mean ± SD and analyzed by unequal variance *t* test. WT, wild-type.

of immune effector cells during sepsis. Toll-like receptor 7 activation is known for its role in human CD4(+) T cell anergy.[21] Thus, we investigated how polymicrobial infection after cecal ligation and puncture affected peritoneal residential cells as well as leukocytes recruited into the peritoneal cavity in both wild-type and Toll-like receptor 7$^{-/-}$ mice. As shown in figure 4A, there was a significant increase in the peritoneal cells in wild-type and Toll-like receptor 7$^{-/-}$ septic mice as compared with their sham counterparts. Comparing the two cecal ligation and puncture groups, Toll-like receptor 7$^{-/-}$ mice had higher peritoneal cells than wild-type ([28.0 ± 17.2] × 10^6 *vs.* [12.4 ± 7.9] × 10^6 cells per cavity, $P = 0.0339$). To identify the peritoneal cell populations, we stained the cells with fluorescence-labeled antibodies for specific leukocyte surface markers and analyzed them using flow cytometry. Supplemental Digital Content figure S1 (http://links.lww.com/ALN/B926) illustrates the gating strategy used to identify the various leukocyte populations. Leukocytes were identified as CD45$^+$ population, neutrophils as CD45$^+$Ly6G$^+$, and small resident macrophage as CD45$^+$F4/80^{low}. With this gating strategy, we found that although the percentage of CD45$^+$ leukocytes was about the same in wild-type and Toll-like receptor 7$^{-/-}$ mice (fig. 4, B and C), Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice had many more CD45$^+$ leukocytes in the peritoneal cavity than that of wild-type–cecal ligation and puncture mice (fig. 4D). Moreover, neutrophil is a major phagocyte important for controlling bacterial dissemination during infection. We found that the percentage as well as the total numbers of the peritoneal CD45$^+$Ly6G$^+$ neutrophils increased in both wild-type and Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice compared with sham mice (fig. 4, E–G). Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice had even higher CD45$^+$Ly6G$^+$ neutrophil

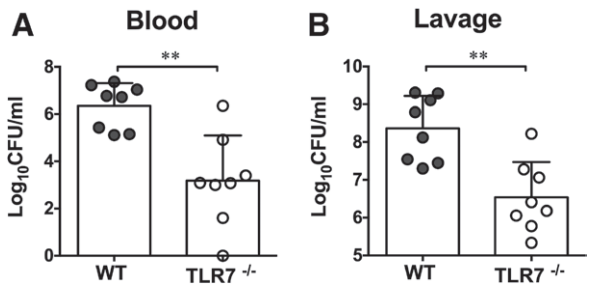

Fig. 3. Bacterial loading in the blood and peritoneal cavity. Both the blood and peritoneal lavage were harvested 24 h after sham or cecal ligation and puncture procedure. After serial dilutions, anticoagulated blood and lavage were incubated on agar plates and bacterial colony-forming units (CFU) per ml were calculated and plotted in a Log_{10} scale. (*A*) Bacterial loading in the blood. (*B*) Bacterial loading in the peritoneal space. $^{**}P < 0.01$. Unequal variance *t* test. N = 8 mice per group. Data are expressed as mean ± SD. TLR7$^{-/-}$, Toll-like receptor 7–deficient; and WT, wild-type.

numbers than that of wild-type cecal ligation and puncture mice ([17.8 ±13.2] × 10^6 *vs.* [6.3 ± 5.7] × 10^6, P = 0.0357; fig. 4G). Another important type of phagocyte in the peritoneal cavity is macrophage. A previous study has identified two distinct peritoneal macrophage subsets, namely large peritoneal macrophages expressing CD11b^{high} F4/80^{high} MHC$^-$ and small peritoneal macrophages expressing CD11b$^+$ F4/80^{low} MHC$^+$.[22] Large peritoneal cells are the major resident macrophages in unoperated naïve mice, whereas the small peritoneal macrophages become the predominate type in lipopolysaccharide-injected mice and are mainly derived from blood monocytes.[22] We noted that large peritoneal macrophages with F4/80^{high} expression were greatly diminished within 24 h in wild-type mice subjected to sham surgery compared with unoperated naïve mice (data not shown), whereas small peritoneal macrophages with F4/80^{low} expression appeared as the predominate population (fig. 4H). As indicated in figure 4I, both wild-type and Toll-like receptor 7$^{-/-}$ cecal ligation and puncture groups had markedly reduced percentage of CD45$^+$/F4/80^{low} peritoneal macrophages compared with their sham controls. Toll-like receptor 7$^{-/-}$ mice, however, appeared to have significantly higher numbers of CD45$^+$/F4/80^{low} macrophages than wild-type mice ([1.7 ± 1.1] × 10^6 *vs.* [4.1 ± 2.8] × 10^6 cells per cavity, P = 0.0343, fig. 4J). We also measured leukocytes in the blood. As indicated in table 1, there was a trend that blood leukocyte counts in Toll-like receptor 7$^{-/-}$ mice were slightly higher than that of wild-type mice ([3.2 ± 1.2] × 10^3 cells per μl *vs.* [4.8 ± 2.1] × 10^3 cells per μl, P = 0.054) after sham procedure. In the mice undergoing cecal ligation and puncture, the blood leukocyte counts dropped significantly in both wild-type and Toll-like receptor 7$^{-/-}$ mice.

Phagocytic Function of the Peritoneal Neutrophils and Macrophages

Next, we tested whether Toll-like receptor 7 signaling plays a role in regulating phagocytic function of the peritoneal leukocytes. We used an *in vivo* phagocytosis assay in which opsonized yellow-green fluorescence-labeled microsphere beads were injected intraperitoneally at 23 h after sham or cecal ligation and puncture procedure and 1 h later, the peritoneal cells were harvested and immediately chilled followed by surface marker staining. As shown in Supplemental Digital Content figure S2 (http://links.lww.com/ALN/B926), the percentage of cells that contained phagocytosed beads was calculated as the percentage of fluorescein-positive cells in leukocytes (CD45$^+$), neutrophils (CD45$^+$Ly6G$^+$), or macrophages (CD45$^+$ F4/80^{low}). As shown in figure 5, A and B, compared with sham mice, the percentage of leukocytes and neutrophils with phagocytosed fluorescent beads increased significantly and equally in both wild-type and Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice. The percentage of phagocytic macrophages increased significantly in wild-type cecal ligation and puncture mice compared with sham but remained the same in Toll-like receptor 7$^{-/-}$ mice (fig. 5C). Next, we measured the capacity of single cell to phagocytose beads, which was expressed as mean fluorescence intensity of microspheres within each cell population. As shown in figure 5D, the mean fluorescence intensity of leukocytes leukocyte (CD45$^+$) dropped significantly in wild-type cecal ligation and puncture mice compared with sham. Toll-like receptor 7$^{-/-}$ mice showed the same bead phagocytosis capacity in single cell and displayed the same degree of decrease as the wild-type cecal ligation and puncture mice. For neutrophils and macrophages, the single cell phagocytosis capacity remains the same in wild-type and Toll-like receptor 7$^{-/-}$ mice between sham and cecal ligation and puncture group (fig. 5, E and F). Finally, we calculated the total number of leukocytes, neutrophils, and macrophages that had phagocytosed beads in the peritoneal cavity to assess the overall phagocytic capacity of the individual cell population. As illustrated in figure 5, G–I, compared with wild-type cecal ligation and puncture mice, Toll-like receptor 7$^{-/-}$ cecal ligation and puncture mice had a marked increase in the number of the phagocytic leukocytes and neutrophils, and significantly more phagocytic macrophages compared with the sham mice.

Effect of Wild-type and Toll-like Receptor 7$^{-/-}$ Cecal Slurry in Wild-type Mice after Peritoneal Injection

The gut microbiota is shaped by both environment and host genetics.[23] To examine the possibility that the difference in colonic microbiome between wild-type and Toll-like receptor 7$^{-/-}$ mice contributes to the beneficial effects seen in Toll-like receptor 7$^{-/-}$ mice after cecal ligation and puncture, we injected an equal amount of cecal slurry from

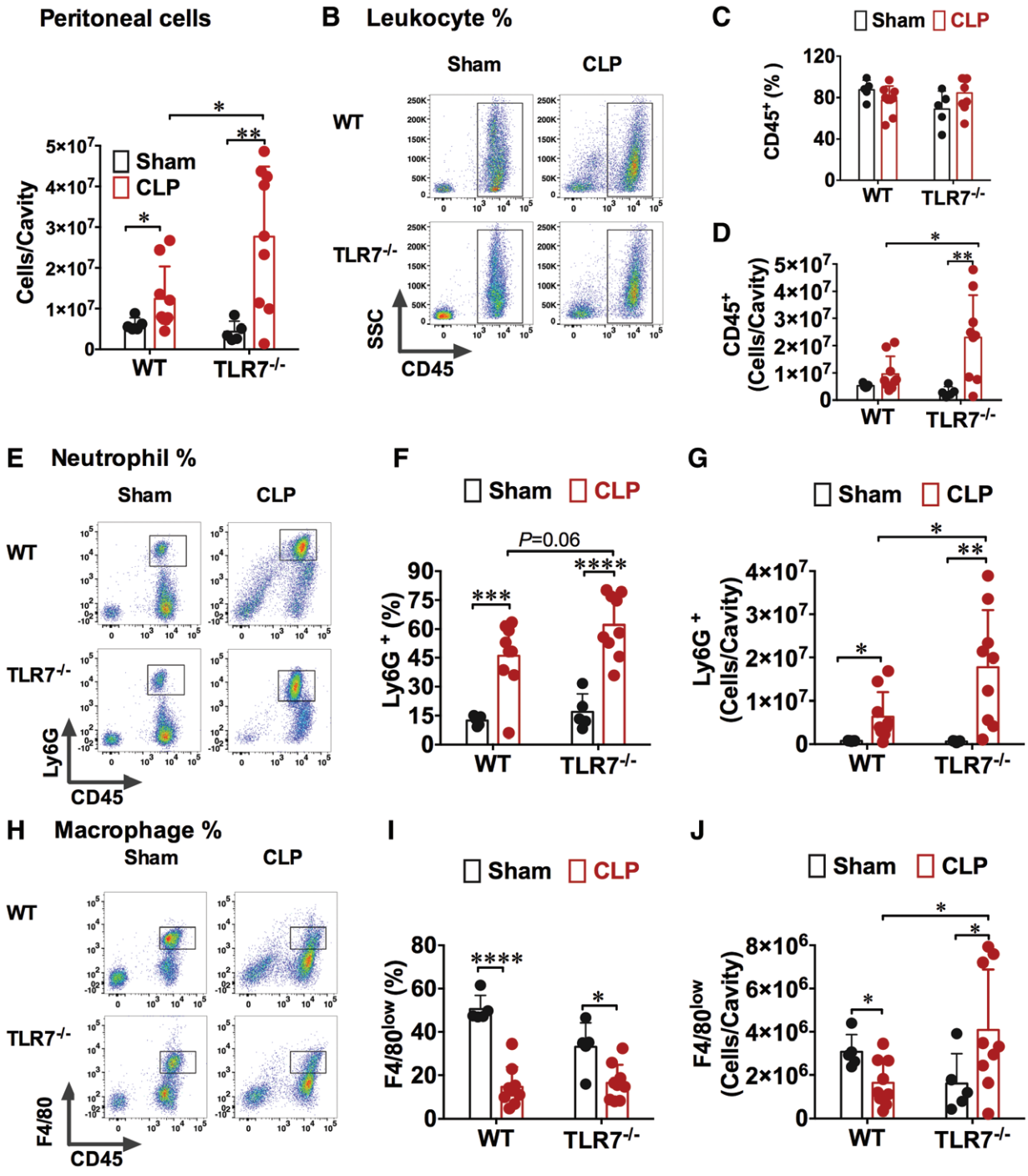

Fig. 4. Absence of Toll-like receptor 7 (TLR7) enhances neutrophil migration to the peritoneal space during polymicrobial sepsis. Twenty-four hours after surgery, peritoneal cells were harvested and analyzed using flow cytometry. Surface marker: $CD45^+$ for leukocyte, $CD45^+Ly6G^+$ for neutrophil, and $CD45^+$ $F4/80^{low}$ for small peritoneal macrophage. (*A*) Total number of cells in the peritoneal cavity at 24 h after sham or cecal ligation and puncture (CLP) procedure. $^*P < 0.05$, $^{**}P < 0.01$. (*B*) Representative flow cytometry plots of leukocytes ($CD45^+$) population. (*C*) Percentage of leukocytes ($CD45^+$) in the peritoneal space. (*D*) Leukocytes increased significantly in the $TLR7^{-/-}$ septic mice as compared with that of wild-type (WT) septic mice. N = 5 to 9, $^*P < 0.05$, $^{**}P < 0.01$. (*E*) Flow cytometry plots depicting the increase in neutrophil percentage in the peritoneal cavity of $TLR7^{-/-}$ septic mice. (*F* and *G*) TLR7-deficiency led to marked neutrophil migration to infection site in both percentage and absolute numbers. N = 5 to 9, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (*H*) Representative flow cytometry plots of small resident macrophages in the peritoneal space. (*I*) Percentage of small resident macrophage decreased following sepsis. N = 5 to 9 per group. $^*P < 0.05$, $^{****}P < 0.0001$. (*J*) TLR7 deficiency increased the small macrophage numbers. N = 5 to 9 per group. $^*P < 0.05$. Unequal variance *t* test. Data are expressed as mean ± SD.

Table 1. Leukocytes in the Blood at 24 h after Procedure

	Leukocytes (10^3/μl)	
Strain	**Sham**	**CLP**
WT	3.2 ± 1.2*	1.5± 0.9**
TLR7$^{-/-}$	4.8 ± 2.1	1.5± 0.8***

N = 9 to 12. Unequal variance *t* test. Data are expressed as mean ± SD. CLP, cecal ligation and puncture; TLR7, Toll-like receptor 7; WT, wild-type.
*P = 0.054 *vs.* TLR7$^{-/-}$-sham, **P = 0.0025 *vs.* WT-sham, ***P = 0.0006 *vs.* TLR7$^{-/-}$-sham.

wild-type or Toll-like receptor 7$^{-/-}$ mice (fig. 6A) to the wild-type recipient mice. Twenty hours later, body temperature, cytokines, and leukocyte migration were detected. As shown in figure 6B, both wild-type and Toll-like receptor 7$^{-/-}$ cecal slurry injection resulted in similarly severe hypothermia in recipient wild-type mice (28.3 ± 1.8 *vs.* 28.2 ± 0.5°C). Injection of wild-type and Toll-like receptor 7$^{-/-}$ cecal slurry also induced a marked increase in cytokine production. Interestingly, whereas the wild-type mice that received Toll-like receptor 7$^{-/-}$ cecal slurry displayed significantly higher plasma interleukin-6 production than the ones that received wild-type cecal slurry (fig. 6C), no difference was observed in peritoneal interleukin-6 and tumor necrosis factor-α production between the two groups (fig. 6D). Moreover, both groups of mice elicited marked (but to the same degree) peritoneal neutrophil migration compared with the vehicle control ([3.20 ± 1.80] × 10^7 *vs.* [3.20 ± 1.70] × 10^7 *vs.* [0.14 ± 0.02] × 10^7 cells per cavity, wild-type *vs.* Toll-like receptor 7$^{-/-}$ *vs.* Vehicle, fig. 6, E and F).

Discussion

In the recent Sepsis-3 definition, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.[24] The new definition emphasizes the primacy of the nonhomeostatic host response to infection. The host response is initiated by pathogen-associated molecular patterns and further amplified by damage-associated molecular patterns during host–pathogen interaction.[20] As a result, excessive inflammatory response contributed to organ injury and mortality, especially in the early stage of sepsis.[20,25] Previous studies from our lab and others' have demonstrated that extracellular RNAs derived from bacteria and host are recognized by Toll-like receptor 7 and modulate host innate and adaptive immune responses.[9–11,21] However, whether or not Toll-like receptor 7 receptor signaling plays a role in sepsis progression and outcomes was unclear. In the current study, we made three major findings. First, Toll-like receptor 7$^{-/-}$ mice had improved survival and attenuated acute kidney injury following cecal ligation and puncture-induced sepsis. Second, Toll-like receptor 7$^{-/-}$ mice had a lower systemic and local cytokine responses but maintained a strong ability to clear bacteria. Third, Toll-like receptor 7$^{-/-}$ mice had enhanced peritoneal leukocyte recruitment and phagocytic activity during polymicrobial sepsis.

Toll-like receptor 7 recognizes viral or other single-stranded RNA.[3] Specific examples include vesicular stomatitis virus, human immunodeficiency virus 1, hepatitis B and C virus,[4,5,26–28] or synthetic guanine-rich RNA sequence analogs such as resiquimod (R848) and imiquimod (R837).[27,29] In addition to its antiviral effects, a number of studies have revealed the important role of Toll-like receptor 7 in pathoimmunology of many diseases where inflammation plays a pivotal role, such as cancer metastasis,[30] systemic lupus erythematosus,[31] juvenile dermatomyositis,[32] and neurodegeneration.[33] For example, a study by Fabbri *et al.*[30] reported that Toll-like receptor 7 binding to extracellular microRNAs released from tumor triggered a prometastatic inflammatory response, which may lead to tumor growth. We have found that cardiac or splenic RNA and synthetic microRNAs rich in uridine induce proinflammatory response including cytokine and complement production *in vitro* and neutrophil migration *in vivo*.[10–12] The proinflammatory responses are mediated through Toll-like receptor 7–myeloid differentiation primary response 88 signaling induced by Toll-like receptor 7 dimerization in the endosome upon ligand binding[34] and by activation of nuclear factor kappa–light-chain-enhancer of activated B cells, mitogen-activated protein kinase, or type I interferon pathway.[10,11,35–37] Toll-like receptor 7 also reportedly mediates cell injury *via* myeloid differentiation primary response 88–independent mechanisms. Park *et al.*[38] found that interaction between Toll-like receptor 7 and transient receptor potential ankyrin 1 is required to recognize extracellular micro-RNAs for activation and excitation of nociceptive sensory neurons in eliciting pain, which does not require myeloid differentiation primary response 88. Sterile α and armadillo motif containing protein 1, but not myeloid differentiation primary response 88, was demonstrated to mediate Toll-like receptor 7/Toll-like receptor 9–induced apoptosis in neurons.[39] In the current study, we found that mice lacking Toll-like receptor 7 displayed significantly attenuated proinflammatory cytokines including interleukin-6, tumor necrosis factor-α, and interleukin-1β, both in blood and in local infection sites during sepsis. Similar findings were also reported with other innate immune receptors, where genetic deficiency of Toll-like receptor 2 or myeloid differentiation primary response 88 confers beneficial effect against polymicrobial sepsis.[40,41] On the other hand, Koerner *et al.*[42] reported that stimulation of Toll-like receptor 7 before the colon ascendens stent peritonitis improved the immune control of the inflammatory response in the mice. Mice treated with R848 (Toll-like receptor 7 ligand) before sepsis induction exhibited a reduction of proinflammatory cytokine in the spleen and alleviated bacterial loading in the peritoneum/spleen as well as decreased thymus and spleen apoptosis, indicating a trend of improvement even though no mortality data was reported. The protective effect may in part

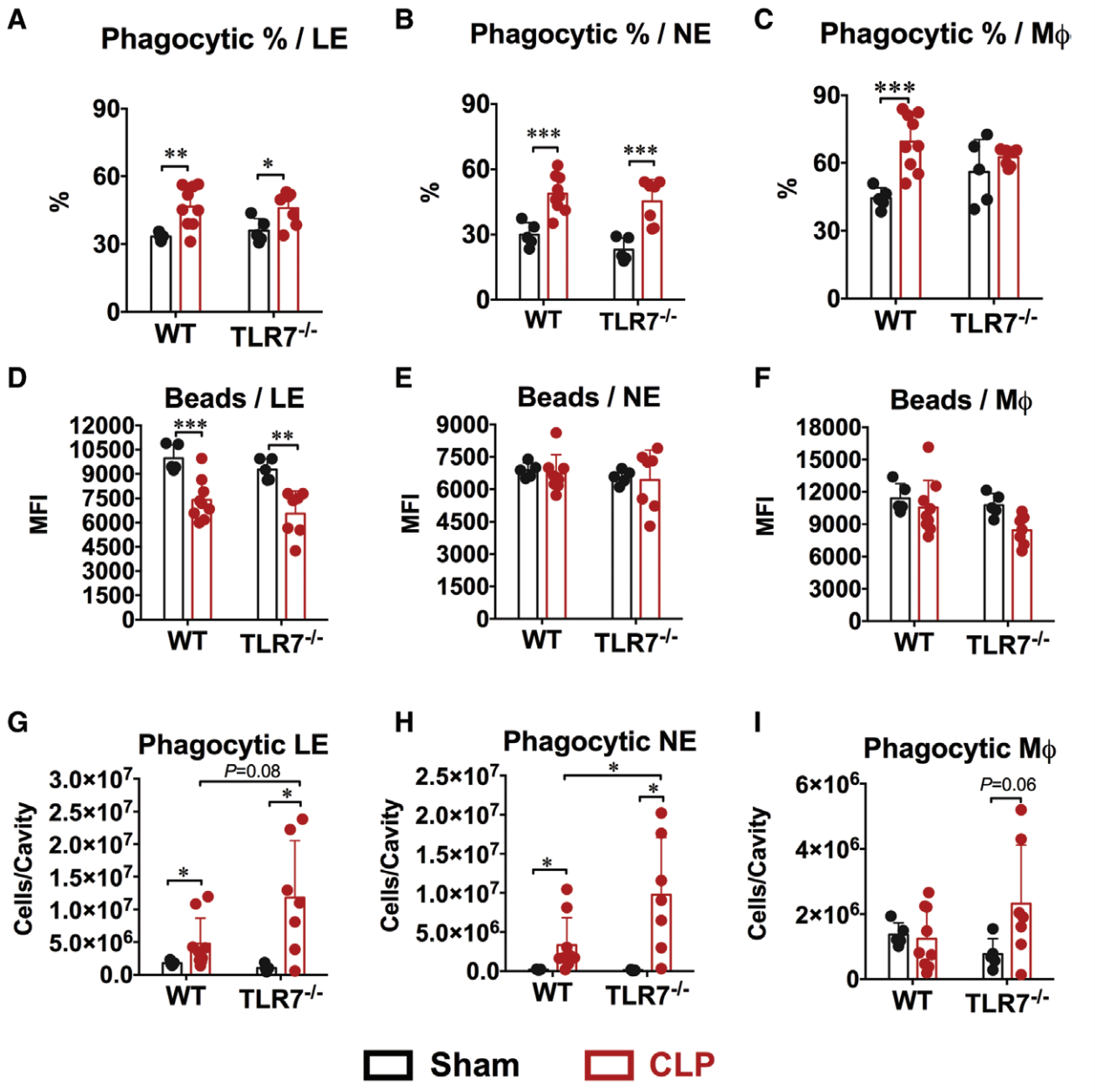

Fig. 5. Toll-like receptor 7 (TLR7) deficiency increased the phagocytic cells after sepsis. 10^8 fluorescein isothiocyanate–labeled carboxylate-modified microspheres (beads) in 200 μl were injected intraperitoneally at 23 h after sham or cecal ligation and puncture (CLP) surgery. One hour later, peritoneal cells were collected and analyzed using flow cytometry. (*A–C*) Percentage of cells with phagocytic function. (*D–F*) Single-cell phagocytic function. (*G–I*) TLR7 deficiency increased phagocytic cells in septic mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N = 5 to 9 mice per group. Unequal variance *t* test. Data are expressed as mean ± SD. LE, leukocytes; MΦ, macrophage; MFI, mean fluorescence intensity; NE, neutrophil; WT, wild-type.

attribute to the Toll-like receptor ligand tolerance effect, in which exposure of innate immune cells to Toll-like receptor ligands induces a state of temporary refractoriness to a subsequent exposure of a Toll-like receptor ligand.

The rate of acute kidney injury is directly proportional to the severity of sepsis. The combination of acute kidney injury and sepsis is associated with a 70% mortality rate when compared with a 45% mortality rate in patients with acute renal failure alone.[43] We found that absence of Toll-like receptor 7 resulted in alleviated sepsis-induced acute kidney injury as evidenced by reduced neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 in

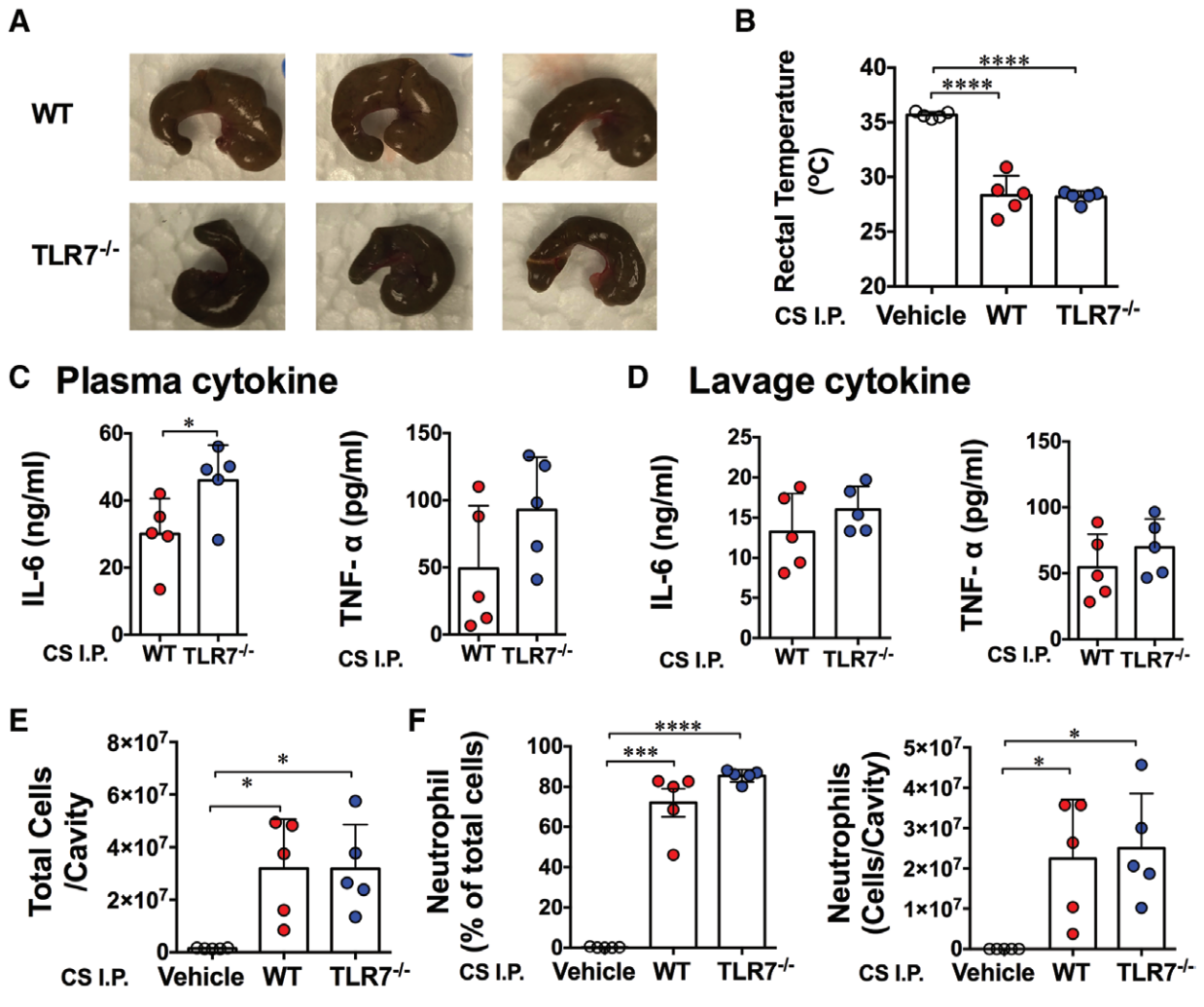

Fig. 6. Cecal slurry of wild-type (WT) and Toll-like receptor 7–deficient (TLR7$^{-/-}$) mice induces systemic and peritoneal inflammation and leukocyte migration in WT mice after peritoneal injection. Cecal slurry collected from WT and TLR7$^{-/-}$ mice was intraperitoneally injected to WT recipient mice. Twenty hours later, rectal temperature, cytokine, and peritoneal cell migration were measured. (*A*) Representative pictures of mouse cecum from WT and TLR7$^{-/-}$ mice. (*B*) Rectal temperature. Data were analyzed by one-way analysis of variance. (*C* and *D*) Cytokine production in the plasma and peritoneal cavity. Unequal variance *t* test. (*E*) Total cell numbers in the peritoneal cavity. Unequal variance *t* test. (*F*) Percentage and absolute number of neutrophil migrated to peritoneal space. Unequal variance *t* test. Each bar represents mean ± SD. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. N = 5 per group. CS, cecal slurry; I.P., intraperitoneal injection; IL, interleukin; TNF, tumor necrosis factor; Vehicle, 5% dextrose in water.

the kidney. This may be secondary to reduced proinflammatory cytokines in Toll-like receptor 7$^{-/-}$ septic mice as Nechemia-Arbely *et al.*[44] demonstrated that inflammatory cytokine such as interleukin-6 promotes peritubular neutrophils accumulation and exacerbates renal injury. Studies have also found a significant expansion of myeloid-derived suppressor cells and severe podocyte injury in glomeruli of kidneys in Toll-like receptor 7 agonist imiquimod-induced lupus mice,[45] implicating a direct role of Toll-like receptor 7 in kidney injury.

One survival factor in bacterial sepsis is successful control of bacterial dissemination. In the present study, we found that Toll-like receptor 7–deficient mice had improved bacterial clearance both in the peritoneal space and in the blood as compared with wild-type mice after cecal ligation and puncture. To identify the possible mechanisms for the enhanced bacterial clearance in Toll-like receptor 7$^{-/-}$ mice, we tested leukocytes migration to the infection site, which is essential for controlling bacterial burden and eliminating systemic spread of infection.[46,47] We found markedly enhanced leukocytes, mainly neutrophils, migration to the peritoneal space in the Toll-like receptor 7$^{-/-}$ mice after cecal ligation and puncture. This may represent one of the potential mechanisms responsible for the improved bacterial clearance and

survival in septic Toll-like receptor $7^{-/-}$ mice. The exact mechanism for the enhanced neutrophil migration to the peritoneal cavity in Toll-like receptor $7^{-/-}$ mice is still unclear. In general, bacterial products, cytokine/chemokine gradients, and phagocyte chemokine receptor expression can all modulate neutrophil migratory responses during sepsis.[48] For example, Toll-like receptor 2 deficiency reportedly enhances neutrophil migratory function in sepsis by promoting neutrophil chemokine receptor CXCR2 while the systemic cytokine levels were lower in Toll-like receptor 2 knockout mice.[49] In addition, impaired neutrophil migration is thought to be attributable to internalization of chemokine receptor CXCR2 in circulating neutrophils of mice or patients with severe sepsis[47,50–52] with involvement of tumor necrosis factor-α.[53] Neutrophils treated with tumor necrosis factor-α exhibit reduced chemotaxis toward chemokine ligand 2.[53] Therefore, we speculate that suppressed production of plasma tumor necrosis factor-α in Toll-like receptor $7^{-/-}$ septic mice may in part contribute to enhanced neutrophil migration.

Different from neutrophils, the percentage of peritoneal macrophages among leukocytes decreased dramatically after sepsis in both wild-type and Toll-like receptor $7^{-/-}$ mice compared with that of sham mice, but the absolute number of macrophages was still significantly higher in Toll-like receptor 7–deficient septic mice, suggesting preserved macrophage ability against bacteria. In line with our findings, Talreja and Samavati[35] reported that Toll-like receptor 7/8–primed macrophages exhibited decreased bacterial clearance when infected with live bacteria. Phagocytic function of immune cells is another important host defense against invading bacteria.[54] Although Toll-like receptor 7 deficiency did not alter the phagocytic function *per se* of the peritoneal neutrophils and macrophages, we did observe an increase in the number of phagocytic neutrophils and macrophages positive with fluorescent beads in these mice. Taken together, the enhanced bacterial clearance in Toll-like receptor $7^{-/-}$ septic mice are likely attributed to enhanced neutrophil and monocyte recruitment into the peritoneal cavity and hence increased phagocytic capability.

There is evidence that changes in the host gene have significant effects on mouse microbiome.[23] For example, the loss of Toll-like receptor 5 altered the gut microbiota and promoted the development of metabolic syndrome in these mice.[55] To determine if the decreased inflammatory response observed in Toll-like receptor $7^{-/-}$ mice was not secondary to less pathogenic virulence of cecal bacteria in these mice, we injected wild-type recipient mice intraperitoneally with cecal slurry harvested from wild-type and Toll-like receptor $7^{-/-}$ mice. Lack of difference in body temperature, peritoneal cytokines, and leukocyte migration between the mice receiving wild-type *versus* Toll-like receptor $7^{-/-}$ cecal slurry strongly suggest that wild-type and Toll-like receptor $7^{-/-}$ mouse slurry exhibit similar virulence and proinflammatory effects, and that any potential difference in the bowel microbiomes in wild-type and Toll-like receptor $7^{-/-}$ mice may not explain the survival benefit and other effects seen in Toll-like receptor $7^{-/-}$ septic mice.

In summary, we have demonstrated that mice lacking Toll-like receptor 7 exhibited attenuated systemic cytokine production, reduced acute kidney injury and bacterial loading, and improved survival compared with wild-type mice after polymicrobial infection. These data suggest that signaling *via* Toll-like receptor 7, a sensor for both pathogen and host single-stranded RNA, may play an important role in sepsis pathogenesis.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Zou: Department of Anesthesiology and Shock Trauma Anesthesiology Research Center, University of Maryland School of Medicine, 660 West Redwood Street, Howard Hall 598, Baltimore, Maryland 21201. lzou@som.umaryland.edu. 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.

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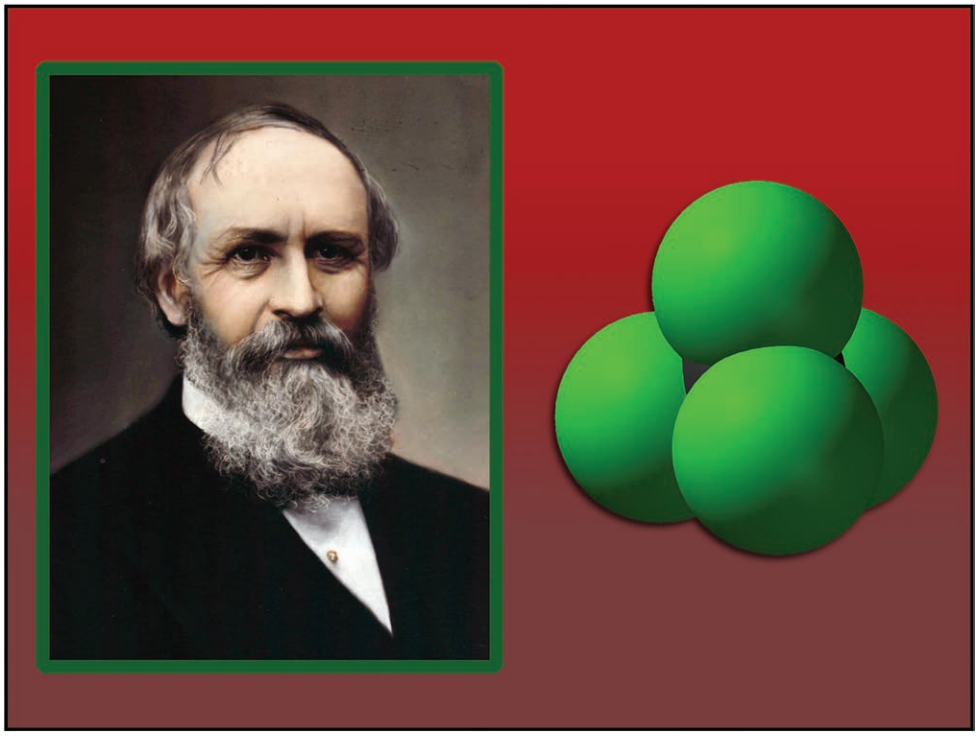

Chicago surgeon Edmund Andrews is best known for encouraging in 1868 the oxygenation of nitrous oxide anesthetics. However, 1 yr earlier, Dr. Andrews had spent time observing CCl_4 or carbon tetrachloride anesthesia in London hospitals. On returning to Chicago, Dr. Andrews noted that the tetrachloride of carbon promised "freedom from nauseating effects," such as those caused by ether. However, when Dr. Andrews's friend, Dr. Sherman, administered the promising new agent to their patient in Chicago, the patient's "pulse and respiration both suddenly ceased." An unnerved Dr. Andrews determined that he himself would "not venture on the use of the article again, unless very extensive experience by others demonstrates its safety." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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