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Toll-like receptor 7 contributes to inflammation, organ injury, and mortality in murine sepsis

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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/6 J background, were used. 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

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AUTHORS CONTRIBUTIONS

LZ and WC conceptualized the idea, designed the study, analyzed the data, and finalized the manuscript. WJ, LG, BW, and LZ performed the experiments and analyzed the data. YF, BW contributed to the experimental design and data interpretation. WJ drafted the manuscript.

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Conflicts of Interest:

The authors declare no competing interests.

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 hours. 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.

Keywords

Toll-like receptor; sepsis; acute kidney injury; bacterial clearance; neutrophil migration; inflammation

INTRODUCTION

Sepsis remains one of the intricate diseases with extremely high mortality. Between 2004-2009, 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 (TLRs) are a pivotal part of host innate immune defense and play a critical role in molecular pattern recognition. Among 10 TLRs, TLR7 and TLR8 were originally identified as the sensor for single-stranded RNA (ssRNA) of viral origins³⁻⁵. Although their genes lie in close proximity on X chromosome⁶, only TLR7 is functional in mice⁷. Besides viral ssRNAs, TLR7 can also recognize nucleic acids released from bacteria. For instance, human primary monocytes and macrophages sense *staphylococcus aureus* ssRNA via TLR8 and induces interferon- β production⁸. Moreover, vaccine composition formulated with a TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*⁹. These findings suggest that TLR7 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 (miRNAs) 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 sepsis¹⁰. *In vitro* and *in vivo* studies show that extracellular RNAs and miRNA mimics can work as host damage-associated molecular patterns to induce a robust proinflammatory response such as cytokine production, immune cell activation, and complement activation¹⁰⁻¹². Most importantly, the proinflammatory role of these extracellular RNAs and miRNAs proves to be dependent of TLR7-myeloid differentiation primary response 88 (MyD88) signaling both in cell cultures and in a peritonitis model *in vivo*¹⁰⁻¹². However, the role of extracellular RNA and TLR7 signaling in sepsis pathogenesis remains unclear.

In the present study, we tested the hypothesis that TLR7 sensing plays an important role in mediating host innate immune responses, tissue injury, and mortality following bacterial sepsis. Taking a *loss-of-function* approach, we examined the impact of TLR7 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 (WT, C57BL/6 J) and TLR7^{-/-} mice ((Tlr7^{tm1Flv}/J) were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice were 8 to 16-week-old and weighed between 22-30g. Animals were housed in an animal facility of Massachusetts General Hospital (MGH) or University of Maryland School of Medicine 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 12h/12h light/dark cycles. All animal protocols were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital (Boston, MA) and Institutional Animal Care & Use Committee of University of Maryland School of Medicine and followed the guideline of the National Institutes of Health (Bethesda, MD). Simple randomization was used to assign animals and the operators of the surgery (WJ and LG) were blinded to the strain information.

Mouse model of polymicrobial sepsis

Cecal ligation and puncture (CLP) model was slightly modified based on what we described previously and was performed in the morning¹³. Briefly, after anesthetization (ketamine 100 mg/kg, xylazine 4 mg/kg), mice were subjected to laparotomy. The cecum was ligated 1.2-1.5 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 CLP. 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 post-operative pain. Rectal temperature was recorded at 24 hours after surgery.

Mortality study

After CLP, mice (n=27/group) were observed every 4 hours for the first 48 hours and every 12 hours for up to 11 days. Experimental operators and observers (WJ and LZ) were blinded to the strain information. Because sham surgery resulted in no mortality¹⁴, 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 (qRT-PCR)

Kidney samples were collected at 24 hours after surgery (N=6-8/group). RNA was extracted from the kidney with TRIzol (Sigma, St. Louis, MO) and cDNA synthesized with reverse transcriptase. Quantitative RT-PCR was performed as described previously¹³. The transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), neutrophil gelatinase-

associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1) were quantified using qRT-PCR with GAPDH as the internal control. The PCR primer sequences are listed below: GAPDH (Forward: 5'-AACTTTGGCATTGTGGAAGG-3', Reverse: 5'-GGATGCAGGGATGATGTTCT-3'); NGAL (Forward: 5'-CTCAGAACTTGATCCCTGCC-3', Reverse: 5'-TCCTTGAGGCCAGAGACTT-3'); KIM-1 (Forward: 5'-CATTTAGGCCTCATACTGC-3', Reverse: 5'-ACAAGCAGAAGATGGGCATT-3'). Transcript expression was calculated using the comparative Ct method normalized to GAPDH (2^{-Ct}) and expressed as the fold difference in the CLP or treatment group over the WT-sham group.

Bacterial colony formation and quantification

Twenty-four hours after sham or CLP procedures, five ml of sterile normal saline was injected into the peritoneal cavities of WT and TLR7^{-/-} mice (N=8/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, Sparks, MD) at 37°C for 36 hours. 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 ELISA analysis.

Cytokine ELISA

Twenty-four hours after procedure, plasma and peritoneal lavages were collected and measured for interleukin 6 (IL-6), interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α) and chemokine ligand 2 (CXCL2) using commercially available DuoSet or Quantikine HS ELISA kits (R&D systems, Minneapolis, MN) 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, Waltham, MA) were opsonized by incubating with Roswell Park Memorial Institute-1640 cell culture medium containing 10% fetal bovine serum (FBS) at 37 °C for 30 mins. The opsonized microspheres were then injected intraperitoneally at 23 hours after sham or CLP surgery in a dose of 10⁸ microspheres/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 fraction of cells (4×10^5) were stained with the following surface markers: CD45-Phycoerythrin (clone 30-F11, 1:1500 dilution, BD Biosciences, San Jose, CA,), Ly6G-Brilliant Violet 421 (clone 1A8, 1:100 dilution, BD Biosciences, San Jose, CA), F4/80-Alexa Fluor 647 (clone T45-2342, 1:100 dilution, BD Biosciences, San Jose, CA) at 4 °C for 30 min in the dark as described previously¹². Cells were washed twice in 4 ml of ice cold phosphate-buffered saline (PBS) buffer and resuspended in PBS containing 5% FBS, 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 CLP surgery, mice (N=9-12/group) were anesthetized as described above. Blood was collected via cardiac puncture and transferred to blood collection tubes containing 3.2% sodium citrate as anti-coagulant (Grenier, FisherScientific, Pittsburgh, PA). Leukocyte count was tested using an automated cell counter (Beckman COULTER® AC-T diff™ Analyzer, Brea, CA).

Cecal Slurry (CS) model of polymicrobial sepsis

Male and age matched WT and TLR7^{-/-} 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, Lake Forest, IL) in water to make a cecal contents slurry at a concentration of 80 mg/ml. This CS was mixed well by vortex and filtered through a 100 µm sterile cell strainer. Mice were received the CS intraperitoneally in a dose of 1.3 mg/g body weight in 500 µl volume. The vehicle group receive 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., La Jolla, CA). 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

TLR7^{-/-} mice have reduced mortality and attenuated acute kidney injury in polymicrobial sepsis

As shown in Fig. 1A, WT CLP mice showed an accumulated mortality of 48% on day 2 and 81% on day 11. In comparison, TLR7^{-/-} CLP 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 models¹⁵. Consistent with the mortality data, WT CLP mice displayed a significantly lower rectal temperature compared with TLR7^{-/-} CLP mice (28.3 ± 0.7 vs. 31.1 ± 1.1 °C, $P=0.0448$) (Fig.1B) at 24 hours after

surgery, suggesting a less severe septic condition in these TLR7^{-/-} mice. NGAL¹⁶⁻¹⁸ and KIM-1¹⁹ are two highly sensitive biomarkers of acute kidney injury (AKI). As illustrated in Fig. 1C, WT CLP mice quickly developed AKI as evidenced by markedly elevated kidney NGAL and KIM-1 gene expression. TLR7^{-/-} CLP mice had substantially lower NGAL (139 ± 41 vs. 307 ± 184 folds, $P=0.0364$) and KIM-1 ($13[4, 223]$ vs. $40 [16, 49]$ folds, $P=0.0704$) gene expression as compared with WT-CLP. Together, these data suggest that lack of TLR7 signaling confers both survival and kidney-protecting benefits in the mouse model of polymicrobial sepsis.

Lack of TLR7 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 pathogens²⁰. To determine the role of TLR7 signaling in host inflammation after polymicrobial sepsis, we measured the proinflammatory cytokines, IL-6, IL-1 β , and TNF- α , and the chemokine CXCL2, known for their key role in sepsis pathogenesis. As illustrated in Fig. 2, we observed significant reduction in IL-6 and TNF- α , and CXCL2, in the plasma and the peritoneal lavages of TLR7^{-/-} mice as compared to that of WT mice after CLP. However, IL-1 β was only reduced in the peritoneal lavages, but not in the plasma, of TLR7^{-/-} CLP mice when compared with WT CLP mice. To determine the role of TLR7 in bacterial clearance, we measured the bacterial loading both in the blood and the peritoneal lavage 24 hours after sham and CLP surgery. Almost no bacterial colonies were identified from sham samples. As shown in Fig. 3, there were marked bacterial loads in both blood and peritoneal lavage of CLP mice. Compared to WT CLP mice, TLR7^{-/-} CLP mice had a marked reduction in bacterial loading in both blood and the peritoneal lavage.

TLR7^{-/-} mice exhibited enhanced neutrophil and small peritoneal macrophage recruitment after CLP

The reduced bacterial loading in TLR7^{-/-} mice as compared to WT mice suggested a possible role of TLR7 activation in functional impairment of immune effector cells during sepsis. TLR7 activation is known for its role in human CD4(+) T cell anergy²¹. Thus, we investigated how polymicrobial infection after CLP affected peritoneal residential cells as well as leukocytes recruited into the peritoneal cavity in both WT and TLR7^{-/-} mice. As shown in Fig. 4A, there was a significant increase in the peritoneal cells in WT and TLR7^{-/-} septic mice as compared with their sham counterparts. Comparing the two CLP groups, TLR7^{-/-} mice had higher peritoneal cells than WT ($[28.0 \pm 17.2] \times 10^6$ vs. $[12.4 \pm 7.9] \times 10^6$ cells/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. Supplement Fig. 1 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 WT and TLR7^{-/-} mice (Fig. 4B-C), TLR7^{-/-} CLP mice had many more CD45⁺ leukocytes in the peritoneal cavity than that of WT-CLP 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 WT CLP and TLR7^{-/-} CLP mice compared with sham mice (Fig. 4 E-G). TLR7^{-/-} CLP mice had even higher CD45⁺Ly6G⁺ neutrophil numbers than that of WT CLP mice ($[17.8 \pm 13.2] \times 10^6$ vs. $[6.3 \pm 5.7] \times 10^6$, $P=0.0357$) (Fig. 4G). Another important type of phagocytes 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 macrophage in unoperated naïve mice, whereas the small peritoneal macrophages become the predominate type in lipopolysaccharide-injected mice and are mainly derived from blood monocytes²². We noted that large peritoneal macrophages with F4/80^{high} expression were greatly diminished within 24 hours in WT mice subjected to sham surgery compared to 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 Fig. 4I, both WT and TLR7^{-/-} CLP groups had markedly reduced percentage of CD45⁺/F4/80^{low} peritoneal macrophages compared with their sham controls. TLR7^{-/-} mice, however, appeared to have significantly higher numbers of CD45⁺/F4/80^{low} macrophages than WT mice ($[1.7 \pm 1.1] \times 10^6$ vs. $[4.1 \pm 2.8] \times 10^6$ cells/cavity, $P=0.0343$, Fig. 4J). We also measured leukocytes in the blood. As indicated in Table I, there was a trend that blood leukocyte counts in TLR7^{-/-} mice were slightly higher than that of WT mice ($[3.2 \pm 1.2] \times 10^3$ cells/ μ l vs. $[4.8 \pm 2.1] \times 10^3$ cells/ μ l, $P=0.054$) after sham procedure. In the mice undergoing CLP, the blood leukocyte counts dropped significantly in both WT and TLR7^{-/-} mice.

Phagocytic function of the peritoneal neutrophils and macrophages

Next, we tested whether TLR7 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 hours after sham or CLP procedure and 1 hour later, the peritoneal cells were harvested and immediately chilled followed by surface marker staining. As shown in Supplement Fig. 2, 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 Fig. 5A-B, compared to sham mice, the percentage of leukocytes and neutrophils with phagocytosed fluorescent beads increased significantly and equally in both WT and TLR7^{-/-} CLP mice. The percentage of phagocytic macrophages increased significantly in WT CLP mice compared to sham but maintained the same in TLR7^{-/-} mice (Fig. 5C). Next, we measured the capacity of single cell to phagocytose beads, which was expressed as mean fluorescence intensity (MFI) of microspheres within each cell population. As shown in Fig. 5D, the MFI of leukocytes leukocyte (CD45⁺) dropped significantly in WT CLP mice compared to sham. TLR7^{-/-} mice showed the same bead phagocytosis capacity in single cell and displayed the same degree of decrease as the WT CLP mice. For neutrophils and macrophages, the single cell phagocytosis capacity remains the same in WT and TLR7^{-/-} mice between sham and CLP group (Fig. 5E-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 Fig. 5G-I, compared

to WT CLP mice, TLR7^{-/-} CLP mice had a marked increase in the number of the phagocytic leukocytes and neutrophils, and significant more phagocytic macrophages compared with the sham mice.

Effect of WT and TLR7^{-/-} cecal slurry in WT mice after peritoneal injection

The gut microbiota is shaped by both environment and host genetics²³. To examine the possibility that the difference in colonic microbiome between WT and TLR7^{-/-} mice contributes to the beneficial effects seen TLR7^{-/-} mice after CLP, we injected equal amount of cecal slurry (CS) from WT or TLR7^{-/-} mice (Fig. 6A) to the WT recipient mice. Twenty hours later, body temperature, cytokines and leukocyte migration were detected. As shown in Fig. 6B, both WT and TLR7^{-/-} CS injection resulted in similarly severe hypothermia in recipient WT mice (28.3 ± 1.8 vs. 28.2 ± 0.5 °C). Injection of WT and TLR7^{-/-} CS 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 IL-6 production than the ones that received WT cecal slurry (Fig. 6C), no difference was observed in peritoneal IL-6 and TNF- α production between the two groups (Fig. 6D). Moreover, both WT and TLR7^{-/-} CS elicited marked (but to the same degree) peritoneal neutrophil migration compared to the vehicle control and ($[3.20 \pm 1.80] \times 10^7$ vs. $[3.20 \pm 1.70] \times 10^7$ vs. $[0.14 \pm 0.02] \times 10^7$ cells/cavity, WT CS vs. TLR7^{-/-} CS vs. Vehicle, Fig. 6E-F).

DISCUSSION

In the recent Sepsis-3 definition, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection²⁴. The new definition emphasizes the primacy of the non-homeostatic host response to infection. The host response is initiated by pathogen-associated molecular patterns and further amplified by danger-associated molecular patterns during host-pathogen interaction²⁰. 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 TLR7 and modulate host innate and adaptive immune responses^{9-11,21}. However, whether or not TLR7 receptor signaling plays a role in sepsis progression and outcomes was unclear. In the current study, we made three major findings. First, TLR7^{-/-} had improved survival and attenuated acute kidney injury following CLP. Second, TLR7^{-/-} mice had a lower systemic and local cytokine responses but maintained a strong ability to clear bacteria. Third, TLR7^{-/-} mice had enhanced peritoneal leukocyte recruitment and phagocytic activity during polymicrobial sepsis.

TLR7 recognizes viral or other single-stranded RNA³. 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 anti-viral effects, a number of studies have revealed the important role of TLR7 in patho-immunology of many diseases where inflammation plays a pivotal role such as cancer metastasis³⁰, systemic lupus erythematosus³¹, Juvenile dermatomyositis³², and neurodegeneration³³. For example, study by Fabbri, *et al.* reported

that TLR7 binding to extracellular miRNAs released from tumor triggered a prometastatic inflammatory response, which may lead to tumor growth³⁰. We have found that cardiac or splenic RNA and synthetic miRNAs rich in uridine induces proinflammatory response including cytokine and complement production *in vitro* and neutrophil migration *in vivo*¹⁰⁻¹². The proinflammatory responses are mediated through TLR7-MyD88 signaling induced by TLR7 dimerization in the endosome upon ligand binding³⁴ and by activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), mitogen-activated protein kinase or type I interferon pathway^{10,11,35-37}. TLR7 also reportedly mediates cell injury *via* MyD88-independent mechanisms. Park, *et al.* found that interaction of TLR7 and transient receptor potential ankyrin 1 is required to recognize extracellular miRNAs for activation and excitation of nociceptive sensory neurons in eliciting pain, which does not require MyD88³⁸. Sterile α and armadillo motif containing protein 1, but not MyD88, was demonstrated to mediate TLR7/TLR9-induced apoptosis in neurons³⁹. In current study, we found that mice lacking TLR7 displayed a significantly attenuated proinflammatory cytokine including IL-6, TNF- α and IL-1 β both in the blood and in local infection sites during sepsis. Similar findings were also reported with other innate immune receptors, where genetic deficiency of TLR2 or MyD88 confers beneficial effect against polymicrobial sepsis^{40,41}. On the other hand, Koerner *et al.*⁴² reported that stimulation of TLR7 before the colon ascendens stent peritonitis improved the immune control of the inflammatory response in the mice. Mice treated with R848 (TLR7 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 attribute to the TLR ligand “tolerance” effect, in which exposure of innate immune cells to TLR ligands induces a state of temporary refractoriness to a subsequent exposure of a TLR 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 alone⁴³. We found that absence of TLR7 resulted in alleviated sepsis-induced acute kidney injury as evidenced by reduced NGAL and KIM-1 in the kidney. This may be secondary to reduced proinflammatory cytokines in TLR7^{-/-} septic mice as Nechemia-Arbely *et al.*⁴⁴ demonstrated that inflammatory cytokine such as IL-6 promotes peritubular neutrophils accumulation and exacerbates renal injury. Studies also found a significant expansion of myeloid-derived suppressor cells and severe podocyte injury in glomeruli of kidneys in TLR7 agonist imiquimod-induced lupus mice⁴⁵, implicating a direct role of TLR7 in kidney injury.

One survival factor in bacterial sepsis is successful control of bacterial dissemination. In the present study, we found that TLR7-deficient mice had improved bacterial clearance both in the peritoneal space and in the blood as compared to WT mice following CLP. To identify the possible mechanisms for the enhanced bacterial clearance in TLR7^{-/-} mice, we tested leukocyte 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 TLR7^{-/-} after CLP. This may represent one of the potential mechanisms responsible for the improved bacterial clearance

and survival in septic TLR7^{-/-} mice. The exact mechanism for the enhanced neutrophil migration to the peritoneal cavity in TLR7^{-/-} mice is still unclear. In general, bacterial products, cytokine/chemokine gradients, and phagocyte chemokine receptor expression can all modulate neutrophil migratory responses during sepsis⁴⁸. For example, TLR2 deficiency reportedly enhances neutrophil migratory function in sepsis by promoting neutrophil chemokine receptor CXCR2 while the systemic cytokine levels were lower in TLR2 KO mice⁴⁹. 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 TNF- α ⁵³. Neutrophils treated with TNF- α exhibit reduced chemotaxis toward CXCL2⁵³. Therefore, we speculate that suppressed production of plasma TNF- α in TLR7^{-/-} septic mice may in part contribute to enhanced neutrophil migration.

Different from neutrophils, the percentage of peritoneal macrophages among leukocytes decreased dramatically following sepsis in both WT and TLR7^{-/-} mice compared to that of sham mice. But the absolute number of macrophages was still significantly higher in TLR7-deficient septic mice, suggesting preserved macrophage ability against bacteria. In line with our findings, Talreja, *et al.* reported TLR7/8-primed macrophages exhibited decreased bacterial clearance when infected with live bacteria³⁵. Phagocytic function of immune cells is another important host defense against invading bacteria⁵⁴. Although TLR7 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 TLR7^{-/-} 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 host gene have significant effects on mouse microbiome²³. For example, the loss of TLR5 altered the gut microbiota and promoted the development of metabolic syndrome in these mice⁵⁵. To determine if the decreased inflammatory response observed in TLR7^{-/-} mice was secondary to less pathogenic virulence of cecal bacteria in these mice, we injected WT mice intraperitoneally with cecal slurry harvested from WT and TLR7^{-/-} mice. Lack of difference in body temperature, peritoneal cytokines and leukocyte migration between the mice receiving WT *vs.* TLR7^{-/-} cecal slurry strongly suggest that WT and TLR7^{-/-} mouse slurry exhibit similar virulence and proinflammatory effects, and that any potential difference in the bowel microbiomes in WT and TLR7^{-/-} mice may not explain the survival benefit and other effects seen in TLR7^{-/-} septic mice.

In summary, we demonstrated that mice lacking TLR7 exhibited attenuated systemic cytokine production, reduced acute kidney injury and bacterial loading, and improved survival compared with WT mice after polymicrobial infection. These data suggest that signaling *via* TLR7, a sensor for both pathogen and host single-stranded RNA, may play an important role in sepsis pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Toll-like receptor 7-deficient mice have improved survival and attenuated acute kidney injury after polymicrobial sepsis.

(A) Survival rate of WT and TLR7^{-/-} mice during sepsis. Mice were subjected to 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 hours after CLP surgery. ****P* < 0.001, *****P* < 0.0001 vs. sham group. Unequal variance t-test, *N* = 11/group. (C) Kidney NGAL and KIM-1 mRNA expression in WT and TLR7^{-/-} mice at 24 hours post-procedure. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 vs. sham group. Unequal variance t-test. *N* = 4-8/group. Each bar represents mean ± SD. NGAL = neutrophil gelatinase-associated lipocalin, KIM-1 = kidney injury molecule-, CLP = cecal ligation and puncture, WT = wild-type.

A Plasma

B Lavage

Figure 2. TLR7^{-/-} mice display lower proinflammatory cytokine production in both blood circulation and peritoneal cavity following CLP procedure.

Twenty-four hours after CLP procedure, cytokines in the plasma and peritoneal lavage were measured by ELISA. (A) Plasma IL-6, IL-1 β, TNF-α, 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-1 β, TNF-α, CXCL2. N=6-8/group. IL-6, IL-1 β 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, CLP = cecal ligation and puncture.

Figure 3. Bacterial loading in the blood and peritoneal cavity.

Both the blood and peritoneal lavage were harvested 24 hours after sham or CLP procedure. After serial dilutions, anticoagulated blood and lavage were incubated on agar plates and bacterial colony-forming units (CFU) /ml were calculated and plotted in a Log₁₀ scale. **(A)** Bacterial loading in the blood. **(B)** Bacterial loading in the peritoneal space. **P<0.01. Unequal variance t-test. N=8 mice/group. Data are expressed as mean ± SD. CLP = cecal ligation and puncture.

Figure 4: Absence of 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 (LE), CD45⁺Ly6G⁺ for neutrophil (NE) and CD45⁺ F4/80^{low} for small peritoneal macrophage (MΦ). **A**) Total number of cells in the peritoneal cavity at 24 hours after sham or 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 to that of WT septic mice. $N = 5-9$, * $P < 0.05$, ** $P < 0.01$. **(E)** Flow cytometry plots depicting the increase in neutrophil percentage in the peritoneal

cavity of TLR7^{-/-} septic mice. **(F, G)** TLR7-deficiency led to marked neutrophil migration to infection site in both percentage and absolute numbers. N=5-9, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. **(H)** Representative flow cytometry plots of small resident macrophages in the peritoneal space. **(I)** Percentage of small resident macrophage decreased following sepsis. N=5-9/group * $P<0.05$, **** $P<0.0001$. **(J)** TLR7 deficiency increased the small macrophage numbers. N=5-9/ group. * $P<0.05$. Unequal variance t-test. Data are expressed as mean $\pm$ SD. WT = wild-type, CLP = cecal ligation and puncture, FSC = forward scatter, SSC = side scatter

Figure 5: TLR7 deficiency increased the phagocytic cells following sepsis.

10^8 fluorescein isothiocyanate (FITC)-labeled carboxylate-modified microspheres (beads) in 200 μ l were injected intraperitoneally at 23 hours following sham or CLP surgery. One hour later, peritoneal cells were collected and analyzed using flow cytometry. (A, B, C) Percentage of cells with phagocytic function. (D, E, F) Single cell phagocytic function. (G, H, I) TLR7 deficiency increased phagocytic cells in septic mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N=5-9 mice/ group. Unequal variance t-test. Data are expressed as mean $\pm$ SD. WT = wild-type, CLP = cecal ligation and puncture, LE = leukocytes, NE = neutrophil, M Φ = macrophage, MFI=mean fluorescence intensity.

Figure 6: Cecal slurry of WT and 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 was analyzed by One-Way ANOVA. (C, 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 $\pm$ SD. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$. N= 5/group. Vehicle=5% dextrose in water, CS=cecal slurry, I.P.=intraperitoneal injection.

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Table 1:

Leukocytes in the blood at 24 hours after procedure

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

* $P=0.054$ vs. TLR7^{-/-}-sham

** $P=0.0025$ vs. WT-sham

*** $P=0.0006$ vs. TLR7^{-/-}-sham. N=9-12. CLP = cecal ligation and puncture. Unequal variance t-test. Data are expressed as mean $\pm$ SD.