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Non-hematopoietic TLR2 contributes to neutrophil and cardiac function impairment during polymicrobial sepsis¹

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Abstract

Toll-like receptor 2 (TLR2) has been implicated in neutrophil and cardiac dysfunction during sepsis. Here we tested the hypothesis that non-hematopoietic (parenchymal) and hematopoietic TLR2 play distinct roles in sepsis pathogenesis. To achieve this, we generated two groups of chimeric mice with TLR2 deletions either in non-hematopoietic cells (KO mice with WT-bone marrow, BM) or in BM cells (WT mice with KO-BM). Polymicrobial sepsis was created by cecum ligation and puncture (CLP). Neutrophil functions, cytokine production, and bacterial clearance were investigated following CLP or sham procedures. Cardiac contractile function was measured in a Langendorff apparatus. Intracellular reactive oxygen species were measured using redox-sensitive dye and flow cytometry. CLP mice had markedly increased peritoneal neutrophil recruitment compared with the sham-operated mice. TLR2 KO mice, regardless their TLR2 phenotypes (WT vs. KO) in their BM-derived hematopoietic cells, had markedly increased neutrophil migration as well as phagocytosis, and reduced cytokine productions compared to TLR2 WT mice following polymicrobial peritonitis. These changes in the chimeric TLR2 KO mice were associated with enhanced blood bacterial clearance and markedly improved cardiac contractile function. Moreover, CLP induced a robust ROS production in the peritoneal leukocytes isolated from WT mice but not from TLR2 KO mice. Taken together, these data indicate that TLR2, particularly that of non-hematopoietic cells, plays a major role in sepsis pathogenesis by impairing neutrophil migratory and phagocytic function, promoting cytokine production, and mediating cardiac contractile dysfunction during polymicrobial sepsis. TLR2 also mediates critical ROS production during polymicrobial sepsis.

Keywords

Sepsis; phagocytosis; cytokine; innate immunity; toll-like receptors; cardiac dysfunction

INTRODUCTION

Sepsis is defined as the systemic inflammatory response syndrome that occurs during infection and is the 10th leading cause of death in the United States (1). It has an estimated prevalence of 751,000 cases each year, more than 50% of whom receive intensive care (2).

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The incidence of sepsis and the overall sepsis-related mortality have increased between 1993–2003 (3). Similarly, the rate of severe postoperative sepsis in surgical patients has more than doubled between 2001–2006 (4).

The immunopathogenic network of sepsis is complex involving multiple components of innate immune system including toll-like receptors (TLRs), complement factors, and inflammatory cells (5–7). The activated inflammatory cells such as neutrophils and monocytes release a large quantity of lipid mediators, cytokines, and reactive oxygen species (ROS). Together, these potent mediators cause profound vasodilatation, capillary leak, and cardiac dysfunction, which lead to refractory hypotension, tissue hypoperfusion and severe hypoxia, multi-organ failure, and death. Cardiac dysfunction is a hallmark of severe sepsis and contributes to its high mortality.

Neutrophil plays a pivotal role in the host innate immune defense (5, 6, 8). In response to invading pathogens, neutrophils quickly migrate to the sites of infection, recognize and engulf microorganisms (phagocytosis), killing them through a complex process involving ion movement across the phagocytic vacuoles and subsequent enzymatic digestion (9). The signaling mechanisms that control these critical neutrophil functions during polymicrobial sepsis remain incompletely understood.

Toll-like receptors (TLRs) represent the essential components of the innate immunity and play an important role in infectious and non-infectious tissue injury (10–12). They recognize invading pathogens and their wall components *via* the pathogen-associated molecular patterns. Each TLR recognizes different groups of pathogens. For example, TLR4 recognizes the component of Gram-negative bacteria, such as lipopolysaccharide (LPS), and TLR2 responds to Gram-positive bacterial such as *Staphylococcus aureus* (13) and the Gram-negative component peptidoglycan-associated lipoprotein (14). Investigations from this and other laboratories have suggested an important role of TLR2 in impairing neutrophil CXCR2 expression, cardiac function, and mortality during polymicrobial sepsis (15, 16). However, how TLR2 signaling impacts on critical neutrophil and cardiac functions during polymicrobial sepsis remains to be investigated.

In an effort to further dissect the role of TLR2 signaling in polymicrobial sepsis, we sought to test the hypothesis that TLR2 signaling from hematopoietic and non-hematopoietic cells plays distinct roles in impairing critical neutrophil functions and in the development of cardiac dysfunction during polymicrobial sepsis. To test this, we generated two groups of mice with chimeric TLR2 deletions either in bone marrow (BM)-derived hematopoietic cells (WT/KO-BM) or in non-hematopoietic cells (KO/WT-BM). Our data demonstrate that that non-hematopoietic (parenchymal) TLR2 signaling plays a predominant role in the impairment of neutrophil migratory and phagocytic functions and in the development of cardiac dysfunction during polymicrobial sepsis. Moreover, we demonstrate that TLR2 activation is sufficient to induce intracellular ROS production in isolated neutrophils and cardiomyocytes and that TLR2 signaling is required for neutrophil ROS production during polymicrobial sepsis.

MATERIALS AND METHODS

Animals

All animal experiments were performed with the approval of the Animal Care Committee of Massachusetts General Hospital. Sixteen to 18 week-old chimeric male mice were used for the studies. All mice were housed in temperature-controlled, air-conditioned facilities with 12/12 h light/dark cycles and fed the same bacteria-free diet. Male C57BL/6 mice were

purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and TLR2^{-/-} mice were generated by Takeuchi *et al* (13).

Generation of TLR2 chimeric mice

Chimeric models were generated by lethal irradiation and bone marrow transplantation as described previously (17). Briefly, recipient mice (8 week-old) were irradiated with 10 Gy of Cesium-137 radiation. Twenty-four hours later, they were injected, *via* tail vein, with bone marrow cells (BM) cells ($1-2 \times 10^7$) prepared from donor mice. Four groups of mice were generated: 1) WT with WT-BM, 2) WT with KO-BM, 3) KO with WT-BM, and 4) KO with KO-BM. As illustrated in Fig. 1, eight weeks after transplantation, successful reconstitution of the donor bone marrow cells in the recipient mice was characterized by 1) genotyping of the recipient BM cells vs. myocardial tissues, 2) TLR2 expression on BM neutrophils, circulating neutrophils, and liver macrophages as determined by flow cytometry (see below), 3) specific TLR2 functional signaling in BM-derived macrophages as tested by TLR2 and TLR4 ligand-induced cytokine production.

Cell isolation and culture

Liver macrophages – Liver macrophages (Kupffer cells) were isolated as described previously with modifications (18). Briefly, the mouse liver was perfused with Hanks balanced salt solution (HBSS) *via* the portal vein. The liver was then removed and minced with scissors. After mixed with 10 ml of HBSS containing 0.05% collagenase (Type IV, Sigma), the hepatic tissue solutions were gently shaken at 37 °C for 30 min and were filtered through a 300-μm mesh followed by a 70-μm cell strainer. The cell suspensions were loaded onto Histopaque discontinuous density gradient (Histopaque-1077 and -1119, Sigma) and centrifuged at 700 g at room temperature for 30 min. The cells at the interface were collected, washed and analyzed by flow cytometry. *Blood neutrophils* – Mouse neutrophils were isolated as described previously (19). Briefly, blood was collected and transferred into a tube containing 3 ml of 1.25% w/v dextran in saline. The tube was then filled with 10 ml of the dextran solution and inverted twice. Erythrocytes were allowed to sediment for 30 min at room temperature. The leukocyte-rich supernatant was collected and cells were washed with PBS and loaded onto Histopaque -1119 and centrifuged at 700g at room temperature for 30 min. The cells at the PBS-Histopaque interface were collected, washed and analyzed. *Bone marrow neutrophils* – Bone marrow cells were removed from both the femur and the tibia as described previously (17). Neutrophils were isolated using Histoaque gradient as described above, and the neutrophil purity was ~ 90% as examined by Gr-1 and Ly-6G antibodies staining and flow cytometry (17). *Bone marrow macrophage cultures* – BM macrophage cultures were performed as described previously (17).

Rat neonatal cardiomyocyte cultures

Neonatal cardiomyocytes were prepared as described previously with minor modifications (20, 21). Briefly, the hearts were isolated, dissected from major vessels, and cut into small pieces. The heart tissues were then incubated in ADS buffer (pH = 7.35, 116 mM NaCl, 20 mM HEPES, 0.8 mM Na₂HPO₄, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO₄) containing 0.4mg/ml collagenase 2 (Worthington, Lakewood, NJ) and 0.6mg/ml pancreatin (Sigma, St. Louis, MO, USA) at 37 °C for 5 min in a shaker. Cell suspension was slowly removed, and the remaining myocardial tissues were further incubated with the enzyme buffer for 4 more times. Cells in suspension were collected, spun, and resuspended in DMEM containing 20% FBS, and 4.5% D-glucose. Fibroblasts were removed by plating cells on 10-cm dishes for 60 min. Neonatal cardiomyocytes were then plated in 6-well plate (3×10^5 cells/well) and incubated in CO₂ incubator at 37 °C for 5 days before experiments were performed.

TLR2 genotyping

TLR2 genotyping was performed as described previously with minor modifications (22). Genomic DNA of BM cells and of myocardial tissues was extracted using Direct Tail extraction kit (Viagen Biotech, Los Angeles, CA). The primers for the TLR2 WT mice were as follows: forward primer, 5'-CTT CCT GAA TTT GTC CAG TAC AGG G-3' and reverse primers, 5'-CTA CGA GCA AGA TCA ACA GGA GAA G-3', which produced a 499-bp product. The primers for the PCR amplification of the TLR2^{-/-} (TLR2-KO) were as follows: forward primer, 5'-CTT CCT GAA TTT GTC CAG TAC AGG G-3' and reverse primers, 5'-GGG CCA GCT CAT TCC TCC CAC TCA T-3', which gave a 334-bp product. PCR was performed with amplification conditions as 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min, followed by 10 min at 72°C.

Flow cytometry detection of TLR2 expression

Bone marrow neutrophils, blood neutrophils, and liver macrophages were stained with anti-TLR2-PE (eBioscience, San Diego, CA) together with Gr-1-APC (BD Biosciences, San Jose, CA, USA) for neutrophils, F4/80-FITC (eBioscience) for macrophages and TLR2 expression was measured by flow cytometry.

RNA extraction and qRT-PCR

Total RNA was extracted from mouse macrophage using TRIzol reagent, and cDNA was synthesized by reverse-transcription reaction. mRNA levels were quantified using qRT-PCR methods as described previously (17, 23). The primer sequences have been reported before (17, 23, 24) and are listed as follows: 18S rRNA, forward primer: 5'-TCATGTGGTGTGAGGAAAGC-3' and reverse primer: 5'-TGGCGTGGATTCTGCATAATG-3'; intercellular adhesion molecule (ICAM), forward: 5'-GTGATGCTCAGGTATCCATCCA-3', reverse: 5'-CACAGTTCTCAAAGCACAGCG-3'; macrophage inflammatory protein-1 (MIP-1), forward: 5'-CACCTCTGTACCTGCTCAACATC-3', reverse: 5'-GGTTCCTCGCTGCCTCCAAGACTCT-3'; interleukin-1 β (IL-1 β), forward: 5'-GCCCATCTCTGTGACTCAT-3', reverse: 5'-AGGCCACAGGTATTTTGTCTG-3'; tumor necrosis factor α (TNF α), forward: 5'-CTGGGACAGTGACCTGGACT-3', reverse: 5'-GCACCTCAGGGAAGAGTCTG-3'. Changes in relative gene expression normalized to 18S rRNA levels were determined using the relative threshold cycle method (17, 23).

Detection of intracellular reactive oxygen species

Cells were pre-incubated with the redox sensitive dyes dichlorodihydrofluorescein diacetate (DCF, Molecular Probes, Invitrogen, Carlsbad, CA, USA) at 37 °C for 30 min. For the *in vitro* studies, Pam3Cys (1–20 μ g/ml), LPS (20 μ g/ml), poly (I:C) (20 μ g/ml), or CpG (20 μ g/ml) was added to cells and incubated at 37 °C for 1 hour (BM neutrophils) or 2 hours (cardiomyocytes). Cultured cardiomyocytes were detached and suspended in PBS. Cell suspensions were then kept on ice and ROS-DCF was analyzed by flow cytometry. For the *ex-vivo* studies, the peritoneal cells were harvested 12 hours after sham and CLP procedures and incubated with DCF as above. DCF was measured in FITC channel of flow cytometry and quantified by mean fluorescence intensity (MFI).

Mouse model of polymicrobial sepsis

Cecal ligation and puncture (CLP) was performed as described previously (16). In brief, the cecum was ligated 1.0 cm from the tip. A through and through puncture was made with an 18-gauge needle and a small amount (droplet) of feces was extruded to ensure the patency of the puncture site before returned it back to the abdominal cavity. The sham-operated mice underwent laparotomy but without CLP. The abdominal wall incision was closed in layers.

After surgery, pre-warmed normal saline (30 ml/kg body weight) was administered subcutaneously. Postoperative pain control was managed with subcutaneous injection of bupivacain and buprenorphine.

Flow cytometry analysis of peritoneal neutrophils

Twenty-four hours after surgeries, 5 ml of normal saline was injected into the peritoneal space and mixed thoroughly by gentle massage of the abdomen. Three ml of the peritoneal lavage fluid was collected and centrifuged. The supernatants were saved for cytokine measurements and the cell pellets 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 CXCR2 monoclonal antibodies (R&D Systems, Minneapolis, MN, USA), respectively. Cells were gated on Gr-1 and CXCR2 for neutrophil percentage in the recruited peritoneal cells. The total neutrophil numbers in the peritoneum were calculated by multiplying the total peritoneal cell number by the percentage of neutrophils.

Phagocytosis assay

Yellow-green fluorescent polystyrene microspheres (FITC) (FluoSpheres; Invitrogen, Carlsbad, CA) was opsonized by incubating with RPMI-1640-containing fetal bovine serum (FBS) for 30 min. The opsonized microspheres were then incubated with 4×10^5 peritoneal cells at 37 °C for 30 min as described previously (25). Cells were washed, stained with Gr-1 antibody, and analyzed with flow cytometry for Gr-1+ (APC) and phagocytic (FITC) neutrophils. The data were expressed as percentage of total Gr-1+ neutrophils.

Multiplex cytokine immunoassays

Twenty-four hours after sham or CLP procedures, mouse blood and peritoneal lavage were collected. Sera and peritoneal supernatants were prepared at 4 °C and stored at -80 °C. Cytokine concentrations of serum and peritoneal supernatants were determined using a fluorescent bead-based multiplex immunoassay (Luminex Co., Austin, TX) as previously described (16).

Bacterial clearance determination

Peritoneal and blood bacterial counts were determined by incubating 50 µl of serially diluted peritoneal lavage and anticoagulated blood on blood agar plates [Trypticase Soy Agar with 5% Sheep Blood (TSA II), BD] at 37°C for 14–16 hours.

Assessment of cardiac function in isolated heart

Cardiac function was assessed in a Langendorff perfusion system as described previously (16, 23). Briefly, mice were heparinized (1000 IU/kg, S.Q.) and euthanized. After the heart was excised, the aorta was quickly cannulated and retrograde-perfused at a constant flow rate (3 ml/min) with modified Krebs-Henseleit buffer. The heart was paced at 7 Hz. After 10 min of perfusion, LVSP (left ventricular systolic pressure), LVEDP (LV end-diastolic pressure), dP/dt_{max} (maximal first derivative of LV developed pressure) were recorded for 20 min. LVDP was calculated as follows: LVDP (LV developed pressure) = LVSP-LVEDP.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA, USA). The distributions of the continuous variables were expressed as the mean $\pm$ SE. Cytokine production was analyzed by Student *t*-test. The *P* values of bacterial clearance analysis was applied on the log₁₀ scale and based on Students' *t*-test. All other data were analyzed by

two-way ANOVA with Bonferroni post hoc tests for statistic significance. The null hypothesis was rejected for $P < 0.05$.

RESULTS

Characterization of TLR2 chimeric models

Eight weeks after bone marrow transplantation, the establishment of donor bone marrow (BM) in recipient mice was first confirmed by genotyping. As indicated in Fig. 1A, WT mice with WT-BM had WT genotype in both the heart and BM cells. WT mice with KO-BM had KO genotype in BM but maintained WT genotype in the heart. Conversely, KO mice with WT-BM had WT genotype in their BM but maintained KO genotype in the heart. As expected, KO mice with KO-BM had KO genotype in both the heart and BM. It is noteworthy that the hearts from both WT/KO-BM and KO/WT-BM chimeric mice also had low levels of TLR2 genotypes consistent with that of their BM genotypes, which might represent those of the BM-derived macrophages residing in the hearts as we demonstrated below that tissue (liver) macrophages had the same TLR2 phenotypes as those of BM. Moreover, we used flow cytometry to determine the TLR2 phenotypes in BM neutrophils (Gr-1+), in circulating neutrophils (Gr-1+), and in liver macrophages (F4/80+). As illustrated in Fig. 1B, FACS detected the differential TLR2 expression between WT and TLR2 KO cells isolated from BM, blood, and liver macrophages (Fig. 1B, *upper panel*). Importantly, in the TLR2 chimeric mice (Fig. 1B, *lower panel*), circulating neutrophils and liver macrophages of the chimeric mice had the same TLR2 phenotypic expression as those of the donor BM. Accumulative flow cytometry data shown in Fig. 1C indicated that circulating neutrophils and liver macrophages had the same TLR2 phenotypes as those of BM-derived cells. It is noteworthy that liver macrophages isolated from WT/KO-BM chimeric mice had slightly higher level of TLR2 expression than those from KO mice, but still much lower than that of WT mice (Fig. 1C, *right panel*). Finally, to specifically test TLR2 signaling function in the BM-derived cells, we cultured BM macrophages from the four groups of mice and treated them with PBS, Pam3cys (TLR2 ligand), or LPS (TLR4 ligand), and tested cytokine gene expression. As indicated in Fig. 2, Pam3cys and LPS induced a robust cytokine mRNA expression in WT cells. TLR2-deficiency completely abolished the cytokine production induced by Pam3cys, but not LPS. Importantly, BM macrophages isolated from the TLR2 KO mice with WT donor BM responded normally to Pam3cys and LPS, whereas BM cells isolated from WT mice with KO donor BM failed to respond to Pam3cys, but responded normally to LPS. Taken together, this series of studies clearly demonstrated that both circulating neutrophils and tissue macrophages had the same TLR2 phenotypes as the donor bone marrow cells and the construction of the chimeric TLR2 deletion models was successful.

Mice deficient of TLR2 in non-hematopoietic cells have increased neutrophil recruitment into the peritoneal cavity

The chimeric mice were subjected to sham or CLP surgical procedures. As illustrated in Fig. 3, all sham control chimeric mice had low levels of neutrophils in the peritoneal space. In response to polymicrobial infection, a significant number of total neutrophils were recruited into the peritoneal cavity in the WT/WT-BM mice ($11.6 \pm 4.9 \times 10^6$) compared to the sham controls of the same chimeric mice ($0.9 \pm 0.3 \times 10^6$) ($P < 0.01$). In comparison, two KO recipient chimeric mice, namely KO/WT-BM and KO/KO-BM, had significantly enhanced total leukocytes and neutrophil recruitment (Fig. 3A–B). The neutrophil recruitment was significantly increased from $11.6 \pm 4.9 \times 10^6$ in WT/WT-BM to $32.9 \pm 6.0 \times 10^6$ in KO/WT-BM and $27.8 \pm 4.8 \times 10^6$ in KO/KO-BM mice. The two groups of chimeric mice were deficient of TLR2 in their non-hematopoietic tissues but had restored (KO/WT-BM) or deficient (KO/KO-BM) TLR2 expression in their BM-derived cells such as neutrophils and

macrophages. In contrast, WT/KO-BM mice, which were deficient of TLR2 in their BM Gr-1+ neutrophils cells, circulating Gr-1+ neutrophils, and partially in liver macrophages, but had normal TLR2 expression in the parenchymal tissues, had the same levels of neutrophil recruitment as the WT/WT-BM mice after CLP surgery (Fig. 3B). Taken together, these data strongly suggest that TLR2 signaling in the non-hematopoietic cells plays a major role in the impairment of the neutrophil recruitment during polymicrobial peritonitis.

Mice deficient of TLR2 in non-hematopoietic cells have enhanced neutrophil phagocytic function during polymicrobial sepsis

Because neutrophil phagocytosis is an important cellular defense mechanism during polymicrobial infection after they migrate to the infectious site, we next investigated how TLR2 signaling would impact on the phagocytic function of neutrophils by measuring their ability to engulf fluorescent beads *in vitro* (25). As indicated in Fig. 4, peritoneal neutrophils isolated from the 4 groups of sham mice had similar levels of phagocytic function (Fig. 4A). However, in septic CLP mice, neutrophils isolated from KO/WT-BM and KO/KO-BM chimeric mice had significantly higher levels of phagocytic function ($49.2 \pm 1.5\%$ and $41.7 \pm 2.3\%$, respectively) compared with WT/WT-BM and WT/KO-BM mice ($27.1 \pm 7.1\%$ and $23.6 \pm 3.9\%$, respectively). The actual numbers of phagocytic neutrophils in the peritoneal space were significantly higher in CLP groups than sham groups and higher in KO recipient groups than WT recipient groups (Fig. 4C). These data suggest that TLR2 signaling in non-hematopoietic tissues plays a significant role in inhibiting overall neutrophil phagocytic function during polymicrobial sepsis.

Mice deficient of TLR2 had better bacterial clearance during polymicrobial sepsis

Given the demonstrated effect of TLR2 on neutrophil migratory and phagocytic functions, we next examined the bacterial loading in the blood and the peritoneal lavage from the mice subjected to sham or CLP surgery. No bacteria grew in all sham samples (data not shown). However, as indicated in Fig. 5, twenty-four hours after CLP, WT/WT-BM mice exhibited significant bacterial counts in the plasma and the peritoneal lavage (in Log₁₀ scale, 7.8 ± 0.4 and 9.2 ± 0.3 , respectively). In comparison, KO/KO-BM chimeric mice displayed a significant decrease in the bacterial counts both in blood and the peritoneal fluid (3.3 ± 0.1 and 6.4 ± 0.03 , respectively) compared with WT/WT-BM chimeric animals. Moreover, both WT/KO-BM and KO/WT-BM mice showed a significant reduction in blood bacterial loading (5.1 ± 0.3 and 5.0 ± 0.2 , respectively), indicating the critical role of both hematopoietic and non-hematopoietic TLR2 in the impairment of blood bacterial clearance during polymicrobial sepsis.

Role of hematopoietic and non-hematopoietic TLR2 signaling in local and systemic cytokine production during polymicrobial sepsis

Local and systemic inflammation is considered as an important contributor to the pathogenesis of sepsis and contributes to organ dysfunction (5). We tested cytokine and chemokine production both in the plasma and the peritoneal lavage. As indicated in Fig. 6, there was a robust increase in the level of both pro- and anti-inflammatory cytokines in the plasma and the peritoneal lavage fluid 24 hours after CLP surgery, whereas the sham mice had minimal level of cytokines (data not shown). Compared to WT/WT-BM mice, both KO/WT-BM and KO/KO-BM mice had markedly attenuated level of the pro-inflammatory cytokines/chemokine, namely, IL-6, TNF α , and Keratinocyte chemoattractant (KC), in the plasma and peritoneal lavage, suggesting that non-hematopoietic TLR2 signaling plays a critical role in both local and systemic cytokine production. Interestingly, WT/KO-BM chimeric mice, which were deficient of TLR2 in hematopoietic cells, had a similar level of the cytokines in the peritoneal lavage but significantly lower IL-6 and TNF α levels in the

plasma compared to WT/WT-BM mice. This may suggest that hematopoietic TLR2 signaling plays minor role in the local cytokine production and some selective roles in the systemic cytokine production. IL-10, an anti-inflammatory cytokine that may play both protective and injurious role during bacterial infection (26–29), was reduced dramatically in the plasma and the peritoneal lavage of WT/KO-BM, KO/WT-BM, and KO/KO-BM mice compared with WT/WT-BM group, indicating that both hematopoietic and non-hematopoietic TLR2 are required for IL-10 production.

Non-hematopoietic TLR2 signaling contributes to cardiac dysfunction during polymicrobial sepsis

Organ failure such cardiac dysfunction represents a main feature of severe sepsis and contributes to its high mortality. To test the contribution of TLR2 signaling to the pathogenesis of septic cardiac dysfunction during polymicrobial sepsis, the hearts were isolated from sham and septic mice and cardiac function was measured in a Langendorff perfusion system. As illustrated in Fig.7, 24 hours after CLP, there was a marked reduction in LV contractile function in WT/WT-BM and WT/KO-BM mice compared to their sham controls as demonstrated by decreased LVDP and $dP/dt_{\max}$ ($P < 0.01$). In comparison, KO/WT-BM or KO/KO-BM mice had significantly improved LV contractile functions (Fig.7).

TLR2 mediates ROS production in isolated cells and during polymicrobial sepsis

ROS plays an important role in proinflammatory cytokine generation (30–32) and contributes to the pathogenesis of sepsis by impairing mitochondrial function and inducing organ failure (33), including cardiac dysfunction (34, 35). We measured ROS production in neutrophils and rat cardiomyocytes in response to TLR ligands. As illustrated in Fig. 8, Pam3Cys, a TLR2 ligand, led to a dose-dependent increase in ROS production in both neutrophils and cardiomyocytes as measured by DCF flow cytometry. TLR2 deletion significantly attenuated Pam3cys-induced ROS production in neutrophils. In contrast, LPS, CpG, or poly (I:C) at the same concentration did not induce ROS production in both cell types. To determine whether or not TLR2 mediates ROS production during polymicrobial sepsis, we subjected WT and TLR2 KO mice to sham and CLP procedures. Twelve hours later, the peritoneal cells were harvested and the intracellular ROS was measured using flow cytometry. As indicated in Fig. 9, there was minimal ROS signal in the peritoneal cells from both WT and TLR2 KO sham mice. However, there was a marked increase in ROS levels in the peritoneal cells harvested from CLP WT mice. In comparison, the intracellular ROS levels were significantly reduced in septic TLR2 KO mice. Of note, 80–93% of the peritoneal cells were identified as Gr-1+ neutrophils in both WT and TLR2^{-/-} CLP mice 12 hours after CLP (data not shown). Taken together, these data suggest that activation of TLR2 is sufficient to induce a specific ROS production in isolated neutrophils and cardiomyocytes and that TLR2 signaling mediates ROS production in the peritoneal leukocytes during polymicrobial sepsis.

DISCUSSION

Using chimeric TLR2 deletion models and an *in vivo* model of polymicrobial sepsis, we demonstrated that TLR2, particularly those in non-hematopoietic cells, is important for the attenuated neutrophil migration, impaired phagocytic function, and impaired bacterial clearance during sepsis. The non-hematopoietic TLR2 also played a critical role in the development of cardiac dysfunction during polymicrobial sepsis. Systemic cytokine production during polymicrobial sepsis is, however, highly dependent on TLR2 signaling of both non-hematopoietic and hematopoietic cells. Finally, TLR2 is essential for intracellular ROS production in leukocytes during polymicrobial sepsis.

In response to bacterial invasion, neutrophils are quickly recruited to the infectious site through a complex process involving mobilization, margination, rolling, adhesion, and transmigration (36). Once they reach the infectious site, activated neutrophils contain bacterial invasion through their phagocytic function and potent microbial killing mechanisms (9). Recent investigations have demonstrated an important role of TLR2 signaling in the impairment of neutrophil migration (15, 16). Aleves-Filho and colleagues propose that bacterial sepsis impairs neutrophil migratory function by down-regulation of the surface expression of neutrophil CXCR2, a chemokine receptor critical for neutrophil chemotaxis function, *via* a TLR2-dependent mechanism. In an *in vitro* system, they demonstrate that direct neutrophil TLR2 activation by its agonist causes CXCR2 down-regulation and the consequent impairment of neutrophil chemotaxis (15). Our *in vivo* data from the chimeric study, however, suggest a predominant role for the non-hematopoietic TLR2 signaling in the impairment of neutrophil migration. In our study, similar to global TLR2 KO mice, chimeric TLR2 KO mice with wild-type TLR2 expression in their hematopoietic cells such as neutrophils had enhanced peritoneal neutrophil recruitment. In contrast, WT mice with TLR2 deletion in their hematopoietic cells had the same levels of neutrophil recruitment as WT mice. It is likely that TLR2 signaling in the microenvironment, in this case the peritoneal cavity modulates neutrophil migration by controlling cytokine/chemokine production locally. The enhanced neutrophil recruitment in these TLR2 KO mice is unlikely due to differential CXCR2 expression on neutrophils since our previous study has shown that both WT and TLR2 KO mice have markedly, but equally, down-regulated CXCR2 expression on their peritoneal neutrophils after CLP (16). It is noteworthy, however, that these chimeric mice also had lower levels of cytokine/chemokine in their peritoneal lavage compared to the WT control, but the reduction of cytokines appeared more profound in the plasma than in the peritoneal cavity (with exception of KC) in these mice. Whether or not the altered cytokine/chemokine levels and the resultant cytokine gradients between the circulation and the peritoneal cavity are responsible for the enhanced peritoneal neutrophil recruitment in the KO chimeric mice remain to be investigated.

Phagocytosis is an important cellular defense mechanism of neutrophil against bacterial infection (37). We investigate whether or not TLR2 signaling plays any role in neutrophil phagocytic function *in vivo* as measured in an *ex-vivo* assay system. Neutrophils isolated from the four groups of sham mice had not been exposed to pathogens and exhibited the same low levels of phagocytic function, suggesting that at baseline, TLR2 *per se* is not involved in the microsphere phagocytosis by the isolated neutrophils and that TLR2 deficiency has no impact on the baseline of phagocytic function *in vivo*. This is expected because the latex microspheres do not contain TLR2 ligands. In contrast, neutrophils isolated from septic mice had been exposed to and activated by bacterial pathogens *in vivo* in the bacteria-infected peritoneal space. Under this condition, the chimeric mice lacking TLR2 in their parenchymal tissues had much higher percentage of phagocytic neutrophils compared to that of WT mice. These data suggest that while TLR2 may not be needed in the initial contact between neutrophils and microspheres during the phagocytosis assay, TLR2 in parenchymal tissues had made an impact on neutrophil phagocytic function *in vivo*, which was detected by the *ex-vivo* phagocytosis assay. Using the *ex vivo* assay, our data clearly indicate that TLR2 deficiency, either globally (*e.g.*, in KO/KO-BM) or locally in the parenchymal tissues (*e.g.*, in KO/WT-BM), leads to a marked increase in the neutrophil phagocytic function. To our surprise, the WT/KO-BM chimeras, the mice lacking TLR2 in their hematopoietic cells such as neutrophils, have the same level of neutrophil phagocytic function as WT controls. These data may suggest that TLR2 signaling in the local peritoneal environment, rather than that of circulating cells such as neutrophils, attenuates the phagocytic function of activated neutrophils. This seems consistent with a recent report that *in vitro*, TLR2-deficiency has no impact on phagocytic function of isolated neutrophils

against purified *S. aureus* (38). It remains to be investigated, however, as to how local residential TLR2 signaling modulates neutrophil phagocytic function in response to polymicrobial infection. It is noteworthy that while the percentage of phagocytic neutrophils in the peritoneal space was the same in the WT CLP mice (*i.e.*, WT/WT-BM or WT/KO-BM) as the sham mice, the total numbers of phagocytic neutrophils in these CLP chimeric mice are actually > 10 times more than that in the sham chimeric mice (Fig. 4C). This is the result of a large influx of circulating Gr-1+ neutrophils into the infectious peritoneal space. This observation is consistent with a recent report by Kelly-Scumpia, which indicates that CLP mice have a higher percentage of phagocytic neutrophils only at early stage of sepsis (12 hours after CLP), but by 48 hours, the percentage of phagocytic neutrophils is at the same level as the sham animals (25).

Bacterial clearance reflects the overall effectiveness of the host immune defense. In this study, we demonstrate that KO/KO-BM mice have lowest bacterial load in the peritoneum and in the blood compared to other three chimeric groups. The enhanced bacterial clearance may be explained by the fact that TLR2 KO mice have markedly increased neutrophil migratory and phagocytic function. Interestingly, chimeric TLR2 deletion, either in hematopoietic or non-hematopoietic cells, leads to a similar decrease in the blood bacterial load. This may suggest the importance of TLR2 of both cell populations in modulating the ultimate host bacterial clearance.

Several laboratories have previously established the role of TLR2 signaling in systemic cytokine responses during polymicrobial sepsis (15, 16, 39). Compared to WT septic mice, TLR2-deficient septic mice have reduced serum pro-inflammatory cytokines such as IL-6 and TNF α . The present study extended these previous findings and demonstrated that non-hematopoietic TLR2 plays a predominant role in both local peritoneal and systemic production of proinflammatory cytokines such as IL-6 and TNF α , whereas hematopoietic TLR2 mainly controls system cytokine production and has minimal role in local proinflammatory cytokine production.

Cardiac dysfunction is one of the most important clinical features of septic shock. While often reversible, septic cardiac dysfunction is one of the main causes of cardiovascular collapse and contributes to the high mortality during severe sepsis. Our previous work has demonstrated that TLR2 signaling plays a critical role in the development of cardiac dysfunction during polymicrobial sepsis (16). However, whether or not polymicrobial sepsis exerts cardiac dysfunction directly through TLR2 signaling is not entirely clear (40). In the current study, we found that non-hematopoietic TLR2 plays an essential role in mediating cardiac dysfunction during sepsis. This was demonstrated by marked improvement of cardiac function in septic mice that were deficient of TLR2 in non-hematopoietic cells. In contrast, mice deficient of TLR2 in hematopoietic cells such as neutrophils were not protected from septic cardiac dysfunction. How could non-hematopoietic TLR2 mediate cardiac dysfunction during polymicrobial sepsis? One might speculate a few potential mechanisms that could link the non-hematopoietic TLR2 signaling and cardiac dysfunction during sepsis. Non-hematopoietic TLR2 may contribute to cardiac dysfunction by mediating abundant cytokine production that are cardiac depressive (41). Myocardial cytokine production during polymicrobial sepsis is TLR2-dependent (16). Activation of myocardial TLR2 during polymicrobial sepsis may also cause a direct cardiac depression *via* mechanisms such as intracellular ROS production (see below). This notion is supported by the previous reports that the pathogenic TLR2 ligand inhibits cardiomyocyte functions such as sarcomere shortening and Ca²⁺ transients *in vitro* (42) and that Gram-positive bacteria such as *Staphylococcus aureus* attenuate left ventricular function in isolated perfused heart (43).

To further probe the mechanisms by which TLR2 contributes to sepsis pathogenesis, we examined the effect of TLR2 on the intracellular ROS production. Emerging evidence have indicated that intracellular ROS plays a critical role in regulating cytokine production (30–32) and in organ failure (33), and contributes to septic mortality (44). We found that TLR2 activation induced a robust ROS production in both neutrophils and cardiomyocytes. This is consistent with a recent report that incubation with *Staphylococcus aureus* induces ROS in neutrophils in a TLR2-dependent manner (38). Moreover, we demonstrated a marked increase in intracellular ROS production in leukocytes during polymicrobial sepsis. Importantly, TLR2 deficiency almost abolished the ROS production during peritonitis sepsis. These data clearly suggest that TLR2 activation is sufficient to induce ROS production and is essential for polymicrobial sepsis-induced ROS production *in vivo*. ROS may contribute to sepsis pathology by regulating cytokine production and by inducing organ dysfunction. Recent studies suggest that ROS can interact with TLR signaling and promotes both NF- κ B- and IRF-3-dependent inflammatory pathways (31). ROS may also contribute to cardiac dysfunction in an endotoxemia model (34, 35). Moreover, *in vivo* administration of superoxide dismutase, a free-radical scavenger, prevents endotoxin-induced cardiac dysfunction (34).

In summary, our data demonstrate that TLR2, particularly that in non-hematopoietic cells, plays a predominant role in the pathogenesis of severe polymicrobial sepsis, which includes impairment of neutrophil migration and phagocytosis and mediating robust inflammatory reaction and intracellular ROS production. Non-hematopoietic TLR2 deficiency is associated with improved blood bacterial clearance and better-preserved cardiac function during polymicrobial sepsis. This study indicates that specific targeting of TLR2 signaling pathway may represent a novel therapeutic strategy in the management of severe sepsis and cardiac dysfunction during polymicrobial sepsis.

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Fig. 1. Characterization of TLR2 chimeric deletion models

A. TLR2 genotyping. Eight weeks after donor bone marrow transplantation, the bone marrow cells and myocardial tissues of the four groups of chimeric mice, namely WT/WT-BM, WT/KO-BM, KO/WT-BM, KO/KO-BM mice, were harvested and DNA extracted for PCR genotyping. The WT allele is shown as a band of 499-bp and the TLR2^{-/-} (KO) allele as 334-bp. **B.** Representative flow cytometry plots of TLR2 phenotypic expression. Six weeks after BM transplantation, BM neutrophils (Gr-1+), circulating blood neutrophils (Gr-1+), and liver macrophages (F4/80+) were isolated from the four groups of mice. Cells were gated on either Gr-1 or F4/80 for neutrophils or macrophages, respectively, and TLR2 expression was analyzed. **C.** Accumulated data of TLR2 expression in BM Gr-1+ neutrophils, blood Gr-1+ neutrophils, and liver F4/80+ macrophages presented as mean fluorescent intensity (MFI). Each error bar represent mean $\pm$ SE, N = 4.

Fig. 2. Cytokine mRNA expression of BM macrophages treated with Pam3cys and LPS

Six weeks after lethal irradiation and BM transplantation, BM cells were isolated from the chimeric mice, namely KO/WT-BM and WT/KO-BM, and differentiated to macrophages in cultures. BM cells isolated from WT and KO mice were used as the positive and negative controls, respectively. Macrophage cultures were treated with PBS, Pam3cys (1 μ g/ml), or LPS (500 ng/ml) for 4 hours. The cytokine mRNAs were analyzed using qRT-PCR. Each error bar represents mean $\pm$ SE, N = 4.

Fig. 3. KO/WT-BM and KO/KO-BM chimeric mice have enhanced neutrophil recruitment to the peritoneal space after CLP

The 4 groups of chimeric mice underwent sham or CLP procedures. Twenty-four hours later, the peritoneal lavage was harvested from the sham and CLP mice. Total peritoneal cells were manually counted. Five $\times 10^5$ of the lavage cells from each mouse were loaded for neutrophil identification by Gr-1- and CXCR2-gated flow cytometry. **A–B.** Total peritoneal cells and Gr-1⁺ neutrophils were harvested from the peritoneal cavity, respectively. 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. Each error bar represents the mean $\pm$ SE, $n = 5$ –6 mice. **C.** Representative flow cytometry plots of the peritoneal leukocytes isolated from the chimeric mice. The cells were gated on Gr-1 and CXCR2, the chemokine KC receptor specific for neutrophils. The cells within each circle represent Gr-1⁺ and CXCR2⁺ neutrophils. The percentage of the neutrophils in the lavage cells is shown next to the circles. Of note, in sham animals, less than 20% of the peritoneal cells are Gr-1-positive. However, in the CLP mice, however, >85–90% of the peritoneal cells are Gr-1-positive neutrophils. Since the same number of cells (5×10^5) were loaded in both sham and CLP groups for each flow cytometry run, the Gr-1-negative cells represent a much larger portion of the cells loaded on FCS in the sham animals, whereas a very small portion in the CLP animals.

Fig. 4. KO/WT-BM and KO/KO-BM septic mice have enhanced neutrophil phagocytic function
Leukocytes were harvested from the peritoneal space of sham and CLP mice and incubated with opsonized fluorescent (FITC) microspheres at 37 °C for 30 min and then stained with APC-labeled Gr-1 antibody. **A:** Percentages of phagocytic neutrophils (FITC-bead+ and APC-Gr-1+) were presented. Each error bar represents the mean $\pm$ SE of 4–6 separate neutrophil preparations. **B:** Representative flow cytometry plots and histograms of Gr-1 positive cells gated on FITC (fluorescent microspheres) and APC (Gr-1). The flow cytometry histograms indicate the two populations of Gr-1+ neutrophils: non-phagocytic (left) and phagocytic (right) cells. The y-axis represents cell numbers of each cell population. **C.** Phagocytic neutrophils in the peritoneal space: The phagocytic neutrophils were calculated as % of phagocytic neutrophils $\times$ total Gr-1+ neutrophils in the peritoneal space.

Fig. 5. Bacterial load in the blood and the peritoneal space following CLP

Both the blood and peritoneal lavage were harvested 24 hours after the CLP surgery. After serially dilution, they were incubated at 37°C for 14–16 hours. 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 $\pm$ SE of the CFU in each mouse group. **A.** CFU in the blood. **B.** CFU in the peritoneal lavage.

A. Peritoneal lavage cytokine production

B. Plasma cytokine production

Fig. 6. Local and systemic cytokine production in TLR2 chimeric mice

Twenty-four hours after CLP procedures, the peritoneal lavage and plasma were collected from the septic mice. Cytokines, namely IL-6, TNFα, KC and IL-10, were measured using a multiplex fluorescent bead-based immunoassay. **A.** Peritoneal lavage cytokines. **B.** Plasma cytokines. Each error bar represents the mean ± SE of 4–7 mice.

Fig. 7. Non-hematopoietic TLR2 deficient septic mice have better-preserved cardiac function after CLP as measured in isolated perfused hearts

The 4 groups of TLR2 chimeric mice underwent sham and CLP surgeries. Twenty-four hours later, the hearts were isolated and perfused with modified Krebs-Henseleit buffer in a Langendorff perfusion system. Left ventricular functions were measured. *LVDP* = left ventricle developed pressure; *dP/dt_{max}* = the maximal first derivative of *LVDP*. Each error bar represents the mean $\pm$ SE. The dash line represents the mean value of LV function in the sham mice.

Fig. 8. TLR2 activation induces ROS production in neutrophils and cardiomyocytes

DCF-loaded mouse neutrophils or rat neonatal cardiomyocytes were stimulated with TLR ligands for 1–2 hours. Intracellular ROS production was measured by flow cytometry and quantified. **A.** Representative plots of flow cytometry illustrating Pam3Cys-induced ROS production in neutrophils (*upper panel*) and cardiomyocytes (*lower panel*). **B.** Pam3Cys induces a dose-dependent ROS production in neutrophils isolated from WT or KO mice (*upper panel*) or cardiomyocytes (*lower panel*). Each error bar represents mean $\pm$ SE, $N = 3$. **C.** Accumulated data of intracellular ROS production in response to various TLRs ligands at the concentration of 20 μ g/ml in neutrophils (*upper panel*) and cardiomyocytes (*lower panel*). Each error bar represents mean $\pm$ SE, $N = 3$. MFI = mean fluorescence intensity; KO = knockout; TLR = toll-like receptor; WT = wild type. *** $P < 0.001$ vs. none; ## $P < 0.01$, ### $P < 0.001$ vs. WT.

Fig. 9. TLR2 mediates ROS production in the peritoneal neutrophils during polymicrobial sepsis WT and TLR2 KO mice were subjected to sham or CLP surgical procedures. Twelve hours later, the peritoneal cells were harvested and counted. The cells were stained with either DCF or Gr-1 antibody and analyzed by flow cytometry for ROS and Gr-1+ (not shown). **A.** Representative plots of flow cytometry illustrating intracellular ROS production in various groups of the peritoneal cells as indicated. **B.** Accumulative ROS data as measured as DCF flow cytometry in various groups of the peritoneal cells.