Importance of Toll-like Receptor 2 in Mitochondrial Dysfunction during Polymicrobial Sepsis

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ABSTRACT

Background: Toll-like receptor 2 (TLR2) contributes to sepsis pathogenesis such as deleterious systemic inflammation, cardiac dysfunction, and high mortality in animal studies. Mitochondrial dysfunction is a key molecular event that is associated with organ injury in sepsis. The role of TLR2 in sepsis-induced mitochondrial dysfunction remains unclear.

Methods: Intracellular hydrogen peroxide (H$_2$O$_2$), mitochondrial superoxide (O$_2^·$), mitochondrial membrane potential ($\Delta$Ψm), and intracellular adenosine triphosphate (ATP) were measured in peritoneal leukocytes. A mouse model of polymicrobial sepsis was generated by cecum ligation and puncture (CLP). Wild-type and TLR2-deficient (TLR2$^{-/-}$) mice were subjected to sham or CLP. Mitochondrial functions including reactive oxygen species (ROS), $\Delta$Ψm, intracellular ATP, and complex III activity were measured.

Results: TLR2/1 activation by Pam3Cys enhanced intracellular H$_2$O$_2$ and mitochondrial O$_2^·$ production in leukocytes, but had no effect on mitochondrial $\Delta$Ψm and ATP production. The effect was specific for TLR2/1 as TLR3 or TLR9 ligands did not induce ROS production. Polymicrobial sepsis induced mitochondrial dysfunction in leukocytes, as demonstrated by increased H$_2$O$_2$ and mitochondrial O$_2^·$ production (CLP vs. sham; H$_2$O$_2$: 3,173 ± 498, n = 5 vs. 557 ± 38, n = 4; O$_2^·$: 707 ± 66, n = 35 vs. 485 ± 35, n = 17, mean fluorescence intensity, mean ± SEM), attenuated complex III activity (13 ± 2, n = 16 vs. 30 ± 3, n = 7, millioptical densities/min), loss of mitochondrial $\Delta$Ψm, and depletion of intracellular ATP (33 ± 6, n = 11 vs. 296 ± 29, n = 4, nmol/mg protein). In comparison, there was significant improvement in mitochondrial function in septic TLR2$^{-/-}$ mice as evidenced by attenuated mitochondrial ROS production, better-maintained mitochondrial $\Delta$Ψm, and higher cellular ATP production.

Conclusions: TLR2 signaling plays a critical role in mediating mitochondrial dysfunction in peritoneal leukocytes during polymicrobial sepsis. (Anesthesiology 2014; 121:1236-47)

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Sepsis is defined as the systemic inflammatory response syndrome that occurs during infection. It has an estimated incidence of 751,000 cases each year. Both the incidence of sepsis and the overall sepsis-related mortality have increased significantly between 1993 and 2003. Similarly, the rate of severe postoperative sepsis in surgical patients has more than doubled between 2001 and 2006. Sepsis is the 10th leading cause of death in the United States.

A major cause of death in patients with severe sepsis is multiple organ failure, but the underlying pathogenesis is not fully understood. Mitochondrial damage and dysfunction has been recognized as an important molecular pathology in sepsis and is linked to the severity of organ dysfunction and possibly outcome of sepsis. The increased production of cellular reactive oxygen species (ROS) of mitochondrial origin during sepsis can cause significant oxidative stress to cells and may severely inhibit oxidative phosphorylation and adenosine triphosphate (ATP) generation, which can potentially cause multi-organ failure.

Although the host innate immune response is necessary to eradicate invading pathogens, excessive inflammatory responses during sepsis is harmful and may lead to tissue injury, in part, by damaging mitochondrial structure and function. Several molecular mechanisms have been proposed responsible for mitochondrial dysfunction. These include attenuated activity of mitochondrial electron transport chain enzyme complexes, inhibitory effects of reactive nitrogen and oxygen species on oxidative phosphorylation and ATP production, increased expression of mitochondrial uncoupling proteins, and the formation of the mitochondrial.
permeability transition pore. However, the upstream signaling that mediates these molecular events leading to mitochondrial dysfunction in sepsis is poorly understood.

Toll-like receptors (TLRs) play an essential role in the host immune and inflammatory responses during sepsis as well as certain noninfectious tissue injury.24-27 TLRs may also play a role in regulating mitochondrial function. Dijarzadeh et al.28 have shown that TLR3 activation attenuates maximal mitochondrial respiration in cultured human hepatocytes. West et al.29 demonstrate that TLR1/2/4 signaling augments macrophage bactericidal activity through mitochondrial ROS production. Yet, others have suggested a dual role for TLR4 signaling in modulating mitochondrial function. TLR4 activation not only triggers endotoxin-induced oxidative stress and mitochondrial DNA (mtDNA) damage but also mediates mitochondrial biogenesis by up-regulation of mitochondrial complex IV and mitochondrial transcription factors.30-32 These data suggest that TLR signaling may have a significant impact on mitochondrial function during bacterial sepsis.

TLR2 forms a heterodimer with either TLR1 or TLR6. The resulting TLR2/TLR1 and TLR2/TLR6 complexes recognize distinct ligands triacyl and diacyl lipoproteins, respectively. We have previously demonstrated the significant contribution of TLR2 signaling to the pathogenesis of polymicrobial sepsis.33-35 TLR2 activation by bacterial wall components induces cardiomyocyte inflammatory response and dysfunction in vitro.26 TLR2 mediates intracellular hydrogen peroxide (H$_2$O$_2$) production29 and contributes to cardiac dysfunction and mortality27 in septic animals. The survival benefit of TLR2 deficiency was also confirmed recently29 and in Pseudomonas aeruginosa sepsis model.30 In the current study, we tested the hypothesis that TLR2 mediates mitochondrial dysfunction during polymicrobial sepsis. Specifically, we tested the effect of TLR activation on mitochondrial function in isolated leukocytes in vitro and determined the impact of TLR2 deletion on mitochondrial dysfunction in a mouse model of peritoneal polymicrobial sepsis.

Materials and Methods

Animals

Eight- to 12-week-old gender-, age-, and strain-matched mice were used for the studies. Wild-type (WT) (C57BL/6J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a animal facility at Massachusetts General Hospital for at least 1 week before experiments. TLR2$^{-/-}$ mice were generated by Takeuchi et al.31 All animals were housed in pathogen-free, temperature-controlled, and air-conditioned facilities with 12 h/12 h light/dark cycles and fed with the same bacteria-free diet. Animal care and procedures were performed according to the protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and were in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health. Simple randomization method was used to assign animals to various experimental conditions.

Reagents

Pam3Cys, poly (I:C), CpG were purchased from Enzo Life Science (Farmingdale, NY). Lipopolysaccharide (Escherichia coli 0111:B4) and lipoteichoic acid (LTA) were from Sigma-Aldrich (St Louis, MO). Dichlorodihydrofluorescein diacetate (H2-DCF-DA), MitoSOX red reagent, and 5,5',6,6'-tetrachloro-1',3',3'-tetraethylbenzimidazolylcarboxyanine iodide (JC-1) were purchased from Invitrogen-Molecular Probes (Eugene, OR). Antimycin A and tetramethylrhodamine ethyl ester perchlorate (TMRE) were purchased from Sigma-Aldrich. ATP bioluminescence assay kit CLS II was purchased from Roche Molecular Biochemicals (Indianapolis, IN). MitoTox OXPHOS Complex III Activity Kit was from Abcam (Cambridge, MA).

Peritoneal Cell Isolation after Thioglycollate Injection

Peritoneal cells were elicited chemically by intra-peritoneal injection of 4% thioglycollate. After 12–16 h, 6 ml of Dulbecco’s phosphate-buffered saline (DPBS) without calcium and magnesium was injected into the peritoneal space and mixed thoroughly by gentle massage. The peritoneal lavage fluid was collected and centrifuged at 1,500 rpm for 5 min. The supernatants were discarded and the cell pellets were suspended in RPMI 1640 containing 0.05% bovine serum albumin. We have previously shown that more than 85% of the peritoneal cells are Gr-1$^+$ neutrophils.32

Peritoneal Cell Collection after Surgery

Twenty-four hours after sham or cecum ligation and puncture (CLP) surgery, 6 ml of ice-cold DPBS without calcium and magnesium was injected into the peritoneal space and mixed thoroughly by gentle massage. Five milliliters of the peritoneal lavage were collected and centrifuged at 1,500 rpm for 5 min. The supernatants were discarded and the cell pellets were suspended in RPMI 1640. We have previously shown that more than 90% of the peritoneal cells from the CLP mice are Gr-1$^+$ neutrophils.32

Reactive Oxygen Species

Total intracellular H$_2$O$_2$ was measured with dichlorodihydrofluorescein diacetate (H2-DCF-DA, Cat. D399, Invitrogen), whereas mitochondrial superoxide (O$_2^-$) was assayed with MitoSOX (Cat. M36008, Invitrogen). Specifically, peritoneal neutrophils were harvested, plated in 96-well plate, and treated with antimycin A (Cat. A8674, Sigma) or TLR ligands as indicated. At the end of treatment, cells were incubated with freshly prepared H2-DCF-DA or MitoSOX at 37°C in the dark for 30 min. Unstained controls were handled similarly except that treatments and dyes were omitted. Dye-loaded cells were resuspended in cold DPBS containing 1% fetal bovine serum and analyzed immediately by flow cytometry at fluorescein isothiocyanate or R-phycocerythin channel. Ten thousand cells were routinely counted by flow cytometry, and data expressed as the median fluorescence intensity in arbitrary units from at least three separate experiments. In some experiments, MitoSOX-stained cells attached
to pre-coated plates (with 5 µg/ml of fibronectin and 20 µg/ml of gelatin) were analyzed for ROS production under fluorescence microscope at Texas Red channel.

**Mitochondrial Membrane Potential**

Two methods were employed to measure mitochondrial membrane potential (ΔΨm). First, we used TMRE (Cat. 87917, Sigma) to measure levels of ΔΨm. TMRE is a cationic dye that is rapidly and reversibly accumulated by healthy mitochondria. Decrease in the levels of TMRE indicates reduction in mitochondrial membrane potential levels. Experimentally, a fraction of cells (5 × 10⁵) from the peritoneal lavage was labeled with freshly prepared TMRE at 37°C in the dark for 30 min. Unstained controls were treated similarly, except that ligand treatment and dyes were omitted. Dye-loaded cells were immediately re-suspended in cold DPBS containing 1% fetal bovine serum and analyzed immediately by flow cytometry at the R-phycocerythrin channel. Ten thousand cells were routinely collected, and data were expressed as the mean fluorescence intensity (MFI) in arbitrary units from the average of at least three separate experiments. Second, we measured mitochondrial ΔΨm using JC-1 dye (Invitrogen, MP 03168). Specifically, peritoneal leukocytes were treated with antimycin A or stimulated with TLR ligands as indicated. At the end of treatments, cells were incubated with 2 µM JC-1 at 37°C for 30 min and washed twice with DPBS. Finally, fluorescence was read at red fluorescence (excitation: 535 nm; emission: 590 nm) and green fluorescence (excitation: 485 nm; emission: 530 nm) using a fluorescence plate reader. The level of ΔΨm was calculated by ratio of red fluorescence to green fluorescence.

**ATP Assay**

Intracellular ATP level was measured by a luciferase-based assay using the ATP Bioluminescence Assay Kit CLS II (Roche Molecular Biochemicals). In brief, intracellular ATP was released using a boiling method. Specifically, peritoneal neutrophils were treated with antimycin A or stimulated with TLR ligands as indicated. At the end of treatments, cells were then harvested, washed twice with ice-cold DPBS, drained and resuspended in boiling buffer (100 mM Tris and 4 mM EDTA, pH 7.75). The suspensions were pipetted, vortexed, and snap frozen in liquid nitrogen. Frozen cells were boiled for 3 min in a water bath, placed on ice for 5 min, and then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and kept on ice until measurement. Finally, 35 µl of luciferase reagent was added to 35 µl of the sample or standard. Experiments were performed in triplicates, and data were standardized to the protein concentration using the Bradford protein assay.

**Mouse Model of Polymicrobial Sepsis**

A mouse model of polymicrobial sepsis was generated by CLP as described previously.²⁷ 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 (50 ml/kg) was administered subcutaneously. Postoperative pain control was managed with subcutaneous injection of bupivacaine (3 mg/kg) and buprenorphine (0.1 mg/kg). Of note, this model of polymicrobial peritonitis in C57BL/6 mice leads to severe sepsis as evidenced by multi-organ dysfunction such as cardiac dysfunction and acute kidney injury with 60–90% of mortality.²⁷,³³

**Mitochondrial Complex III Activity Assay**

Mitochondrial complex III activity was measured using MitoToxOXPHOS Complex III Activity Kit (Cat. ab109905, Abcam) according to the manufacturer’s protocol with some modifications. Briefly, cells were lysed by sonication and mitochondrial fractions were re-suspended in ice-cold DPBS. Complex III activity was then measured in a mixture (1:1 ratio) of cell suspension and assay solution containing succinate, rotenone, potassium cyanide, cytochrome c by monitoring complex III-sensitive cytochrome c reduction (λ = 550 nm). Data were collected every 20 s for 5 min after initiation of the reaction.

**Mitochondrial Gene Expression**

Mitochondrial transcript factor A (Tfam) and cytochrome c oxidase subunit II (COX-2), both coded by mtDNA, were quantified by real-time quantitative reverse transcription polymerase chain reaction. The following primers were used: Tfam, forward 5′-CATTATGTTATCTGAAGCCTTC-3′, reverse 5′-CTCTTTCCCCGACCTTATTC-3′; COX-2, forward 5′-ACCGAGTCTGTTCTGCCAATA-3′, reverse 5′-GCTTGATTAGTGCAGGCCTTG-3′; Glyceraldehyde 3-phosphate dehydrogenase, forward 5′-AACCCTTGACCTGGAAGG-3′, reverse 5′-GGATGCAGGATGATGTTCT-3′.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). The distributions of the continuous variables were expressed as the mean ± SEM. Data were analyzed by one-way ANOVA with Tukey or two-way ANOVA with Bonferroni post hoc tests for statistic significance. Of note, the sample sizes were based on our prior experiences rather than a formal statistical power calculation. The null hypothesis was rejected for P value of less than 0.05 with the two-tailed test.

**Results**

**TLR2 Activation Leads to Intracellular and Mitochondrial ROS Production in Peritoneal Leukocytes**

To establish a system that is reliable and sufficiently sensitive to detect cellular ROS production, we first tested the effect
of antimycin A on intracellular \( \text{H}_2\text{O}_2 \) and mitochondrial \( \text{O}_2^- \) production in the peritoneal leukocytes. Antimycin A is a potent inhibitor of the mitochondrial respiratory chain enzyme complex III and known for its ability to induce mitochondrial \( \text{O}_2^- \) production as demonstrated in figure 1A.

As illustrated in figure 1B and C, antimycin A treatment led to both intracellular \( \text{H}_2\text{O}_2 \) and mitochondrial \( \text{O}_2^- \) production as measured by flow cytometry and fluorescent microscopy. To determine whether or not TLR signaling induces ROS production, we next stimulated leukocytes with various TLR ligands. Similar to antimycin A, Pam3Cys (a TLR1/2 ligand, 20 \( \mu \text{g/ml} \)) induced a significant increase in both intracellular \( \text{H}_2\text{O}_2 \) and mitochondrial \( \text{O}_2^- \) levels as demonstrated by flow cytometry (\( \text{H}_2\text{O}_2 \)): con vs. Pam3, 531 \( \pm \) 57 vs. 2426 \( \pm \) 89; \( \text{O}_2^- \): con vs. Pam3, 848 \( \pm \) 38 vs. 1,621 \( \pm \) 91, MFI) (fig. 2A–D) and fluorescent microscopy (fig. 1). In contrast, at the same concentration, LTA (a TLR2/6 ligand), poly (I:C) (a TLR3 ligand) or CpG (a TLR9 ligand) had no effect on intracellular or mitochondrial ROS production.

Lipopolysaccharide (TLR4 ligand) only induced a modest increase in mitochondrial \( \text{O}_2^- \) level (fig. 2A–D). The effect of Pam3Cys was dose-dependent and partially mediated via TLR2 as Pam3Cys-induced mitochondrial \( \text{O}_2^- \) production was significantly attenuated in TLR2-deficient leukocytes (WT vs. knockout [KO], 2,766 \( \pm \) 259 vs. 2,044 \( \pm \) 57, MFI) (fig. 2E–G).

**TLR2 Activation Has No Impact on Mitochondrial Membrane Potential and Intracellular ATP Production**

The mitochondrial membrane potential (\( \Delta \Phi \text{m} \)) is generated by protons transport across the mitochondrial inner membrane. This process is catalyzed by the enzyme complexes I, III, and IV of the electron transport chain and produces the proton motive force to generate ATP. Previous studies have shown that a positive correlation exists between \( \Delta \Phi \text{m} \) reduction and ROS production \(^{34-36} \) and that ATP depletion represents a hallmark of mitochondrial dysfunction. \(^{37} \)

We therefore analyzed \( \Delta \Phi \text{m} \) response to TLR ligands using...
two mitochondrial membrane potential-sensitive fluorescent probes, namely TMRE and JC-1. As illustrated in figure 3A and B, although antimycin A, a complex III inhibitor, induced a dose-dependent reduction in the mitochondrial ΔΨm, most TLR ligands tested, i.e., Pam3Cys, LTA, lipopolysaccharide, and CpG, had no effect on ΔΨm. Poly (I:C) led to a higher ΔΨm. Consequently, antimycin A led to marked reduction in ATP production in leukocytes (fig. 3C and D). Moreover, absence of glucose in culture media markedly reduced ATP production in the untreated cells (control) and further abolished ATP production in the antimycin A-treated leukocytes (fig. 3C vs. fig. 3D). Similar to ΔΨm data, Pam3cys and lipopolysaccharide did not reduce cellular ATP production in leukocytes (fig. 3C and D). These

Fig. 2. Effect of various Toll-like receptor ligands on intracellular H2O2 and mitochondrial O2− production in peritoneal leukocytes. (A, B) Effect of TLR ligands on intracellular H2O2 generation. Thioglycollate-elicited peritoneal leukocytes were treated with TLR ligands as indicated: Pam3Cys, LTA, or LPS (20 μg/ml) for 1 h, incubated with 10 μM of DCF and analyzed for intracellular H2O2 with flow cytometry. Representative histograms are presented in A and combined MFI data in B. n = 3 in each group. ***P < 0.001 vs. control. The experiments were performed twice with the similar results. (C, D) Effect of TLR ligands on mitochondrial O2− production. Cells were treated with TLR ligands as indicated: Pam3Cys, LTA, LPS, poly (I:C) or CpG (20 μg/ml) for 1 h, incubated with 2.5 μM of MitoSOX and analyzed for mitochondrial O2− production. Representative histograms are presented in C and combined MFI data in D. n = 3 in each group. *P < 0.05, ***P < 0.001 vs. control. The experiments were performed four times. (E) TLR2 activation induces a dose-dependent mitochondrial O2− production. n = 3 in each group. *P < 0.05, ***P < 0.001 vs. untreated control. The experiments were performed twice. (F, G) TLR2 mediates Pam3Cys-induced mitochondrial O2− production. Peritoneal leukocytes harvested from WT or TLR2−/− mice were treated with Pam3Cys (20 μg/ml) for 1 h and analyzed for mitochondrial O2− production with flow cytometry. Representative histograms are presented in F and combined MFI data in G. n = 3 in each group. ***P < 0.001 vs. control. ## P < 0.01 vs. WT. Each error bar represents mean ± SEM. DCF = dichlorodihydrofluorescein diacetate; H2O2 = hydrogen peroxide; LPS = lipopolysaccharides; LTA = lipoteichoic acid; MFI = mean fluorescence intensity; Mito O2− = mitochondrial superoxide; TLR2KO = TLR2 knockout; WT = wild type. The experiments were performed twice with similar results.
data suggest that unlike the complex III blocker antimycin A, TLR activation is not sufficient to induce mitochondrial dysfunction.

**TLR2 Mediates Mitochondrial ROS Production in Leukocytes during Polymicrobial Sepsis**

Next, we tested whether or not TLR2 plays a role in leukocyte mitochondrial ROS production in sepsis. We subjected WT and TLR2−/− mice to sham or CLP procedure, a clinically relevant animal model of peritoneal polymicrobial sepsis. Twenty-four hours after the procedures, the peritoneal cells were harvested and the intracellular H2O2 and mitochondrial O2− were measured using flow cytometry. As indicated in figure 4, there was a basal level of ROS signal in the peritoneal leukocytes isolated from sham mice. However, in leukocytes harvested from WT septic mice, there was a significant increase in cellular H2O2 and mitochondrial O2− levels. In comparison, both intracellular H2O2 and mitochondrial O2− were markedly reduced in TLR2−/− septic mice (intracellular H2O2: 3,173 ± 498 vs. 1,628 ± 324; mitochondrial O2−: 707 ± 66 vs. 451 ± 37, WT-CLP vs. TLR2 KO-CLP, MFI) (fig. 4).

These data clearly suggest that TLR2 signaling plays an important role in mediating cellular H2O2 and mitochondrial O2− production in the peritoneal leukocytes during polymicrobial sepsis.

**TLR2 Signaling Contributes to Mitochondrial Dysfunction during Polymicrobial Sepsis**

Given the role of mitochondrial ΔΨm and ATP production in mitochondrial ROS generation, we tested the mitochondrial ΔΨm and intracellular ATP concentration in peritoneal leukocytes of animals with polymicrobial peritonitis. We found that there was a marked reduction in the mitochondrial ΔΨm and ATP generation in septic WT mice as compared with the sham-operated controls (ΔΨm: 4,455 ± 400 vs. 1,694 ± 352, MFI; ATP: 296 ± 29 vs. 33 ± 6 nmol/mg protein; sham vs. CLP in WT, MFI) (fig. 5). TLR2 deletion significantly improved the mitochondrial ΔΨm and ATP production (ΔΨm: 1,694 ± 352 vs. 2,866 ± 167, MFI; ATP: 33 ± 6 vs. 71 ± 8, nmol/mg protein; WT-CLP vs. TLR2KO-CLP) (fig. 5). These results suggest that TLR2 signaling contributes to the leukocyte mitochondrial dysfunction during polymicrobial sepsis.
Polymicrobial Sepsis Inhibit Mitochondrial Complex III Activity in Peritoneal via a TLR2-independent Mechanism

Studies have demonstrated that complex III is one of the principal sites responsible for mitochondrial ROS generation. We next examined the complex III activities in leukocytes isolated from sham and septic animals and tested the impact of TLR2 deficiency on their activities during polymicrobial sepsis. As illustrated in figure 6, there was a marked reduction in the complex III activity in WT CLP mice as compared with the sham control mice (30 ± 3 vs. 13 ± 2, sham vs. CLP in WT, millioptical densities/min). However, TLR2-deficient mice did not have improved complex III function as compared with WT mice after CLP. These data clearly suggest that TLR2 signaling mediates ROS production and mitochondrial dysfunction during polymicrobial sepsis via a complex III-independent mechanism.
Polymicrobial Sepsis Induces Mitochondrial Tfam and COX-2 Depletion

Mitochondrial oxidative stress can lead to mtDNA damage and depletion. The mtDNA is reported to be more susceptible to oxidative stress than nuclear DNA. Studies have demonstrated that lipopolysaccharide induces mitochondrial oxidative stress and mtDNA depletion. We examined the effect of polymicrobial sepsis on the expression of the two mitochondrial molecules, namely mitochondrial transcript factor A (Tfam) and COX-2, both coded by mtDNA. As shown in figure 7A, compared with sham mice, CLP led to significantly lower Tfam and COX-2 gene expression in the liver. This effect seemed more prominent in the liver as CLP did not significantly impact on Tfam and COX-2 expression in the heart or peritoneal leukocytes within the same period of time (24 h) (fig. 7B and C). Similar to mitochondrial complex III activity shown in fig. 6, TLR2 deficiency did not reverse the reduced Tfam and COX-2 gene expression in the septic liver (fig. 7A).

Discussion

The current study demonstrates a pivotal role of TLR2 signaling in mediating mitochondrial ROS production as well as mitochondrial dysfunction in a clinically relevant mouse model of severe polymicrobial sepsis. First, we found that activation of TLR1/2, but not TLR2/6, TLR3, TLR4, or TLR9, was capable of inducing a robust intracellular and mitochondrial ROS production in leukocytes. We also found that while the inhibition of mitochondrial respiratory complex III reliably caused mitochondrial dysfunction as evidenced by decreased mitochondrial ΔΨm and cellular ATP production, TLR1/2 activation appeared insufficient to induce mitochondrial dysfunction in isolated leukocytes. Second, we found that polymicrobial peritonitis sepsis led to a marked mitochondrial dysfunction in peritoneal leukocytes with increased intracellular and mitochondrial ROS, decreased mitochondrial ΔΨm, reduced intracellular ATP, and markedly inhibited mitochondrial complex III activity. In comparison, mice deficient of TLR2 had significantly improved mitochondrial function with markedly reduced intracellular and mitochondrial ROS production, and significantly improved mitochondrial ΔΨm, and intracellular ATP production. However, TLR2 deficiency had no impact on mitochondrial complex III activity in both sham and sepsis animals. Finally, we found that polymicrobial sepsis in mice led to depletion of mitochondrial Tfam and COX-2 gene expression in the liver and this process seems independent of TLR2.

We have observed that TLR1/2 activation by Pam3cys leads to a robust production of both cellular H$_2$O$_2$ and mitochondrial O$_2^-$. Cellular ROS (including O$_2^-$ and H$_2$O$_2$) is generated not only via nicotinamide adenine dinucleotide phosphate oxidase-dependent respiratory burst but also through mitochondrial oxidative phosphorylation process. Mitochondria are a major site for ROS production. During the normal respiratory process, ROS is produced as a...
by-product when high-energy electrons escape before they reach the final acceptor oxygen. The first ROS produced in mitochondria is the highly reactive superoxide anion ($O_2^-$), which can mediate oxidative damage to cells. Superoxide dismutase, an intrinsic antioxidant defense system, converts $O_2^-$ into a much more stable ROS, $H_2O_2$. $O_2^-$ has very limited membrane permeability, but $H_2O_2$ can diffuse across membranes and leave mitochondrion to cytosol. Therefore, it is very much likely that the increased mitochondrial ROS production contributes to a portion of the increased intracellular ROS in the leukocytes after TLR1/2 stimulation or during polymicrobial sepsis.

Our previous study shows that activation of TLR1/2, but not TLR3, TLR4, or TLR9, induces a marked intracellular $H_2O_2$ production in rat cardiomyocytes and mouse bone marrow-derived neutrophils. Consistent with this, the current study demonstrates a highly selective and robust effect for TLR1/2 in its ability to induce mitochondrial $O_2^-$ production in neutrophils. A similar finding has been reported in macrophages, where TLR1/2 activation induces mitochondrial ROS production via a mechanism involving TRAF-6 mitochondrial translocation and interaction with a complex I-associated protein evolutionarily conserved signaling intermediate in Toll pathways. Interestingly, under the same conditions and unlike antimycin A (a complex III inhibitor), TLR1/2 activation by Pam3cys is incapable of causing mitochondrial dysfunction. Pam3cys treatment has no effect on mitochondrial $\Delta \Psi_m$ and intracellular ATP production, which has been linked to mitochondrial $O_2^-$ production. Importantly, while mitochondria may produce more ROS at higher membrane potential, lower $\Delta \Psi_m$ and decreased activity of the respiratory chain during mitochondrial dysfunction is associated with a simultaneous increase in ROS production as we have demonstrated in antimycin A-treated leukocytes. These data suggest that TLR1/2 activation alone does not induce depolarization of mitochondrial $\Delta \Psi_m$ and subsequent impairment of oxidative phosphorylation and thus is insufficient to impair mitochondrial function. Mitochondrial ROS generation has been linked with several key cellular processes, such as cell death, cellular oxidative stress, inflammatory cytokine production, and macrophage bactericidal activity.

We demonstrate that polymicrobial sepsis leads to a robust increase in intracellular and mitochondrial ROS production in leukocytes isolated from the infectious peritonitis site. Moreover, TLR2 deficiency markedly reduces ROS production in the peritoneal leukocytes compared with WT mice, suggesting that TLR2 signaling may contribute to leukocyte ROS production during polymicrobial sepsis. To further probe the underlying mechanisms, we tested the effect of TLR2 on mitochondrial function and identified that polymicrobial infection led to marked mitochondrial dysfunction in leukocytes with significantly reduced mitochondrial $\Delta \Psi_m$ and intracellular ATP production. In comparison, mice deficient of TLR2 had preserved mitochondrial $\Delta \Psi_m$ and significantly improved intracellular ATP production. These data suggest that TLR2 signaling may play a contributory role in mitochondrial dysfunction and subsequent mitochondrial ROS production during polymicrobial sepsis. As demonstrated before, TLR2-deficient mice have markedly improved neutrophil migratory and phagocytic function, enhanced blood bacterial clearance and reduced systemic cytokine productions compared with WT mice during polymicrobial sepsis. Collectively, these studies demonstrate that TLR2 signaling plays a central role in regulating mitochondrial function, cellular ROS production, leukocyte migration, and phagocytosis during polymicrobial sepsis.

Antimycin A is a specific inhibitor of mitochondrial complex III. It inhibits succinate and nicotinamide adenine dinucleotide phosphate oxidase, and mitochondrial electron transport between cytochromes b and c. The inhibition of electron transport causes the production of ROS and results in a collapse of the proton gradient across the mitochondrial inner membrane, thereby breaking down the mitochondrial $\Delta \Psi_m$ and reducing intracellular ATP generation. Distinctly different from antimycin A, TLR 2 activation exhibits no effect on mitochondrial $\Delta \Psi_m$ and ATP production even it leads to increased ROS production. This implies that TLR2-mediated mitochondrial ROS production is not associated with mitochondrial dysfunction including that of complex III activity. Similarly, in vivo, septic mice exhibit marked reduction in mitochondrial complex III activity and reduced gene expression of Tfam and COX-2. However, TLR2 deficiency does not protect against complex III activity inhibition or mtDNA depletion during polymicrobial sepsis although it does improve mitochondrial $\Delta \Psi_m$ and intracellular ATP production. Further investigation will be needed to understand the molecular mechanisms by which TLR2 signaling mediates mitochondrial ROS generation in healthy condition and then contributes to mitochondrial dysfunction and ROS production during severe polymicrobial sepsis.

A significant amount of work has been done in determining the role of oxidative stress and mitochondrial dysfunction in sepsis-induced organ injury. Lowes et al. found that mitochondria-targeted antioxidant Mito Q reduces...
ROS production in lipopolysaccharide-treated endothelial cells, arguments mitochondrial membrane potential in major organs, and reduces acute liver and kidney dysfunction after lipopolysaccharide–peptidoglycan administration. Moreover, in vivo administration of superoxide dismutase, a free-radical scavenger, prevents endotoxin-induced cardiac dysfunction. These studies appear to suggest that cell oxidative stress and mitochondrial dysfunction during endotoxia can lead to organ dysfunction. However, the role of mitochondrial ROS in organ dysfunction during polymicrobial sepsis is less clear. Although we have demonstrated the importance of TLR2 in mitochondrial dysfunction as well as cardiac dysfunction in polymicrobial sepsis, whether mitochondrial dysfunction and oxidative stress induce cardiac functional impairment remains to be investigated.

Different animal models of sepsis have been created and categorized as three classes: (1) bacterial infusion models, (2) endotoxin models, and (3) polymicrobial peritonitis models. Infusion models utilize bolus or short-term infusion of bacteria. These models do not correlate well with the clinical situations where in most cases, there is a focus of infection providing continuous dissemination of bacteria. Endotoxin model simulates the clinical situation of hyperinflammation and septic shock. Endotoxin models are highly reproducible and can provide great insight into inflammatory processes. However, these models lack an infectious focus and do not closely mimic the pathophysiology observed in septic patients. Bacterial peritonitis models closely resemble the clinical condition of sepsis after bowel perforation. The most widely used peritonitis model is CLP. Similar to many clinical cases of sepsis, CLP model induces polymicrobial sepsis, but the model has a wide variability in terms of the host inflammatory and physiological responses, and the degree of bacteremia and mortality rates. Another limitation of the CLP model is the lack of clear information on the specific pathogens and the associated pathogen components in the pathogenesis of sepsis as the models involve a mixture of several types of bacteria including both Gram-positive and Gram-negative organisms.

In summary (fig. 8), our data suggest that TLR1/2 activation by Pam3cys is capable of inducing intracellular H$_2$O$_2$ and mitochondrial O$_2^-$ production although it seems insufficient to cause mitochondrial dysfunction. In a mouse model of severe polymicrobial sepsis and employing TLR2-deficient mice, we demonstrate that TLR2 signaling contributes to intracellular and mitochondrial ROS production and mitochondrial dysfunction as evidenced by depleted ATP production and loss of mitochondrial membrane potential (ΔΨm) in leukocytes. However, sepsis induced other mitochondrial dysfunction in leukocytes such as complex III dysfunction in leukocytes and mtDNA reduction in the liver seems to be TLR2-independent. Nevertheless, this study illustrates an important role of TLR2 in mitochondrial dysfunction, which might contribute to the pathogenesis of organ failure during severe sepsis.

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**Competing Interests**

The authors declare no competing interests.

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**References**


2. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: Epidemiology of severe sepsis in the United...

3. Dombrovskiy VY, Martin AA, Sundaram J, Paz HL: Rapid increase in hospitalization and mortality rates for severe sep

4. Bateman BT, Schmidt U, Berman MF, Bittner EA: Temporal
trends in the epidemiology of severe postoperative sep
sis after elective surgery: A large, nationwide sample. Anesthesiology 2010; 112:917–25

5. Minňauo AM, Heron MP, Smith BL: Deaths: Preliminary data

6. Crouser ED: Mitochondrial dysfunction in septic shock and
multiple organ dysfunction syndrome. Mitochondrion 2004;
4:729–41

7. Exline MC, Crouser ED: Mitochondrial mechanisms of sepsis-
induced organ failure. Front Biosci 2008; 13:5030–41

8. Azevedo LC: Mitochondrial dysfunction during sepsis.

420:885–91

10. Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R,
Davies NA, Cooper CE, Singer M: Association between
mitochondrial dysfunction and severity and outcome of sep-

11. Brealey D, Karyampudi S, Jacobs TS, Novelli M, Stidwill R,
Taylor V, Smolenski BT, Singer M: Mitochondrial dysfunction
in a long-term rodent model of sepsis and organ failure. Am
J Physiol Regul Integr Comp Physiol 2004; 286:R941–7

12. Galley HF: Oxidative stress and mitochondrial dysfunction in

produced by liver mitochondria of rats in sepsis. Arch Biochm
Biophys 1995; 316:70–6

14. Andrades M, Ritter C, de Oliveira MR, de Souza J, Pereira DC, Dal-
Pizzol F: Antioxidant treatment reverses oxidative stress produced by liver mitochondria of rats in sepsis. Arch
Biochem Biophys 1995; 316:70–6

15. Ritter C, Andrades ME, Reinke A, Menna-Barreto S, Moreira
JC, Dal-Pizzol F: Oxidative stress and mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am
J Physiol Regul Integr Comp Physiol 2004; 286:R941–7

JC, Dal-Pizzol F: Treatment with N-acetylcysteine plus defer-
 oxamine protects rats against oxidative stress and improves survival in sepsis. Crit Care Med 2004; 32:342–9

17. Ritter C, Andrades ME, Reinke A, Menna-Barreto S, Moreira
JC, Dal-Pizzol F: Treatment with N-acetylcysteine plus defer-
 oxamine protects rats against oxidative stress and improves survival in sepsis. Crit Care Med 2004; 32:342–9


Dis Rep 2004; 6:361–6

20. Feng Y, Chao W: Toll-like receptors and myocardial inflam-

21. Chao W: Toll-like receptor signaling: A critical modulator of
cell survival and ischemic injury in the heart. Am J Physiol
Heart Circ Physiol 2009; 296:H1–12

receptor-3-induced mitochondrial dysfunction in cultured

23. West AP, Brodsky IE, Rahner C, Woor DK, Erdjument-Bromage
H, Tempst P, Walsh MC, Choi Y, Shadel GS, Ghosh S: TLR sig-
nalling augments macrophage bactericidal activity through

24. Suliman HB, Welty-Wolf KE, Carraway MS, Schwartz DA,
Hollingsworth JW, Pantidosi CA: Toll-like receptor 4 medi-
ates mitochondrial DNA damage and biogenic responses

L: TLR4-mediated AKT activation is MyD88/TRIF dependent
and critical for induction of oxidative phosphorylation and
mitochondrial transcription factor A in murine macrophages. J
Immunol 2012; 188:2847–57

26. Zhu X, Bagchi A, Zhao H, Kirschning CJ, Hajar RJ, Chao W,
Hellman J, Schmidt U: Toll-like receptor 2 activation by bac-
terial peptidoglycan-associated lipoprotein activates cardi-
omyocyte inflammation and contractile dysfunction. Crit Care
Med 2007; 35:886–92

27. Zou L, Feng Y, Chen YJ, Si R, Shen S, Zhou Q, Ichinose F,
Scherrer-Crosbie M, Chao W: Toll-like receptor 2 plays a criti-
cal role in cardiac dysfunction during polymicrobial sepsis.
Crit Care Med 2010; 38:1335–42

toll-like receptor 2 contributes to neutrophil and cardiac func-
tion impairment during polymicrobial sepsis. Shock 2011;
36:370–80

29. Bergt S, Wagner NM, Heidrich M, Butschkau A, Nöldege-
Schomburg GE, Vollmar B, Roesner JP: Hydrocortisone
reduces the beneficial effects of toll-like receptor 2 defi-
ciency on survival in a mouse model of polymicrobial sepsis.
Shock 2013; 40:414–9

Hachem C, Martin C, Belaïdouni N, Balloy V, Mira JP,
Cheich JD: Toll-like receptor 2 deficiency increases resis-
tance to Pseudomonas aeruginosa pneumonia in the setting
of sepsis-induced immune dysfunction. J Infect Dis 2012;
206:932–42

31. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa
T, Takeda K, Akira S: Differential roles of TLR2 and TLR4 in
recognition of gram-negative and gram-positive bacterial cell
wall components. Immunity 1999; 11:443–51

MyD88 signaling modulates neutrophil function and isch-
299:C760–9

Thurman JM, Wu X, Atkinson JP, Chao W: Complement factor
B is the downstream effector of TLRs and plays an important
role in a mouse model of severe sepsis. J Immunol 2013;
191:5625–35

34. Korshunov SS, Skulachev VP, Starkov AA: High protonic
potential activates mitochondrial DNA damage and biogenic

Thurman JM, Wu X, Atkinson JP, Chao W: Complement factor
B is the downstream effector of TLRs and plays an important
role in a mouse model of severe sepsis. J Immunol 2013;
191:5625–35

36. Ly JD, Grubb DR, Lawen A: The mitochondrial membrane
potential (deltapsi(m)) in apoptosis; an update. Apoptosis
2003; 8:115–28

37. Kirkinezos IG, Moraes CT: Reactive oxygen species and mito-

38. Turrens JF: Reactive oxygen species and mito-
ochondrial dysfunction in sepsis. J Physiol 2003;
206:932–42

Mira JP, Vannier M, Vannier L: MyD88 signaling modulates
neutrophil function and ischemic myocardial injury. Am J
Physiol Cell Physiol 2010; 299:C760–9

Thurman JM, Wu X, Atkinson JP, Chao W: Complement factor
B is the downstream effector of TLRs and plays an important
role in a mouse model of severe sepsis. J Immunol 2013;
191:5625–35

41. Corboz G, Gluckman PD, Vranken P, Franchi I, Cadiot G,
Baudouin S, Nalysnyk L, Nuñez G: MyD88/TRIF-dependent
activation of NF-κB and ATK in monocytes: upstream and

42. Fridovich I: Superoxide radical and superoxide dismutases.


