 DOI: 10.5114/ceji.2016.60989

The role of myeloid differentiation factor 88 on mitochondrial dysfunction of peritoneal leukocytes during polymicrobial sepsis

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Abstract

Objective: *To investigate the role of myeloid differentiation factor 88 (MyD88) on mitochondrial dysfunction of peritoneal leukocytes during polymicrobial sepsis.*

Material and methods: *Polymicrobial peritonitis, a clinically relevant mouse model of sepsis, was generated by cecum ligation and puncture (CLP) in both male C57BL/6J wild-type (WT) and MyD88 knockout (MyD88$^{-/-}$) mice. Twenty-four hours after surgeries, peritoneal leukocytes were collected and four parameters of mitochondrial function, including total intracellular and mitochondrial ROS burst, mitochondrial membrane depolarization and ATP depletion, were measured by flow cytometry or ATP assay, and then compared.*

Results: *Polymicrobial sepsis led to a marked mitochondrial dysfunction of peritoneal leukocytes with total intracellular and mitochondrial ROS overproduction, decreased mitochondrial membrane potential and reduced intracellular ATP production. In comparison, there was no significant difference in the extent of mitochondrial dysfunction of peritoneal leukocytes between WT and MyD88$^{-/-}$ septic mice.*

Conclusions: *MyD88 may be not sufficient to regulate mitochondrial dysfunction of peritoneal leukocytes during polymicrobial sepsis.*

Key words: *polymicrobial sepsis, peritoneal leukocyte, mitochondrial dysfunction, MyD88.*

(Cent Eur J Immunol 2016; 41 (2): 153-158)

Introduction

Sepsis, defined as a systemic response to infection with the presence of some degree of an organ dysfunction, is the main cause of death in the intensive care unit, and can produce mortality rates as high as 30~50% [1]. The pathogenesis of sepsis is very complicated and not fully understood yet. In recent years, studies have shown that the progresses of sepsis and its induced septic shock or multiple organ dysfunction syndrome (MODS) are closely related to mitochondrial dysfunction. During sepsis, overwhelming inflammatory responses with the release of a large number of inflammatory cytokines and excessive reactive oxygen species (ROS) production result in mitochondrial dysfunction, which includes mitochondrial membrane damage, mitochondrial respiratory chain damage, mitochondrial DNA damage and mitochondrial calcium overload and finally leads to cell apoptosis. Therefore, mitochondrial dysfunction is one of the reasons of sepsis induced-MODS [2].

Toll-like receptor (TLR) is one of the key proteins involved in innate immune, a growing number of studies have found that TLR plays an important role in regulating mitochondrial function. Myeloid differentiation factor 88 (MyD88) as a major adaptor protein of TLR signaling pathways, may transduce crucial signals in the TLR modulated-mitochondrial dysfunction process [3-6]. This study intends to select MyD88 knockout (MyD88$^{-/-}$) mice as the research object and employ a mouse model of cecum ligation and puncture (CLP) to generate polymicrobial sepsis; then observe whether mitochondrial dysfunction can be induced in peritoneal leukocytes and analyze whether there is any difference in the extent of mitochondrial dysfunction between WT and MyD88$^{-/-}$ mice; thus discuss preliminarily whether MyD88 has a critical influence on polymicrobial sepsis-induced mitochondrial dysfunction in peritoneal leukocytes.

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Submitted: 14.10.2015, Accepted: 13.01.2016

Material and methods

Animals

Eight- to twelve-week-old gender- and age-matched mice were used for the experiments. WT C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). MyD88$^{-/-}$ mice were generated by Kawai and colleagues [7] and had been backcrossed > 10 generations into the C57BL/6J strain. All animals were housed at the Massachusetts General Hospital (MGH) animal facility for at least one week before the experiments were performed. All animals were housed in pathogen-free, temperature-controlled, and air-conditioned facilities under a 12 h/12 h light/dark cycle and were fed the same bacteria-free diet. All animal care and procedures were performed according to the protocols approved by the Subcommittee on Research Animal Care of MGH and were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Mouse model of polymicrobial sepsis induced by cecum ligation and puncture (CLP)

A mouse model of polymicrobial sepsis was generated by CLP as described previously [8, 9]. In brief, the cecum was ligated at 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 the cecum was returned to the abdominal cavity. The sham-operated mice underwent laparotomy without CLP. The abdominal wall incision was closed in layers. After surgery, pre-warmed normal saline (50 ml/kg body weight) was administered subcutaneously. Postoperative pain control was managed with subcutaneous injections of bupivacaine (2 ml/kg body weight) and buprenorphine (2 ml/kg body weight).

Peritoneal leukocytes collection

Twenty-four hours after sham or 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 [10].

Reactive oxygen species measurement

Total intracellular H_2O_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 incubated with freshly prepared H2-DCF-DA or MitoSOX at 37°C in the dark for 30 min. Unstained controls were handled similarly but dyes were omitted. Dye-loaded cells were re-suspended in cold DPBS containing 1% fetal bovine serum and analyzed immediately by flow cytometry at fluorescein isothiocyanate or R-phycoerythrin channel. Ten thousand cells were routinely counted by flow cytometry, and data were expressed as the median fluorescence intensity (MFI) in arbitrary units from at least three separate experiments.

Mitochondrial membrane potential measurement

TMRE (Cat. 87917, Sigma) was used to measure levels of $\Delta\Psi m$. TMRE is a cationic dye that is rapidly and reversibly accumulated by healthy mitochondria. A decrease in the levels of TMRE indicates a reduction in mitochondrial membrane potential levels. Experimentally, a fraction of cells (5×10^5) from the peritoneal lavage was labeled with freshly prepared TMRE at 37°C in the dark for 30 min. Unstained controls were treated similarly but 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-phycoerythrin channel. Ten thousand cells were routinely collected, and data were expressed as the mean fluorescence intensity in arbitrary units from the average of at least three separate experiments.

ATP assay

The intracellular ATP level was measured using a luciferase-based assay using an ATP Bioluminescence Assay Kit CLS II (Roche Molecular Biochemicals, 1699695). In brief, intracellular ATP was released using a boiling method. Specifically, splenocytes from sham or septic mice were harvested, washed twice with ice-cold DPBS, drained, and re-suspended 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 supernatants were transferred to fresh tubes and kept on ice until measurements were performed. Finally, 35 µl of luciferase reagent was added to 35 µl of the sample or standard. Experiments were performed in triplicate, and the data were standardized to the protein concentrations of the samples using Bradford protein assay reagent (Bio-Rad, 500-0006).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA). Continuous variables are expressed as the mean ± SEM. One-way ANOVA with post-hoc (Tukey) test was used to determine the statistically significant difference between the groups.

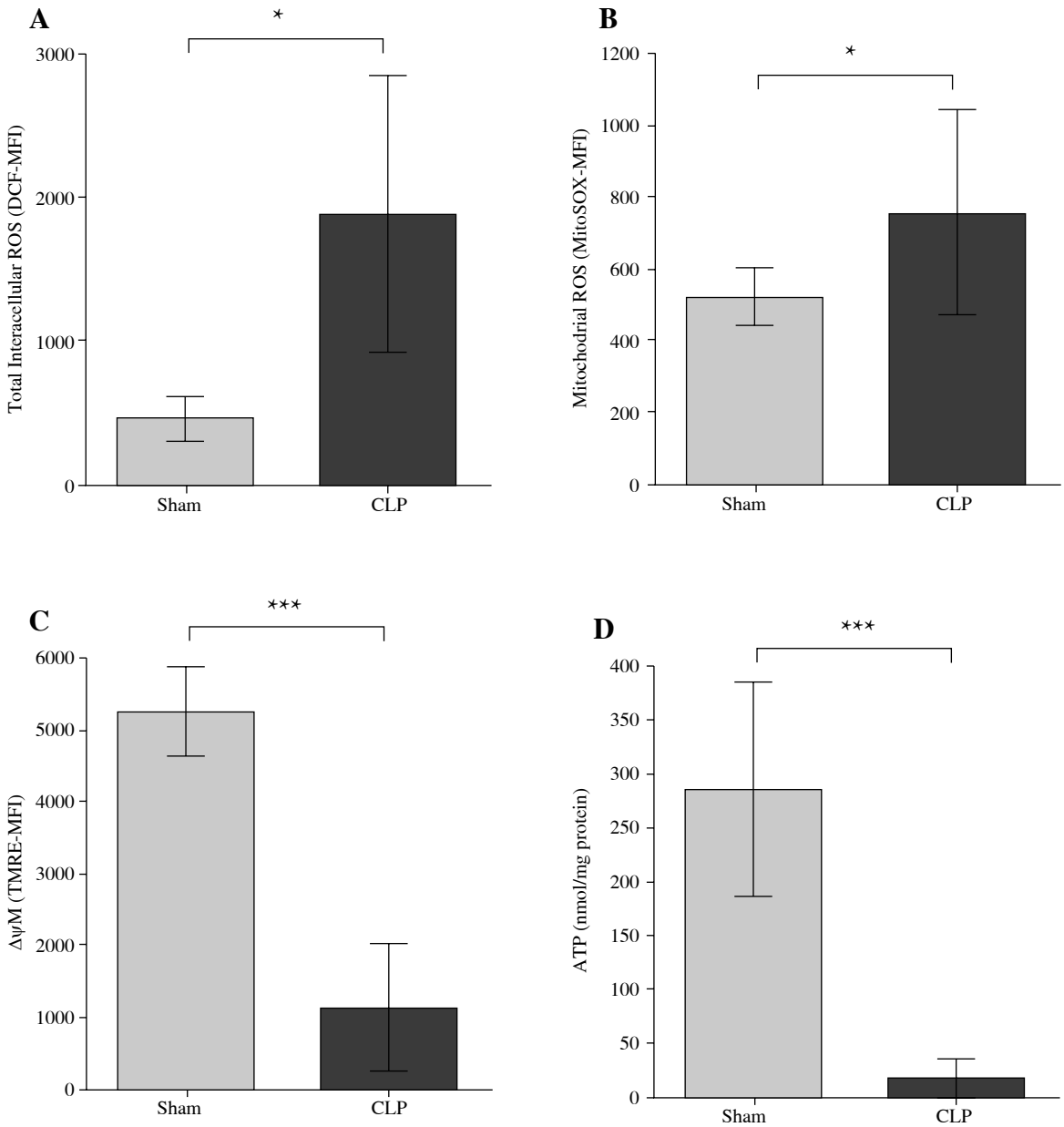

Fig. 1. Polymicrobial sepsis induces mitochondrial dysfunction in peritoneal leukocytes. A) Polymicrobial sepsis induces total intracellular ROS production in peritoneal leukocytes. WT mice were subjected to sham or CLP surgery. Twenty-four hours later, peritoneal leukocytes were harvested, stained with DCF and analyzed with flow cytometry for total intracellular ROS production. B) Polymicrobial sepsis induces mitochondrial ROS production in peritoneal leukocytes. WT mice were subjected to sham or CLP surgery. Twenty-four hours later, peritoneal leukocytes were harvested, stained with MitoSOX and analyzed with flow cytometry for mitochondrial ROS production. C) Polymicrobial sepsis decreases mitochondrial membrane potential in peritoneal leukocytes. WT mice were subjected to sham or CLP surgery. Twenty-four hours later, peritoneal leukocytes were harvested, stained with TMRE and analyzed with flow cytometry for mitochondrial membrane potential. D) Polymicrobial sepsis decreases intracellular ATP production in peritoneal leukocytes. WT mice were subjected to sham or CLP surgery. Twenty-four hours later, peritoneal leukocytes were harvested and analyzed for intracellular ATP level by ATP bioluminescence assay. The data are presented as the mean ± SEM. $n > 3$ mice/group. $^{*}P < 0.05$ vs. sham

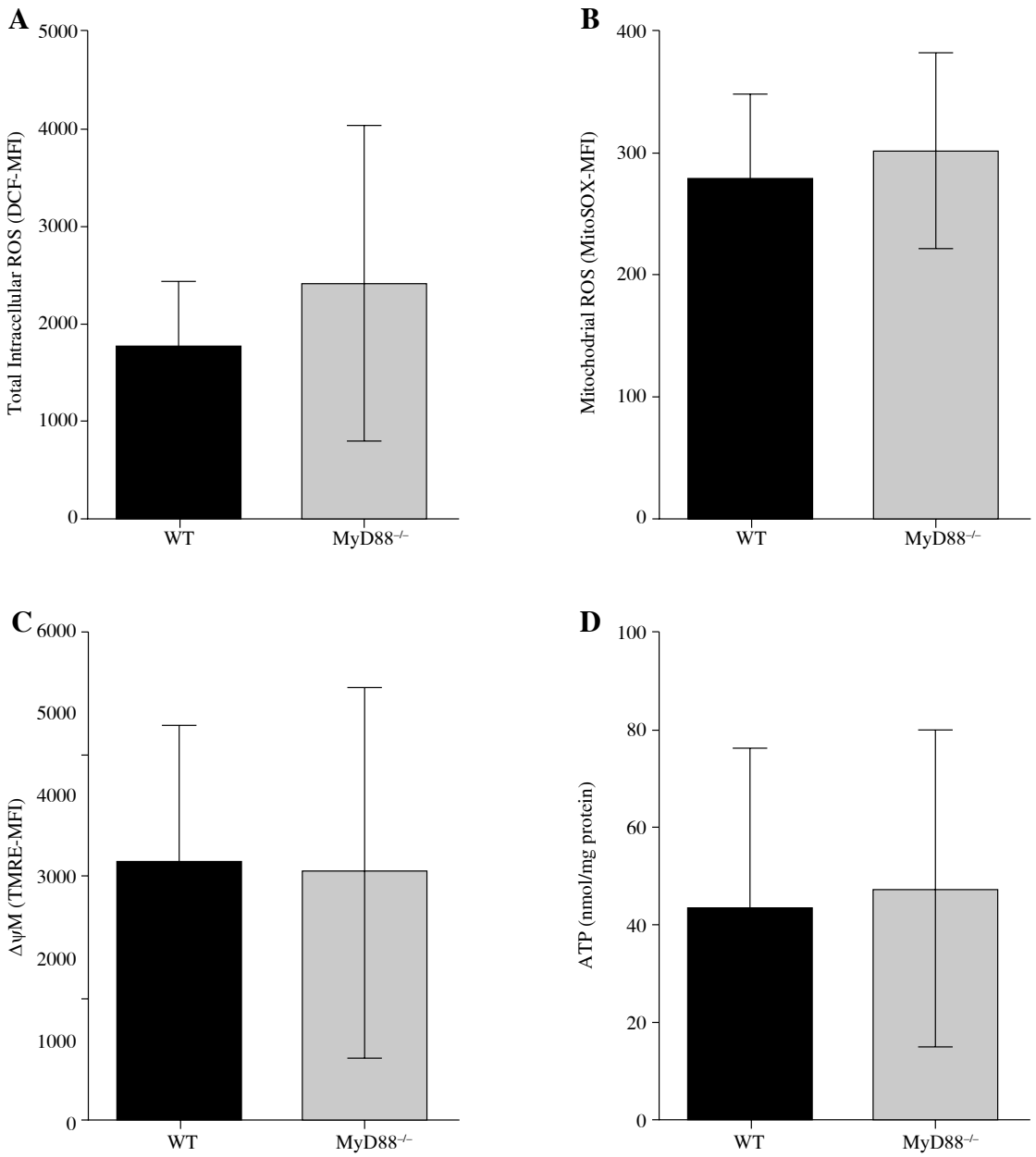

Fig. 2. MyD88 deletion plays an unimportant role in leukocyte mitochondrial dysfunction during polymicrobial sepsis. A) MyD88 plays an unimportant role in total intracellular ROS production in peritoneal leukocytes during polymicrobial sepsis. WT and MyD88$^{-/-}$ mice were subjected to CLP surgery. After twenty-four hours, peritoneal leukocytes were harvested, stained with DCF and analyzed with flow cytometry for total intracellular ROS production. B) MyD88 plays an unimportant role in mitochondrial ROS production in peritoneal leukocytes during polymicrobial sepsis. WT and MyD88$^{-/-}$ mice were subjected to CLP surgery. After twenty-four hours, peritoneal leukocytes were harvested, stained with MitoSOX and analyzed with flow cytometry for mitochondrial ROS production. C) MyD88 plays an unimportant role in mitochondrial membrane potential in peritoneal leukocytes during polymicrobial sepsis. WT and MyD88$^{-/-}$ mice were subjected to CLP surgery. After twenty-four hours, peritoneal leukocytes were harvested, stained with TMRE and analyzed with flow cytometry for mitochondrial membrane potential. D) MyD88 plays an unimportant role in intracellular ATP production in peritoneal leukocytes during polymicrobial sepsis. WT and MyD88$^{-/-}$ mice were subjected to CLP surgery. After twenty-four hours, peritoneal leukocytes were harvested and analyzed for an intracellular ATP level by ATP bioluminescence assay. The data are presented as the mean ± SEM. $n > 3$ mice/group. $^{***}P < 0.001$ vs. sham

Results

Polymicrobial sepsis induces mitochondrial dysfunction in peritoneal leukocytes

Sham and CLP operation was performed in WT mice, the parameters of mitochondrial dysfunction were detected in peritoneal leukocytes, including total intracellular ROS production, mitochondrial ROS production, mitochondrial membrane potential and intracellular ATP production. As shown in Fig. 1, twenty-four hours later, there was a marked increase in total intracellular and mitochondrial ROS production (Fig. 1A, B), meanwhile there was an obvious reduction in mitochondrial membrane potential and intracellular ATP production (Fig. 1C, D), in WT septic mice as compared with the sham control mice. These data clearly suggest that polymicrobial sepsis leads to mitochondrial dysfunction in peritoneal leukocytes.

Polymicrobial sepsis-induced mitochondrial dysfunction in peritoneal leukocytes via a MyD88-independent mechanism

Furthermore, we subjected WT and MyD88$^{-/-}$ mice to CLP operation and 24 hours later, the above four parameters of mitochondrial dysfunction were measured in peritoneal leukocytes. As indicated in Fig. 2, there was no significant difference in all four parameters of mitochondrial dysfunction in peritoneal leukocytes, including total intracellular and mitochondrial ROS production, mitochondrial membrane potential and intracellular ATP production, between WT and MyD88$^{-/-}$ septic mice. These results indicated that MyD88 signaling has no impact on polymicrobial sepsis-induced mitochondrial dysfunction in peritoneal leukocytes.

Discussion

So far, mitochondrial dysfunction has been regarded as the central part of the development of sepsis induced-MODS. But, due to no relevant molecular mechanism, clinical application of mitochondrial dysfunction in therapy of sepsis has certain limitations. The current study preliminarily surveys the possible role of MyD88 in mediating mitochondrial dysfunction of peritoneal leukocytes in a clinically relevant mouse model of polymicrobial sepsis, by comparing the extent of mitochondrial dysfunction in WT or MyD88$^{-/-}$ septic mice, including total intracellular and mitochondrial ROS overproduction, decreased mitochondrial membrane potential and reduced intracellular ATP production. Our results displayed that polymicrobial sepsis induced an obvious mitochondrial dysfunction of peritoneal leukocytes although MyD88 may be not sufficient to modulate it because the four parameters of mitochondrial dysfunction were significantly different just between WT sham and WT septic mice, but not between WT septic and MyD88$^{-/-}$ septic mice.

Bacterial peritonitis models closely mimic the clinical symptoms of sepsis after bowel perforation, and the most widely used peritonitis model is CLP. Similar to numerous clinical cases of sepsis, the CLP model induces polymicrobial sepsis [11]. Hotchkiss *et al.* have proved that both Gram-positive and Gram-negative bacteria exist in blood cultures from CLP-operated mice, including *Proteus mirabilis*, *Streptococcus faecalis*, *Enterobacter cloacae*, *Escherichia coli*, *Bacillus* and so on [12]. A lot of research have confirmed the importance of TLRs family members in the pathogenesis of sepsis through the CLP model. During polymicrobial sepsis induced by CLP, TLR2 deficiency remarkably reduced the mortality rate, improved the cardiac function or inhibited mitochondrial dysfunction of peritoneal leukocytes, particularly lowered mitochondrial ROS production and maintained mitochondrial membrane potential [9, 13, 14]. Interestingly, TLR4 deficiency brought completely opposite results, including a higher mortality rate, deterioration of the cardiac function or increased mitochondrial ROS production of peritoneal leukocytes [15]. TLR2 mainly recognizes peptidoglycan of Gram-positive bacteria but TLR4 typically identifies Gram-negative bacteria and their products, which gives us a clue that TLR2 and TLR4 are involved in polymicrobial sepsis induced by CLP through their respective signal transduction pathway, and lead to two distinct outcomes: TLR2 signaling pathways are destructive whereas TLR4 signaling pathways are protective. In addition, mice deficient in TLR9, a receptor for unmethylated CpG motifs present in bacterial DNA, were resistant to lethal polymicrobial sepsis and have improved the survival rate [16]. TLR7, regarded as a receptor for viral RNA, pharmacological activation of it prior to induction of sepsis, improved the host's capacity to cope with pathogens [17]. MyD88, serving as one of the key adaptors for all TLRs signaling pathways, except TLR3 which transduces its signal through TRIF. Given the above, we may conclude that MyD88 is not efficient to affect sepsis-induced mitochondrial dysfunction of peritoneal leukocytes because of the final integrated result of all MyD88-dependent TLR signaling pathways.

Feng and colleagues found that in comparison with WT mice, MyD88$^{-/-}$ mice had prolonged survival with polymicrobial sepsis induced by CLP [18], which implied that MyD88 may play a bad role during the pathogenesis of sepsis. Significantly, Peck-Palmer and colleagues discovered that compared to WT mice, MyD88$^{-/-}$ mice had worse survival with polymicrobial sepsis induced by CLP [19], which indicated that MyD88 may serve as a good role during the pathogenesis of sepsis. Although both of the two research groups used the same model of sepsis, there were some differences in details. Feng's group used an 18-gauge needle and showed a mortality rate of up to 80% within 3 days in WT mice, but Peck-Palmer's group used a 27-gauge needle

and exhibited that WT mice reached 80% mortality on day 7 after the CLP procedure. We can conclude that MyD88 performs different functions in high-grade and low-grade lethal CLP models. Mitochondrial dysfunction of peritoneal leukocytes may be dependent on MyD88 signaling pathway in a low-grade lethal model of sepsis, and its specific mechanism needs further discussion.

To sum up, although MyD88 does not play a key role in CLP-induced mitochondrial dysfunction of peritoneal leukocytes in mice, MyD88 cannot be ruled out for participating in regulating clinical characteristics of sepsis, especially mitochondrial dysfunction caused by multiple organ dysfunction syndrome. Therefore, further revealing and exploring the molecular mechanism will provide a new strategy for clinical intervention of sepsis.

This work was supported financially by Guangzhou Medical University (2011C43), the Third Affiliated Hospital of Guangzhou Medical University (2012Y20), and Department of Education of Guangdong Province (2015KQNCX137).

The authors declare no conflict of interest.

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