EFFECT OF TLR4/MYD88/NF-KB SIGNALLING ON RENAL INJURY CAUSED BY SEPSIS

PENG WANG1, FUHAI JI2,*
1Department of Anesthesiology, The First Affiliated Hospital of Soochow University, Suzhou 215006, P.R. China - 2Department of Anesthesiology, The First Affiliated Hospital of Soochow University, Suzhou 215006, P.R. China

ABSTRACT

Objective: The purpose of this study was to explore the effects of TLR4/MyD88/NF-κB on the kidneys during sepsis.

Methods: In this experiment, three types of mice—normal mice, TLR4 gene-deficient mice and MyD88 gene-deficient mice—were used to establish a kidney sepsis injury model by cecal ligation puncture (CLP). The animals were divided into four groups: (a) Control group (normal mice without any treatment), (b) WT group (normal mice with CLP treatment), (c) TLR4-/- group (mice with TLR4 gene defect and CLP treatment), and (d) MyD88-/- group (mice with MyD88 gene defect and CLP treatment). We observed the effect of CLP-induced sepsis on the survival rate and intraabdominal bacteria found in different groups, reporting on the level of serum creatine anhydride and blood urea, renal tubule necrosis and the expression of genes related to renal injury. In addition, we analysed the activity of NF-κB p65 in different groups of mice with CLP-induced sepsis using the immunohistochemical technique.

Results: (1) After 192 h of CLP-induced sepsis, the MyD88 gene defect group had the highest survival rate, but also the highest number of bacteria in the abdominal cavity. The total survival time of TLR4-/- group was longer than that of the WT group, and the number of bacteria in the abdominal cavity was less than that of the WT group. (2) Concentrations of serum creatinine and blood urea were significantly reduced in the MyD88-/- gene defect group as compared with the WT group (P<0.001), while serum creatinine and blood urea concentrations in the TLR4-/- group were improved, this improvement was not statistically significant (P>0.05). Compared with the WT mice, the expression of kidney damage molecular-1 (KIM-1) gene in TLR4-/- group was significantly decreased (P<0.01), and the expression of KIM-1 was significantly decreased in MyD88-/- mice (P<0.001). (3) The kidney tissue of mice in the MyD88-/- group was similar to that in the control group, with almost no damage. Compared with WT mice, the expression of TLR4-related ligands HMGB1 and HSP70 in the MyD88-/- group was also significantly reduced. (4) Compared with the WT group, mRNA expressions of IL-1β, IL-6 and TNF-α in the TLR4-/- and MyD88-/- groups were lower, with statistical significance (P<0.05). (5) Immunohistochemical results showed that the absence of TLR4 and MyD88 affected the activity of NF-κB p65 in sepsis mice, and the translocation of NF-κB p65 into the nucleus was significantly protected.

Conclusion: The deletion of the TLR4/MyD88 gene can reduce the effects of sepsis on renal injury, can reduce the expression of related inflammatory factors and can inhibit the activity of NF-κB p65. Therefore, it is possible that the functional mechanism of sepsis on renal injury is related to the TLR4/MyD88/NF-κB signalling pathway.

Keywords: Sepsis, TLR4, Myd88, NF-κB, kidney injury.

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Introduction

Severe sepsis is the main reason for acute kidney injury (AKI)1. In recent years, while researchers have made a number efforts to better understand this condition, little progress has been made. Most researchers have focused on providing evidence of AKI being caused by changes in the renal hemodynamic; nevertheless, a growing number of researchers believe that non-hemodynamic factors, such as immunological factors, might play an important role in the development AKI2-3. Renal damage caused by sepsis is likely due to the recognition of bacterial products by toll-like receptors (TLRs), which trigger subsequent inflammatory responses in sepsis4.

The signal transduction effect of TLRs is partially mediated by the TLR cohesion protein, myeloid differentiation factor 88 (MyD88). MyD88 binds to the TIR domain and recruits signalling proteins that activate transcription factors, such as
NF-κB and activator protein 1 (AP-1), resulting in the overexpression of inflammatory factors, such as IL-6, IL-1β, tumour necrosis factor-α, and so on\(^{(5-6)}\). Although numerous studies have shown that sepsis is strongly correlated with TLRs, the relationship between AKI and TLRs requires further study. During sepsis, TLR signalling is closely related to inflammation and immunity. This signalling can also be activated through bacteria and endogenous ligands (e.g. HGB-1 and HSP70), which are released during cellular stress and interact with the immune system in the extracellular environment; however, these dangerous signalling molecules can aggravate renal inflammation during sepsis\(^{(7-8)}\). By studying the process of TLR4/MyD88 gene deletion in a mouse model of sepsis, observing renal damage caused by sepsis, and through the analysis of different indicators, this study aims to explore the cause kidney damage in sepsis and TLR4/MyD88 NF-κB signalling pathways. The findings of this study may have implications for the future treatment of renal injury caused by sepsis, while at the same time providing a theoretical basis for the development of new drugs.

**Materials and methods**

**Experimental animals**

Thirty-two male mice (C57BL/6) of different types with a weight of about 20-28 g at 6-8 weeks were used. Types included normal mice and mice with TLR4 and MyD88 gene defects, respectively. Animals were provided by the Animal Experimental Model Development Centre for Medicine and Biology, Nanjing University. Mice were kept in cages with up to five other mice under artificial light/darkness for 12 h at a constant temperature of 22 °C (±2 °C) and supplemented with water and food.

**Experimental methods**

**Establishment of Sepsis Mouse Model**

TLR4 and MyD88 gene-deficient mice, plus a sample of normal mice (WT group) were subjected to cecal ligation puncture (CLP) to establish a sepsis kidney injury model\(^{(9)}\). The procedure was as follows: the cecum of the animal was punctured twice with a size 23 needle and then gently pressed to ensure the clearance of the intestinal contents, thus leading to sepsis.

**Experimental Grouping**

The 32 two mice were randomly divided into four groups: (a) Control group (normal mice without any treatment), (b) WT group (normal mice with CLP treatment), (c) TLR4-/- group (mice with TLR4 gene defect and CLP treatment), and (d) MyD88-/- group (mice with MyD88 gene defect and CLP treatment). Eight samples were assigned to each group.

**Survival Rate of Different Kinds of Sepsis Mice**

Survival rates at 192 h after CLP induction were observed twice a day and recorded.

**Count of Bacteria in Abdominal Cavity**

Quantitative bacterial culture of celiac colony forming unit (CFU) of mice in the control group and quantitative bacterial culture of celiac cavity 24 h after sepsis caused by CLP were conducted. CFU was determined after continuous dilution. AGAR was inoculated with 1x106 CFU and incubated at 37oC in an incubator for 18 h.

**Histological and Quantitative Analysis of Acute Tubular Necrosis**

Haematoxylin and Eosin staining was used to conduct quantitative analysis of acute renal tubular necrosis (ATN), and the computer program Image Pro Plus for Windows (USA), combined with an optical microscope (Olympus BX40F-3, Olympus Corporation, Japan), was used to collect and digitise images of the evaluation area. In quantitative analysis, the results are expressed by the ratio of the affected area (selected) to the total measured area (0.073 cm2). Five animals in each group were studied.

**PCR Was Used to Detect the Expression of Genes Related to Renal Function**

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). CDNDA was synthesised using an ML-V reverse transcriptase kit (Promega, Madison, Wisconsin, USA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<tbody>
<tr>
<td>HPRT</td>
<td>GGGCTGTATTCCTGCCCATCG</td>
<td>CCACTGGAACACTGACGCTG</td>
</tr>
<tr>
<td>KIM-1</td>
<td>TCAAGTCTCCCAATCCCTGGT</td>
<td>AAACGGGAGTGGGGTTCAG</td>
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<tr>
<td>HMGB1</td>
<td>ACCCGGATCCCCCTGTTACCTTCTCAACTCTT</td>
<td>ATACGAGCCTGTCAGGAGGCTTCAG</td>
</tr>
<tr>
<td>HSP70</td>
<td>GCCAAGTGCAAGAAGCTCAAGGTCACAGGCAAA</td>
<td>AAAATGTCACAAGGCTGACACAGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGGGTCTGCGGCGCCTATCTG</td>
<td>TGCTCTCTGCTGCGGGAATAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATGGCATGGCTGATACCCACC</td>
<td>GAGGCGCAATTGCTGCTGCCCTCCA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGAAGGAGCAAGAAGTACAGGCC</td>
<td>TCTCTGAGTATTGCCAGA</td>
</tr>
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**Table 1:** Sequence of genes of the primer.
Reverse transcription polymerase chain reaction (PCR) was performed using a TaqMan probe. mRNA expression was normalised to HPRT. Refer to references (9-10) for steps. Primer sequences of related genes are shown in Table 1.

Data statistics
Data is represented as average and standard deviation (SD), or median, upper and lower limits (histomorphometric analysis). T-test and long-rank test were used for analysis and comparison. PCR results were expressed by the ratio of calibrated gene HPRT. The difference was statistically significant (p<0.05). To study survival, animals were monitored twice a day for 8 d (192 h) after CLP. The long-rank test was used for survival curve analysis. All statistical analyses were performed using GraphPad PRISM software.

Results

The impact of CLP-induced sepsis in different types of mice on their survival rate
The survival rate of rats with sepsis (CLP) induced AKI at 192 h is shown in Figure 1. Compared with other groups, the survival rate of mice in the MyD88-/- group was the highest (p<0.05) (Figure 1a). Compared with WT, TLR4-/- mice had a longer overall survival time. This indicated that the survival rate of the MyD88-/- group of AKI mice was greatly improved. In addition, we performed a bacterial count for the abdominal cavity of mice, finding that the MyD88-/- group had a higher bacterial count than that of the control group, and that the bacterial count in TLR4-/- group was lower than that of either WT or MyD88-/- groups (Figure 1b).

Effects of CLP-induced sepsis in different types of mice on renal function
The results show that the MyD88-/- mice were completely unaffected by renal dysfunction caused by sepsis. Compared with the control group, serum creatinine and blood urea concentrations in the WT and TLR4-/- groups were significantly increased. Compared with the WT group, serum creatinine and blood urea concentrations in the MyD88-/- mice was significantly reduced (P<0.001), while TLR4-/- mice showed only modest improvement, not statistically significant (Figure 2a, 2b). In addition, the expression of the KIM-1 gene was significantly increased in WT mice, significantly decreased in TLR4-/- mice (P<0.01) and significantly decreased in MyD88-/- mice (P<0.001) (Figure 2c).

Effects of sepsis on acute tubular necrosis in TLR4 and Myd88 deficient mice
The experimental results show that MyD88 deficiency had a protective effect on renal injury in sepsis.

Figure 1: Effect of TLR4 and MyD88 deletion on survival after acute renal injury in CLP animals. (a) Survival rate of CLP infection in WT, TLR4-/- and MyD88-/- mice. Mice were evaluated twice daily until 192 h after surgery. (b) Abdominal cavity bacterial count 24 h after infection with sepsis.

Figure 2: (a) Effects of CLP-induced sepsis on renal function in different types of mice. (b) Renal function of WT, TLR4-/- and MyD88-/- groups at 24 h after CLP, assessed by serum creatinine and urea levels. (c) Expression of KIM-1 gene in the kidneys of the control group, WT, TLR4-/- and MyD88-/- groups 24 h after CLP. Compared with WT group, ***: p<0.0001; **: p<0.01; *: p<0.05.

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Figure 3: Influence of sepsis on ATN in TLR4 and MyD88 deficient mice. Histological changes of ATN in (a) control group, (b) WT, (c) TLR4 and (d) MyD88 mice 24 h after CLP. (e) Quantitative analysis data statistics. Representative results of five animals in each group. Compared with WT group, ***: p<0.0001.
The renal histological results show that CLP-induced sepsis led to ATN in WT mice, while renal tissue showed peripheral defects, vascular degeneration and severe injury. In the TLR4-/- group, kidney tissue damage was mild, while kidney tissue damage was not observed in the MyD88-/- group, which was similar to the results seen in the control group, indicating that the lack of MyD88 had a strong protective effect on AKI (Figure 3a–3d). Quantitative analysis of histological results showed that compared with WT group, ATN injury in TLR4-/- and MyD88-/- groups was significantly reduced (P<0.001) (Figure 3e).

**Expression of pro-inflammatory cytokines in kidney of mice with TLR4 and Myd88 gene deletion**

Activation of TLR4 through MyD88 leads to the nuclear translocation of NF-κB and AP-1, leading to the expression of genes related to inflammatory responses. TNF-α major pro-inflammatory factor activated in experimental models of sepsis are thought to be one of the main mediators of AKI. The results showed that, compared with the WT group, the mRNA expressions of IL-1β, IL-6 and TNF-α in the TLR4-/- and MyD88-/- groups were all relatively low and statistically significant (P<0.05), and the mRNA expressions of IL-1β, IL-6 and TNF-α in the MyD88-/- group were the most significantly reduced (P<0.001), with the expression of IL-6 being close to that of the control group (Figure 5a–5c).

**Effects of CLP-induced sepsis on renal NF-κB activity in mice with TLR4 and Myd88 defects**

Results 2.5 showed that the mRNA expressions of IL-1β, IL-6 and TNF-α were significantly decreased in the TLR4-/- and MyD88-/- groups. The immunohistochemical results shown in Figure 6a–6d with respect to WT mice clearly show NF-κB p65 transposition to the nucleus (Figure 6b). Compared with the WT group, mice in the TLR4-/- and MyD88-/- groups showed significantly reduced NF-κB p65 transposition to the nucleus. Therefore, the lack of TLR4 and MyD88 affected NF-κB p65 transposition to the nucleus under the protection of the obvious, which eventually lead to inflammation of the lower molecular transcription. According to the statistics shown in Figure 6e, compared with WT group, the activities of NF-κB p65 were significantly decreased in the TLR4-/- and MyD88-/- groups (P<0.01).
Discussion

This study found that TLR4 and MyD88 plays an important role in CLP-induced sepsis in mice. Firstly, the survival rate of mice with normal sepsis is lower than that of mice without TLR4 and MyD88 genes (Figure 1), indicating that TLR4 and MyD88 genes play an important role in the mouse anti-sepsis process. Sepsis is an important cause of kidney injury. Our results show that, in the absence of TLR4 and MyD88 gene sepsis in mice, the results of the renal injury caused by also have different, we found that in the normal sepsis mice (WT), the concentration of serum creatinine and blood urea concentration is higher, compared with the WT group, mice in the lack of TLR4 and MyD88 group, the concentration of the concentration of serum creatinine and blood urea significantly reduced, explain the lack of TLR4 and MyD88D reduced renal function injury (Figure 2). In addition, the results also show that the lack of sepsis in TLR4 and MyD88 mice resulted in relatively little tubular renal damage, while the sepsis seen in normal mice (WT) resulted in relatively serious the renal tubular necrosis. In addition, the expression of the KIM-1 gene in WT mice increased significantly, while gene expression was low in the TLR4 and MyD88 group, having relatively similar levels to the control group (Figure 3).

In addition, we found that the absence of TLR4 and MyD88 resulted in the low expression of related inflammatory cytokines in the kidneys of septic mice, such as IL-1β, IL-6 and TNF-α (Figure 5).

Previous studies of MyD882/2 mice have shown that renal function improved and that serum TNF levels decreased in mouse models after sepsis as compared with health controls(12). Studies have also shown that the levels of TNF-α, IL-1β and IL-6 in sepsis patients are significantly elevated(13), a finding consistent with the experimental results of this study. TNF-α major pro-inflammatory factor activated in experimental models of sepsis is considered to be one of the main mediators of AKI (11). Endotoxin in the kidney causes mesangial cells to release t TNF-α(14, 15). In this study, the activity of NF-κB p65 was analysed by immunohistochemistry. However, in the normal group (WT group), the activity of NF-κB p65 was significantly higher, and in the control group, there was almost no NF-κB p65 activity, indicating that sepsis enhances NF-κB p65 activity, thus leading to an increase in the expression of inflammatory factors (Figure 6).

Studies have shown that hypotension during sepsis, thus leading to tissue hypoxia, which can activate the renal tubules and endothelial cells, and eventually lead to the release of cytokines and chemokines, which in turn leads to increased vascular permeability, and to subsequent renal tubular cell necrosis or apoptosis(16). Hypoxia is considered to be a key factor in blood-brain barrier dysfunction during sepsis. In addition to being a hypotensive, NF-κB also regulates the expression of HIF1-α. Analysis of HIF1-α gene expression and qualitative analysis of renal hypoxia suggests that TLRs, especially MyD88, is involved in renal injury(17). During sepsis, some red flag molecules may also activate TLRs. Previous studies have shown that HMGB1 and HSP70 levels are increased under conditions of cell damage and inflammation(4, 7, 8). In this study, we found that these ligands were highly expressed in WT mice and reduced in knockout mice after sepsis (Figure 4).

These ligands can lead to the production of large amounts of cytokines, resulting in the development of AKI in infected mice. In recent years, the role of apoptosis in the pathogenesis of sepsis has been explored. The overexpression of caspase inhibitors or anti-apoptotic protein BCL-2 significantly increased the survival of CLP-induced sepsis mice(18-20).

TLRs are important for tissue defence against bacteria, but they can be harmful during ongoing infections. This study has shown that TLR4 and MyD88 in sepsis caused by kidney injury plays an important role in the immunological process, with the lack of any TLR4 or MyD88 genes in limiting their expression in response to mouse kidney damage, while also significantly reducing the activity of the NF-κB p65. Inhibiting the expression of TLR4...
and MyD88 can reduce the role of sepsis in renal damage, and can inhibit the activity of the NF-κB, thereby reduce the expression of TNF-α and IL-1β. Therefore, the TLR4/MyD88 NF-κB signalling pathway is likely to be an important mechanism in sepsis leading to the development of AKI.

References


Corresponding Author: Fuhai Ji
Email: tyjn2p@163.com (China)