Original Article
The role and difference of TLR2 and TLR4 in rhabdomyolysis induced acute kidney injury in mice

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Abstract: To investigate the role of toll like receptors (TLRs) 2 and 4 in rhabdomyolysis (RM)-related acute kidney injury (AKI). Wild-type (WT) mice and TLR2 knockout (TLR2^{-/-}) or TLR4 knockout (TLR4^{-/-}) mice were injected with either saline (sham) or glycerin (to induce RM-related AKI). Samples were collected for detection of 0 h 24 h (Cr) creatinine, urea nitrogen (BUN), creatine kinase (CK), and PAS staining of renal tissues. Serum Cr and BUN level was significantly increased in TLR2^{-/-} and TLR4^{-/-}AKI groups more than those in the control group and the WT mice in AKI group. TLR4^{-/-}AKI group Cr, BUN level, and the pathological damage was lightest. The expression levels of signal transduction proteins in TLR2^{-/-} and TLR4^{-/-}AKI group were higher than in the control group, but was lower than that in the wild AKI group, with the TLR4^{-/-}AKI group having the lowest levels. The expression level of inflammatory factor mRNA in TLR2^{-/-} and TLR4^{-/-}AKI groups was higher than that in control group, but was lower than that in wild AKI group, with TLR4^{-/-}AKI group displaying lowest levels. Knockout of TLRs 2 and 4 decreased kidney inflammation and improved RM-related AKI.

Keywords: Toll like receptor 4, toll like receptor 2, acute kidney injury, rhabdomyolysis, inflammation

Introduction

Rhabdomyolysis (RM) is a life-threatening syndrome resulting from the breakdown of striated muscle cell membrane in cases of muscle trauma, ischemia, inflammation, metabolic abnormalities, and systemic poisoning [1, 2]. During RM, large quantities of muscular cell constituents, which can lead to persistent renal injury and intractable inflammatory conditions, are released into the extracellular fluid and the circulation [3, 4]. Today, RM muscle cell leakage has been identified as one of the leading causes of acute kidney injury (AKI) [5]. It is reported that 13-50% of the RM patients develop AKI [6]. In the United States, 7-10% of the AKI are caused by RM [7]. These RM-related AKI patients have a mortality of approximately 20% [8, 9]. According to Sever et al. (2006), after both natural disasters (e.g. earthquakes and hurricanes) and man-made catastrophes (e.g. wars and mining accidents), crush syndrome-induced RM-related AKI, apart

from direct trauma, is the most frequent cause of death [3]. Therefore, understanding the pathogenic mechanism of RM-related AKI is important for early intervention and appropriate treatment.

In retrospect, the muscle leakage of myoglobin, an 18,800-Dalton oxygen carrier that can cause glomeruli obstruction and renal dysfunction, has been proposed as a major contributor to the development of RM-related AKI [10, 11]. Interestingly, in recent years, more and more evidence indicates that, apart from myoglobin-induced renal injury, inflammation also plays a key role in the development and progression of RM-related AKI [12-14]. After RM, the released cell constituents activate pattern recognition receptor (e.g. TOLL like receptors, TLRs) and cause immediate and significant inflammatory responses [2]. The inflammatory activation, which can cause endothelial injury, induce leukocyte entrapment and decrease microvascular blood flow. This causes damage

Table 1. Primer sequences for RT-PCR

Gene	Forward Primers (5'-3')	Reverse Primer (3'-5')
INF-β	CGTGGGAGATGTCCTCAACT	AGATCTCTGCTCGGACCACC
MIP-1α	TGCCAAGTAGCCACATCGAG	GAGATGGGGGTTGAGGAACG
IL-6	CAACGATGATGCACTTGCAGA	CAACGATGATGCACTTGCAGA
TNF-α	AGGCACTCCCCCAAAAGATG	CCCTCACACTCACAAACCAC

to both the medulla and the cortex of the kidney [15-18]. Since TLRs 2 and 4, which are constitutively expressed on renal epithelium, can recognize endogenous ligands during tissue injury and trauma [19, 20], it would be interesting to investigate the roles of TLRs 2 and 4 in the development of RM-related AKI. Indeed, studies have shown that TLRs are involved in the progress of ischemia-reperfusion injury (IRI) in heart, lung, brain, and liver [21, 22]. Chen et al. [23] reported that the TLRs contribute to renal injury during ischemic-induced AKI.

In this study, the role of TLRs 2 and 4 in RM-related AKI was investigated using developing RM-related AKI in wildtype mice as well as in mice with TLR2 or 4 knockout.

Materials and methods

All of the procedures conducted in this study were approved by the Animal Ethical Committee of Chinese PLA General Hospital (Beijing, China).

Mice model of RM-related AKI

Wildtype (WT, n = 10), TLR2 knockout (TLR2$^{-/-}$, n = 10) and TLR4 knockout (TLR4$^{-/-}$, n = 10) mice (all males, 8-week-old, with body weights of 20-25 g) were obtained from Nanjing University Model Animal Research Center (Nanjing, China). Mice from each of these three groups were equally divided into two treatments: sham and RM-related AKI. For sham mice, physiological saline was injected through thigh muscle (both side) at a dose of 8 ml/kg body weight. For RM-related AKI development, mice were injected with 50% glycerin solution (purchased from US Sigma-Aldrich Corporation, 8 ml/kg body weight) in the same way as the sham group. All of the mice were raised in a SPF grade animal house under controlled temperature (22 ± 2°C) and lighting (12:12-h light/dark) with free access to food and water during this study. Twenty-four

hours after injection, 10 mice from each treatment were randomly selected and carefully anesthetized (2% pentobarbital) and euthanized (pick eyeball blood after anesthesia) for serum isolation [blood collected via eye bleeding, centrifuged and stored at -80°C for the determination of creatinine (Cr), urea nitrogen (BUN) and creatine kinase (CK) levels] and kidney (fixed using 10% formalin for Schiff's staining) samples. Twenty-four hours after injection, 10 mice from each treatment were sampled for serum and kidney (stored at -80°C for Western Blot and RT-PCR analysis).

The evaluation of RM and AKI status

Serum levels of Cr, BUN and CK were determined by commercial ELISA kits according to manufacturer's instructions. The mice were considered as RM when the serum CK level was increased to 5 times of the WT-sham treatment. The mice were considered as RM-related AKI when the serum Cr level was increased to 1.5 times of the WT-sham treatment.

Formalin fixed kidney samples were subjected to Schiff's (periodic acid-Schiff PAS) staining. An optical microscope (Olympus Optical Company, Japan) was used to examine the stained slides and results were expressed as photograph objects. The kidney necrosis (ATN) score was determined as previously described [24].

Western blot

Western blot was conducted as previously described [25]. The primary rabbit anti-mouse TLR2, TLR4, myeloid differentiation factor 88 (MYD88, lot number), tumor necrosis factor receptor-associated factor 3 (TRAF-3, please add lot number), interferon regulatory factor (IRF-3, please add lot number), TRAF-6 (please add lot number), activating protein-1 (AP-1, please add lot number), nuclear factor kappa B (NF-KB, please add lot number) and β-actin (please add lot number) antibodies were purchased from American Abcam company (Cambridge, MA, USA). Horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (please add lot number) were purchased from Abcam company. The band density was quantified using Bandscan analysis software and then normalized to β-actin content, because the levels of β-actin did not differ between experimental groups.

Table 2. Serum levels of Cr, BUN and CK in different time points of mice with rhabdomyolysis

Mice	Treatment	Time	Cr (μmol/L)	BUN (mmol/L)	CK (U/L)
WT	N = 10	Sham	12.6 ± 0.73	8.23 ± 0.39	439.57 ± 102.74
	AKI	24 H	212.95 ± 22.15[*]	68.7 ± 1.14[*]	2615.15 ± 292.25[*]
	AKI	48 H	105.92 ± 20.01	54.27 ± 13.4	538.58 ± 127.55
TLR2^{-/-}	N = 10	Sham	13.87 ± 1.39	8.23 ± 1.61	468.3 ± 101.56
	AKI	24 H	155.49 ± 30.42[*,&]	51.57 ± 8.64[*,&]	2637.49 ± 166.59[*,&]
	AKI	48 H	28.96 ± 12.76	14.73 ± 9.24	869.44 ± 189.74
TLR4^{-/-}	N = 10	Sham	13.15 ± 1.25	8.10 ± 1.24	459.15 ± 154.35
	AKI	24 H	63.76 ± 33.01[*,&,$]	36.02 ± 8.54[*,&,$]	2564.6 ± 203.53[*,&]
	AKI	48 H	14.09 ± 2.66	8.27 ± 1.09	790.53 ± 141.93

Note: compare with WT sham, [*]$P < 0.05$; compare with WT AKI group 24 h, [&]$P < 0.05$ (Cr, BUN), [&]$P > 0.05$ (CK); compare with TLR2^{-/-}AKI group, [$]$P < 0.05$.
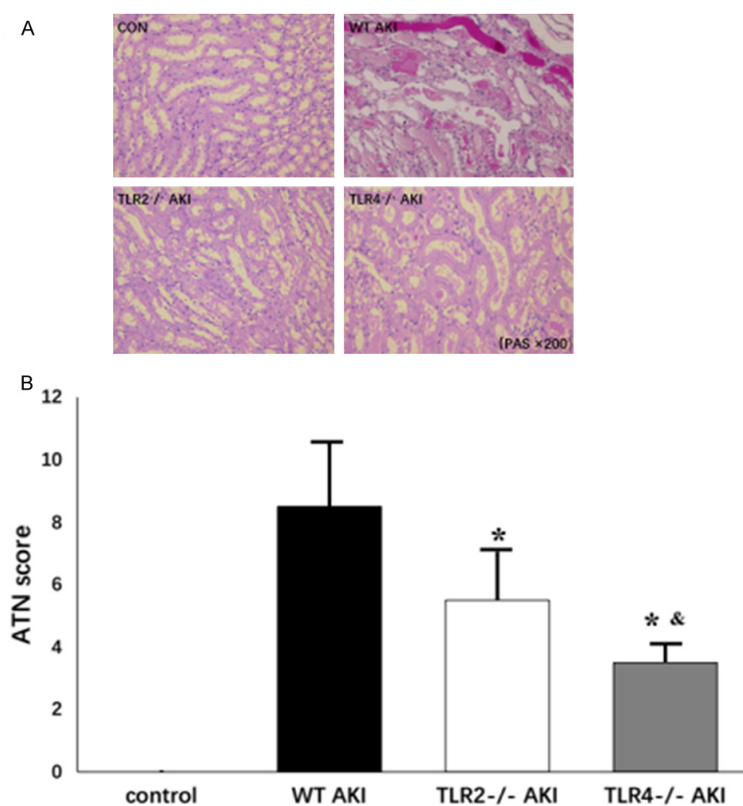
Figure 1. Schiff's staining and renal ATN score 24 h after injection. Compared with WT AKI group, *$P < 0.05$; compared with TLR2^{-/-}AKI group, &$P < 0.05$.

RT-PCR

The sequences of endogenous interferon-β (INF-β), macrophage inflammatory protein-1α (MIP-1α), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an internal reference) were obtained from Gen-Bank. Primer premier 5 software was used to design primers for RT-PCR. Primers (**Table 1**) were synthesized by Huada Gene Company (Shenzhen, Guangdong, China). RT-PCR was performed using TaqMan probes (sample was analyzed in triplicate). The relative mRNA expression was calculated using the 2[-ΔΔCT] method and the results were expressed as fold-change relative to GAPDH.

Statistical analysis

Data were analyzed using SP-SS 16 software (IBM Corp., Chicago, IL). Data are shown as mean ± standard error of the mean (SEM). Two-tailed independent Student's *t*-test was conducted to compare the differences between two groups. Significant differences were considered at $P \leq 0.05$.

Results

Establishment of RM-related AKI

Our results found that at 24 h in the WT AKI group, TLR2^{-/-}AKI group and TLR4^{-/-}AKI group, serum creatinine, urea nitrogen and creatine kinase values were significantly higher than those in the control group. Meanwhile, rhabdomyolysis and acute kidney injury were observed in mice at after 24 h injection, suggesting successful establishment of rhabdomyolysis acute renal injury model mice. The levels of
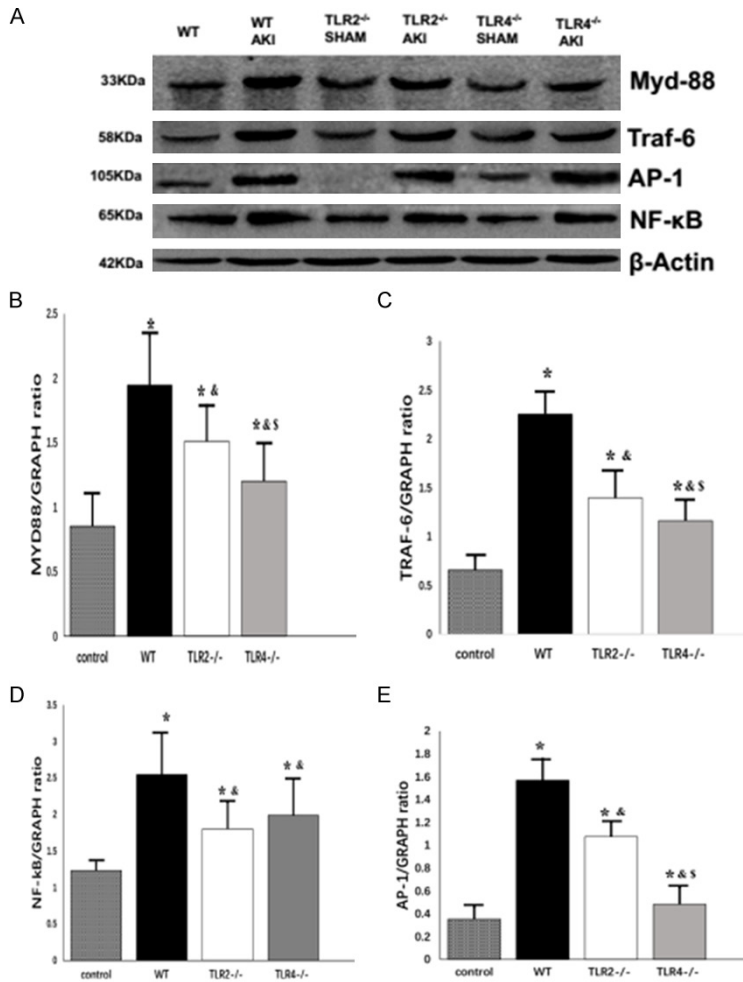
Figure 2. Renal protein expression of MYD88, TRAF-6, NF-kB and AP-1. Compared with WT SHAM group, *P < 0.05; compared with WT AKI group, &P < 0.05; compared with TLR2-/-AKI group, $P < 0.05.

consistent with no different effects on acute kidney injury induced by rhabdomyolysis degree (**Table 2**).

As shown in **Figure 1**, under light microscope, the structure of glomerular and renal tubules in WT-sham mice was intact 24 hour after saline injection. However, in WT-AKI mice, the renal tubular epithelial cells were edematous with vacuolar and granular degeneration, the brush border of the proximal renal tubule was detached, and the basement membrane of renal tubules was exposed. Compared to WT-AKI mice, the TLR2-/-AKI and TLR4-/-AKI mice had decreased epithelial cells edema, decreased vacuolar degeneration, and granular degeneration, as well as decreased basement membrane exposed. No renal ATN was scored in WT-sham mice. However, an average ATN score of 8 was observed in WT-AKI mice. The TLR2-/-AKI (lower) and TLR4-/-AKI (lowest) mice had decreased renal ATN scores compared with WT-AKI mice.

Renal protein expression of MYD88, TRAF-6, NF-kB, AP-1, TRAF-3, and IRF-3

In the study of dependence on the MyD88 channel, we found that compared with WT sham group, the TLR2-/-AKI group and TLR4-/-AKI group presented significantly increased MyD88, TRAF-6, AP-1, NF-kappa B protein expression, suggesting MyD88 dependent downstream signal transduction protein expression was increased. TLR2 and TLR4 are inflammatory signal transduction mediators through their MYD88 dependent pathways and might be involved in skeletal muscle during the dissolution of acute kidney injury. However, compared with the WT AKI group, the TLR2-/-AKI and TLR4-/-AKI groups displayed significantly decreased MYD88, TRAF-6, AP-1, and NF-kappa B protein levels, indicating that TLR2 and

serum creatinine and urea nitrogen in mice from TLR2-/-AKI group and TLR4-/-AKI group were significantly lower than those in group WT and group 24 h, indicating that the degree of acute kidney injury in the TLR2-/-AKI group was lower than that in WT AKI group, with the TLR4-/-AKI group displaying lower kidney injury compared with TLR2-/-AKI group at 24 h. The level of serum creatinine and urea nitrogen in TLR4-/-AKI group was significantly lower than that in TLR2-/-AKI group, indicating that the degree of 24 h acute renal injury in TLR4-/-AKI group was the lightest among these groups. However, WT AKI group, TLR2-/-AKI group, and TLR4-/-AKI group mice in 24 h displayed no significant difference of serum creatine kinase in the degree of injury to the three groups of striated muscle, rhabdomyolysis,
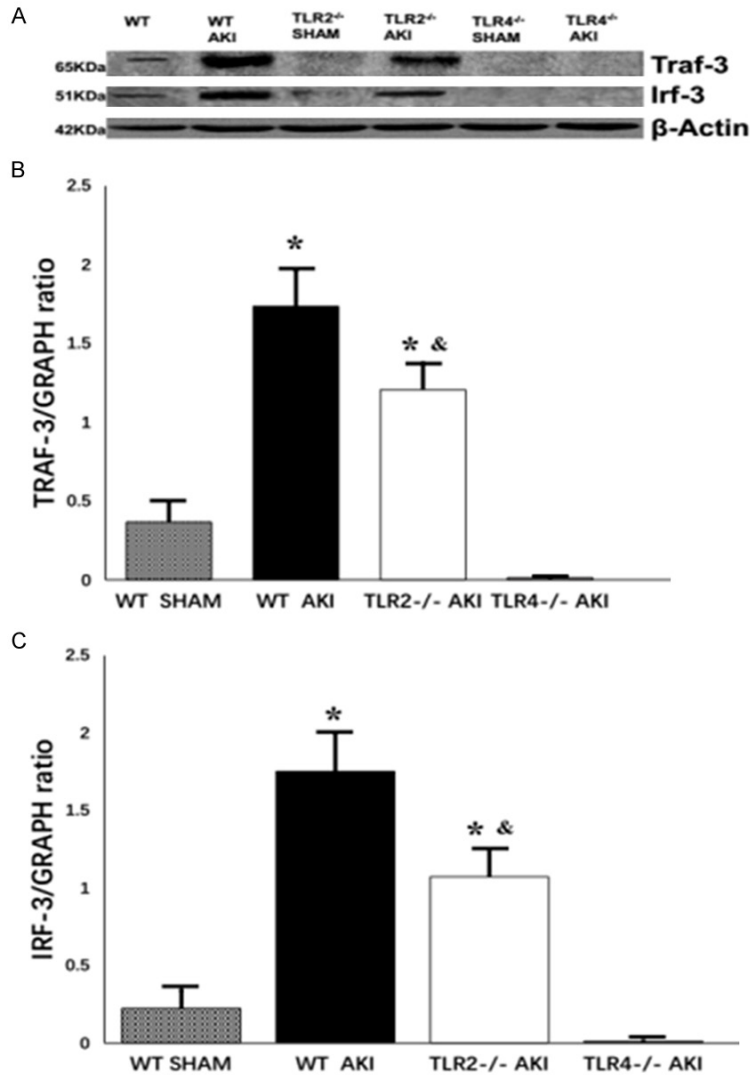
Figure 3. Renal protein expression of TRAF-3 and IRF-3. Compared with WT SHAM group, *P < 0.05; compared with WT AKI group, &P < 0.05.

pathway. Compared with the WT AKI group, expression levels of TRAF-3 and IRF-3 in TLR2-/-AKI group were decreased, indicating that the expression level of proteins downstream of IRF-3 signaling pathway are down-regulated after knockdown of TLR2 (**Figure 3**).
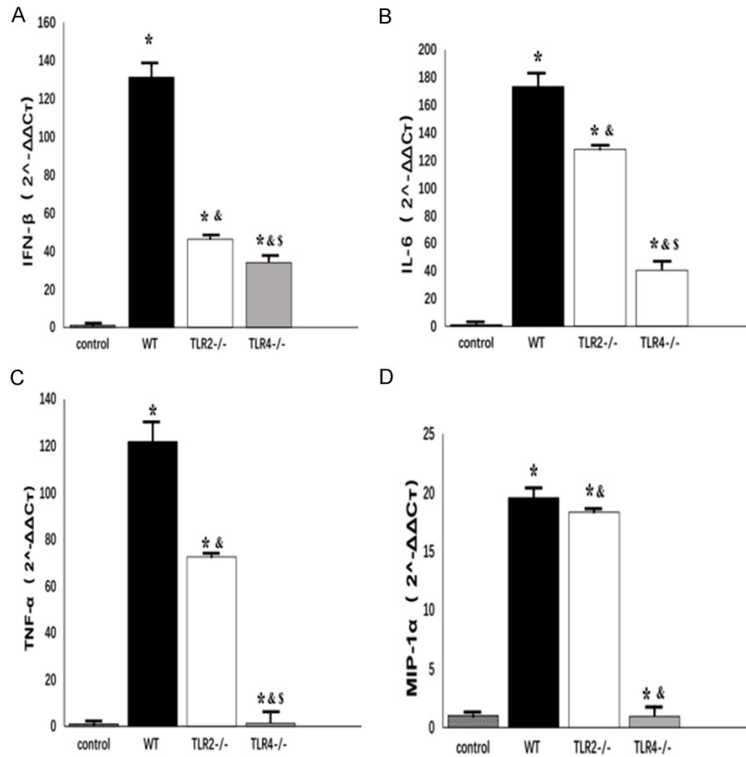
Renal mRNA expression of INF-β, MIP-1α, IL-6 and TNF-α

Analysis of the expression of inflammatory factors found that compared with WT sham group, the WT AKI group and TLR2-/-AKI group showed significantly increased IL-6, inflammatory factor IFN-β MIP-1 and TNF-alpha mRNA levels and the TLR4-/-AKI group displayed significantly increased IFN-β and IL-6 levels, indicating TLR2 and TLR4 are involved in the injury mediated by rhabdomyolysis after. Compared with the WT AKI group, the TLR2-/-AKI and TLR4-/-AKI groups, presented significantly reduced IL-6, inflammatory factor IFN-β, MIP-1α and TNF-α mRNA expression levels, indicating knockdown of TLR2 or TLR4 could downregulate the secretions of inflammatory factors, reduced the degree of inflammation of kidney. Compared with TLR2-/- AKI group, the expression levels of IFN-β, IL-6, MIP-1, TNF-α in TLR4-/- and AKI groups were significantly lower, indicating that TLR4 had a stronger effect on the inhibition of the expression of inflammatory cytokines than TLR2 (**Figure 4**).

Discussion

In this study, RM-related AKI was successfully developed in WT, TLR2-/- and TLR4-/- mice 24 hours after glycerin injection. However, while all the mice experienced the same level of RM (using serum CK level as an indicator), TLR2-/- and TLR4-/- mice had improved AKI status (us-

TLR4 might be involved in the signal transduction pathway of MYD88 dependent inflammation. Interestingly, compared with the TLR2-/-AKI group, expression of MYD88, TRAF-6, and AP-1 expression in TLR4-/-AKI group was significantly decreased (**Figure 2**).

In a study of IRF-3 dependent channels we found that, compared with the WT sham group, the WT AKI group and TLR2-/-AKI group showed significantly increased TRAF-3 and IRF-3 protein expression. However, no expression of TRAF-3 and IRF-3 was observed in the TLR4-/- AKI group, suggesting that TLR4 might transduce inflammatory signals through IRF-3, while TLR2 does not depend on the IRF-3 signaling

Figure 4. Renal mRNA expression of INF-β, IL-6, TNF-α and MIP-1α. Compared with WT SHAM group, *P < 0.05; compared with WT AKI group, &P < 0.05; compared with TLR2$^{-/-}$AKI group, $P < 0.05.

membrane explosion, and kidney ATN score) compared with TLR2$^{-/-}$-AKI mice. These results suggest that TLR2 and TLR4 play different roles in RM-induced inflammatory response. Basically, TLR signaling pathway can be divided into two sub-pathways: MYD88-dependent (both TLR2 and TLR4) and MYD88-independent (TLR4 but not TLR2). In the MYD88-dependent pathway, TLRs-induced MyD88 dimerization and interleukin-1 receptor-associated kinase (IRAK) activation initiates recruitment of TRAF-6 and TRAF-3 [27]. TRAF-6 activates transcription factors like NF-kB and AP-1. TRAF-3 is instrumental for the recruitment of TRAF family member-associated-binding kinase 1 (TBK-1) and the production of type I IFNs and IL-10. In the MYD88-independent pathway, TLR 3 and 4 can activate transcription factor IRF-3 and thereafter induce the produc-

ing serum Cr as an indicator) when compared to WT mice. Similarly, Schiff's staining and renal ATN scores indicate that, at the same time (24 hours after glycerin injection), WT mice experienced more severe kidney injury compared with TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. These results clearly demonstrate that TLR 2 and 4 contribute to the development and progression of glycerin-induced RM-related AKI. In recent years, RM-induced inflammation response has been proposed as a major cause of AKI [10, 13]. Since TLR2 and TLR4 are key pattern recognition receptors for endogenous ligands recognition [15, 16], the current results were as expected. In a study conducted by Rusai et al. (2010), knockout of TLR2 or/and TLR4 could protect mice from renal ischemia/reperfusion injury which was basically characterized as inflammation and cell death [26].

Of particular interest in this study, is that within the TLR knockout AKI groups, TLR4$^{-/-}$-AKI mice had improved AKI status (indicated by serum Cr level, epithelial cell edema, vacuolar and granular degeneration, tubular basement

tion of IFN-β [28]. In this study, glycerin injection increased the renal expression of proteins (MYD88, TRAF-6, NF-kB, and AP-1) in the MYD88-dependent pathway in WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. These results suggest significant inflammatory responses were triggered in the kidney after the development of RM. Interestingly, compared with WT-AKI mice, renal expression of MYD88-dependent pathway proteins were decreased in both TLR2$^{-/-}$-AKI and TLR4$^{-/-}$-AKI mice. Thus, it would be safe to hypothesize that knockout of TLR 2 and 4 reduce RM-induced renal inflammatory response and therefore alleviate RM-related AKI. In addition, TLR4$^{-/-}$-AKI mice had lower levels of MYD88 pathway protein (MYD88, TRAF-6 and AP-1) expression compared with TLR2$^{-/-}$-AKI mice, suggesting TLR4 plays major roles in the RM-induced MYD88-dependent kidney inflammation. As expected, glycerin-induced RM-related AKI also increased the renal expressions of TRAF-3 and IRF-3 in WT and TLR2$^{-/-}$ but not TLR4$^{-/-}$ mice. These results are consistent with the current understanding that TLR4 plays key roles in the recruitment and/or activa-

tion of TRAF-3 and IRF-3 [28]. Interestingly, TLR2$^{-/-}$-AKI mice had decreased renal TRAF-3 and IRF-3 expression compared with WT-AKI mice, indicating that TLR2 also participates in the development of RM-related AKI through MYD88-independent pathway. In the literature, the MYD88-independent pathway (or the IRF-3 pathway) has been proposed as unique to TLR 3 and 4 [28]. However, similar to our results, Shigeoka et al. (2007) reported that TLR2 participated in ischemic renal injury through both MyD88-dependent and -independent pathways [29]. Future investigation is required to confirm the roles of TLR2 in the MYD88-independent pathway.

Glycerin injection up-regulated mRNAs expression of INF-β, IL-6, TNF-α and MIP-1α in the kidney of both WT and TLR2$^{-/-}$-mice. However, in TLR4$^{-/-}$-mice, glycerin induced increased INF-β and IL-6 expression and decreased TNF-α and MIP-1α expression.

In summary, TLR 4 and 2 involve in the renal inflammatory response in the development of glycerin-induced RM-related AKI through MYD88-dependent and -independent pathways. Targeting TLR4 and TLR2 may provide novel therapies for the treatment of RM-related AKI.

Acknowledgements

Disclosure of conflict of interest

None.

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