Sleep and Breathing (2019) 23:503–513
https://doi.org/10.1007/s11325-018-1704-9

SLEEP BREATHING PHYSIOLOGY AND DISORDERS • ORIGINAL ARTICLE

Toll-like receptor-4 deficiency alleviates chronic intermittent hypoxia-induced renal injury, inflammation, and fibrosis

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Received: 22 March 2018 / Revised: 16 July 2018 / Accepted: 30 July 2018 / Published online: 11 August 2018

Abstract

Background Obstructive sleep apnea (OSA)-associated chronic kidney disease is mainly caused by chronic intermittent hypoxia (CIH) triggered renal damage. This study aims to investigate the role of toll-like receptor-4 (TLR4) in underlying mechanism involved chronic intermittent hypoxia (CIH)-induced renal damage.

Methods C57BL/6J mice with normal TLR4 (TLR4 WT) or deficient TLR4 (TLR4 KO) were divided into four groups and exposed to normal air (NA) and CIH: TLR4 WT + NA, TLR4 KO + NA, TLR4 WT + CIH, and TLR4 KO + CIH. CIH lasted for 8 h/day and 7 days/week for 6 weeks. Renal injury and inflammation were evaluated by histology and ELISA. Renal tubular apoptosis, macrophages, and fibroblasts recruitment were determined by TUNEL assay, immunofluorescence, and western blot.

Results In response to CIH, TLR4 deficiency alleviated renal histological injury, renal dysfunction, and fibrosis. TLR4 deficiency ameliorated renal dysfunction (serum BUN and creatinine) and tubular endothelial apoptosis determined by immunofluorescence staining of CD31 and TUNEL, and western blot of apoptotic protein (caspase-3, c-caspase-3, and Bax/Bcl-2 ratio). Furthermore, we also found TLR4 deficiency abrogated CIH-induced macrophages (CD68) and fibroblasts (α-SMA) recruitment, further reducing expression of extra-cellular matrix protein (collagen I and collagen IV) and inflammatory cytokines release (IL-6, TNF-α, and MCP-1). Finally, we used immunohistochemistry to demonstrate that TLR4 deficiency attenuated increased expression of MyD88 and NF-kB p65 after CIH treatment.

Conclusions Our data suggest that TLR4 plays a vital role in CIH-induced renal injury, inflammation and fibrosis, and inhibition of TLR4 probably provides a therapeutic potential for CIH-induced kidney damage.

Keywords Obstructive sleep apnea · Toll-like receptor 4 · Chronic intermittent hypoxia · Renal damage

Introduction

Obstructive sleep apnea and hypoxia syndrome (OSAHS) is a disease characterized by repetitive upper airway collapse and recurrent hypoxia during sleep, which trigger a subthreshold wake-up reaction due to a temporary shortage of oxygen to the organs.

Emerging evidence indicates that chronic kidney disease (CKD) is highly a prevalent complication in untreated OSAHS patients with symptoms of polyuria and proteinuria [1, 2]. Meanwhile, the prevalence of OSAHS in CKD patients ranges several fold higher than the general population [3]. The underlying mechanism of association between OSAHS and chronic renal injury remains unclear. Chronic intermittent hypoxia (CIH), the most characteristic pathophysiological change of OSAHS, often causes inflammatory processes and oxidative stress, contributing to damage of various tissues and organs [4].

Toll-like receptor-4 (TLR4), a type-I transmembrane receptor protein that plays a crucial role in innate and adaptive immune response. One of the most important molecular pathways is through the main adaptor protein, myeloid differentiation factor 88 (MyD88). The ligation of TLR4 ligands results in the recruitment of MyD88 and subsequently leads to activation of NF-kB and MAPK, which drive the expression of pro-inflammatory genes. Studies have demonstrated TLR4 activation in ischemia/reperfusion injury and hemorrhagic shock which are associated with oxidative stress [5, 6]. Our previous studies have found that TLR4 played a protective

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role in LPS and ventilator-induced acute lung injury [7–9]. The pathology and mechanism of repetitive short cycles of desaturation followed by rapid reoxygenation is somewhat analogous to ischemia/reperfusion (I/R). Studies have shown that intermittent hypoxia can promote the expression of TLR4 in the heart and hypothalamus, and atorvastatin can attenuate CIH-induced myocardial remodeling and hippocampal neuronal damage by inhibiting TLR4 and its downstream pathways [10, 11]. In various mouse models of chronic kidney injury, the mutant non-functional TLR4 or TLR4 knock-out protects against kidney dysfunction, inflammatory injury, and fibrosis [12–14]. Interestingly, monocytes from OSAHS patients represent a significant increase in TLR4 surface expression [15]. The role of monocytes/macrophages in the development of tissue fibrosis has been increasingly recognized [16]. There is vast conclusive evidence that the accumulation of macrophages is correlated with renal dysfunction in different fibrosis models [17, 18]. In addition, myofibroblasts are associated with fibrosis in several models of renal injury and are known to express α-smooth muscle actin (α-SMA) abundantly [19]. Therefore, we hypothesized that the TLR4 signaling pathways might be involved in CIH-induced renal injury and macrophage-mediated fibrosis and inflammation. The purpose of our study was to determine (1) the role of TLR4 in CIH-induced renal injury, (2) whether TLR4 deficiency blunts CIH-induced renal fibrosis, and (3) whether TLR4 deficiency abrogates macrophages recruitment-mediated inflammatory response and fibrosis.

Materials and methods

Animal model

TLR4 wild-type C57BL/6J mice ($n = 20$, 6–8 weeks) and TLR4 knockout mice ($n = 20$, 6–8 weeks) were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were male and had B6 background. These animals were housed in departmental animal chambers and were fed standard chow, tap water, and libitum in day-night quarters at 20–25 °C. The animal protocol was approved by the Animal Care Committee of Xiangya Hospital, Central South University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All effects were made to minimize animal suffering. Normal TLR4 were randomly divided into the following two experimental groups of ten animals each: the normal air (TLR4 WT + NA) group and the chronic intermittent hypoxia (TLR4 WT + CIH) group. Another 20 TLR4 KO mice were randomly divided into normal air (TLR4 KO + NA) group, and intermittent hypoxia (TLR4 KO + CIH) group. Mice with CIH treatment were placed in two identical designed chambers. Nitrogen (100%) was delivered to the chambers for 45 s to reduce the ambient fraction of inspired oxygen to 6–8% for 20–25 s. Then, oxygen was infused for 20 s so that the oxygen concentration returned to 20~21% for 35 s. This complete cycle took about 2 min, 8 h/day for 7 days/week for 6 weeks. The oxygen concentration was measured automatically using an oxygen analyzer (Jian He Electronic Company, Foshan, China). At the end of CIH exposure, all the mice were euthanized 15 h after the last hypoxia circle. Their blood and kidneys were collected. Blood samples were obtained from the right atrium. Renal function was calculated as serum creatinine and blood urea nitrogen (BUN), which were measured in the clinical laboratory of Xiangya Hospital (Changsha, China).

Kidney histology

Both kidneys of each animal were perfused with saline solution through the abdominal aorta until they were free of blood. Kidneys were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded specimens were cut into 4-um-thick sections and stained with H&E staining and then observed under a light microscope to make precise pathological diagnosis by a blinded scoring method. Renal injury was examined by the modified 0–5 Jablonski grading scale: 0 represents normal; 1 represents occasional degeneration and necrosis of individual cells; 2 represents degenerative cells and necrosis of individual tubules; 3 represents degeneration and necrosis of all cells in adjacent proximal convoluted tubules with survival of surrounding tubules; 4 represents necrosis confined to the distal third of the proximal convoluted tubules, with a band of necrosis extending across the inner cortex; and 5 represents necrosis affecting all the three segments of the proximal convoluted tubules, as described previously [20]. Interstitial fibrosis was measured by Masson- and Sirius red-stained paraffin sections.

Immunofluorescence staining

For immunofluorescence staining, 4-um paraffin sections were dewaxed in xylene and dehydrated in a graded ethanol series. Antigen was retrieved by microwave-citrate buffer antigen retrieval method and blocked with 3% BSA and 0.1% Triton-100, then incubate with primary antibody overnight at 4 °C. The following primary antibodies were used: mouse anti-CD31 antibody (1:100, Servicebio, China), which has been viewed as a specific marker of endothelial cells [21], rabbit anti-SMA (1:200, CST, USA), mouse anti-CD68 (1:100, Abcam, UK). The following secondary antibodies were used: Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:300, Servicebio, China) and Alexa Fluor-488-conjugated goat anti-rabbit IgG (1:400, Servicebio, China). To visualize apoptotic changes during CIH-induced apoptosis of renal endothelial cells, TUNEL staining was carried out using a Roche Situ Cell Death Detection Kit based on

immunostaining of endothelial cells marker (CD31+). The mean intensity immunostaining was calculated by imageJ software. TUNEL-positive (TUNEL+) and DAPI positive (DAPI+) cells were counted at × 200 magnification with a fluorescence microscopy, respectively. The number of apoptotic cells was calculated as TUNEL+/DAPI+ cells in random ten fields per section for quantification.

Immunohistochemistry

For immunohistochemistry, 4-um paraffin sections were dewaxed in xylene and dehydrated in a graded ethanol series. The two-step biotin staining kit (Zhong Shan Golden Bridge, China) was used for detection of MyD88 and NF-kB p65 expressions after antigen retrieval. Positive cells were stained yellow after DAB reaction. Rabbit anti-MyD88 and anti-NF-kB p65 antibody (Santa Cruz, USA) were used in these experiments.

Western blot

Total proteins were extracted from animal kidney tissues by homogenization in cell lysis buffer containing a mixture of protease and phosphatase inhibitors, and then centrifuged at 13,000*g* for 20 min at 4 °C. The proteins (30 μg) were separated with SDS-PAGE on 10% gels and transferred to polyvinylidene fluoride membrane (PVDF). The membranes were incubated with appropriate primary antibodies diluted in blocking solution overnight at 4 °C and corresponding secondary antibody for 2 h at room temperature. The primary antibodies and the dilutions were as follows: collagen I, collagen IV, and CD68(1:1000, Abcam, UK), and anti-α-SMA, anti-Caspase 3, anti-Caspase3 cleaved, anti-caspase 9, anti-Bax and anti-Bcl-2, anti-GAPDH, anti-β-actin, anti-tubulin (1:1000,CST, USA), and the secondary goat anti-rabbit and goat anti-mouse immunoglobulin G (1:5000, Invitrogen, USA). The band intensities were quantified using ImageJ software (NIH).

Enzyme-linked immunosorbent assay

Mouse serum was isolated from the blood after centrifugation at 14000 rpm for 20 min at 4 °C. After centrifugation, serum was frozen at − 80 °C until enzyme-linked immunosorbent assay (ELISA) analyses were performed. The levels of inflammatory mediators (TNF-α, IL-6, MCP-1 from RayBiotech, USA) in the serum samples were measured in triplicate according to the manufacturer's instructions.

Statistics

Data were presented as mean ± SEM and analyzed by Graphpad Prism (7th edition). The comparisons between multiple groups were determined using ANOVA for parametric data and Kruskal-Wallis test for nonparametric data. The differences between two groups were determined by *t* test for parametric data and Mann-Whitney Rank Sum Test for nonparametric data. A p value < 0.05 was considered statistically significant for all analysis.

Results

TLR4 deficiency blunts the effect of CIH-induced renal dysfunction and histological damage

Examination of renal histological damage was stained with hematoxylin-eosin (HE) staining; NA group mice showed almost normal glomerular and tubular structures, while CIH resulted in prominent tubular atrophy, tubular degeneration, tubular necrosis and cast formation, and tubulointerstitial inflammatory cell infiltration. However, TLR4 deficiency mice with CIH displayed less extensive features of tubule epithelial swelling, tubular atrophy, and tubular lumens without significant changes in distal convoluted tubule (Fig. 1a). Injury score was further evaluated by the modified 0–5 Jablonski grading scale and presented by bar graph (Fig. 1b). Consistent with histological damage, the serum BUN as well as creatinine levels were significantly elevated in the TLR4 WT + CIH group, but TLR deficiency significantly attenuated the increased BUN and creatinine under CIH treatment (Fig. 1c, d). These results demonstrated that TLR4 deficiency attenuated CIH-induced renal histopathological damage and serum parameters of renal function.

TLR4 deficiency attenuates CIH-induced renal tubular endothelial cell apoptosis

Presently, the renal tubular endothelial cell injury has been recognized as a critical factor in the pathogenesis of several chronic kidney diseases (CKD). In order to demonstrate the effect of CIH on renal tubular endothelial cells, we performed immunofluorescence of CD31 and TUNEL staining to evaluate apoptotic rate of the renal tubular endothelial cells. DAPI was used to visualize cell nuclei, thus merged immunofluorescent TUNEL/DAPI staining depicted the proportion of apoptosis (Fig. 2a). In normal kidneys, CD31+ cells clearly stained in the wall of renal proximal and distal tubules did not express TUNEL+ cells. In contrast, CIH significantly reduced CD31 positive expression in renal tubular endothelial cells and peritubular capillary endothelium, indicating that chronic intermittent hypoxia caused severe endothelial injury. In addition, TUNEL+ cells were widely increased in the CIH group. Colocalization of CD31/TUNEL immunofluorescent staining depicted apoptosis of endothelial cells and the percentage of apoptotic endothelial cells was significantly

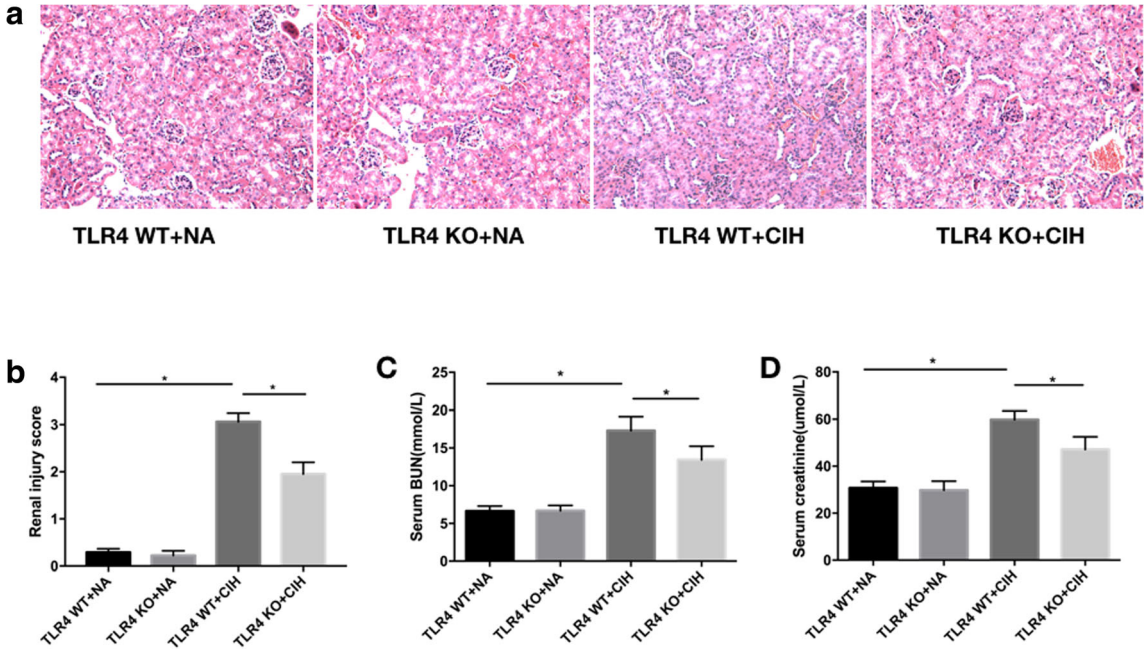

Fig 1 TLR4 deficiency alleviates CIH-induced histological damage and renal dysfunction. Representative kidney sections are stained by H&E. In light microscopic examination, tubular degeneration, tubular necrosis and cast formation, and tubulointerstitial inflammation, and tubular dilations with flattened epithelium are more remarkable in the kidney tissues of the TLR4 WT + CIH group compared to the TLR4 WT + NA group, while TLR4 KO + CIH apparently shows mild dilatation of tubular lumen absence of severe inflammatory infiltrations (**a**, magnification × 200). Sections are graded based on the 0–5 Jablonski grading scale to average the values from ten fields per kidney under microscopy (**b**). Renal function is measured by serum creatinine (**c**) and BUN (**d**). Data are presented as the mean ± SEM. *$p < 0.05$, $n = 10$

increased following CIH exposure. However, TLR4 deficiency showed specific costaining for CD31 and lessened TUNEL+ cells in the merged picture compared with wild mice, indicating that TLR4 deficiency ameliorated CIH-induced endothelial injury (Fig. 2b). Furthermore, the endothelial cell apoptosis is reflected by apoptotic proteins and the balance between Bax and Bcl-2 proteins. In contrast to NA group, CIH upregulated expression of the proapoptotic protein Bax, whereas anti-apoptotic protein Bcl-2 was significantly decreased (Fig. 2c). Our histological results were further supported by Bax/Bcl-2 protein ratio (Fig. 2e), suggesting that CIH-promoted propensity to apoptosis and TLR deficiency alleviated the CIH-induced apoptosis. In addition, CIH increased renal caspase-3 and cleaved caspase-3(c-caspase-3) protein expression in WT mice, which were attenuated by TLR4 deficiency (Fig. 2d, f–g). These data demonstrated that tubular endothelial cell apoptosis played a crucial role in CIH-induced renal injury and TLR4 deficiency alleviated the renal endothelial cell damage.

TLR4 deficiency suppresses CIH-induced renal fibrosis

We also detected the renal fibrosis by Masson and Sirius staining. As shown in Fig. 3, in NA treatment groups, little collagen expression is observed by Masson staining; in the CIH group with wild-type TLR4, strong collagen expression occurred in renal tissue; however, expression intensity was attenuated in CIH with TLR4 deficiency group (Fig. 3a). The same result was observed in renal sections subjected to Sirius red staining and illuminated with polarized light, which indicated that the CIH group presented an increased degree of fibrosis compared with the NA group as well. Notably, the TLR4 deficiency during CIH process showed a significant reduction in the expansion of extracellular matrix proteins (Fig. 3b). We investigated the deposition of extracellular matrix (ECM) protein expression, collagen I and collagen IV. There was no difference in the expression of these ECM proteins in normal air treated mice with or without normal TLR4. However, these ECM proteins significantly increased in mice with wild-type TLR4 in response to CIH treatment compared to NA groups and TLR4 deficiency receiving CIH; the levels were much lower than that observed in mice with wild-type TLR4 with the CIH group (Fig. 3c–e).

TLR4 deficiency blunts CIH-induced macrophages and fibroblasts accumulation and inflammatory cytokines release

Inflammation initiates and sustains renal tubulointerstitial fibrosis; therefore, we detected colocalization of pro-inflammatory macrophages and fibroblasts. We examined immunofluorescence of CD68, a representative marker pro-inflammatory macrophage phenotype (M1), and α-SMA,

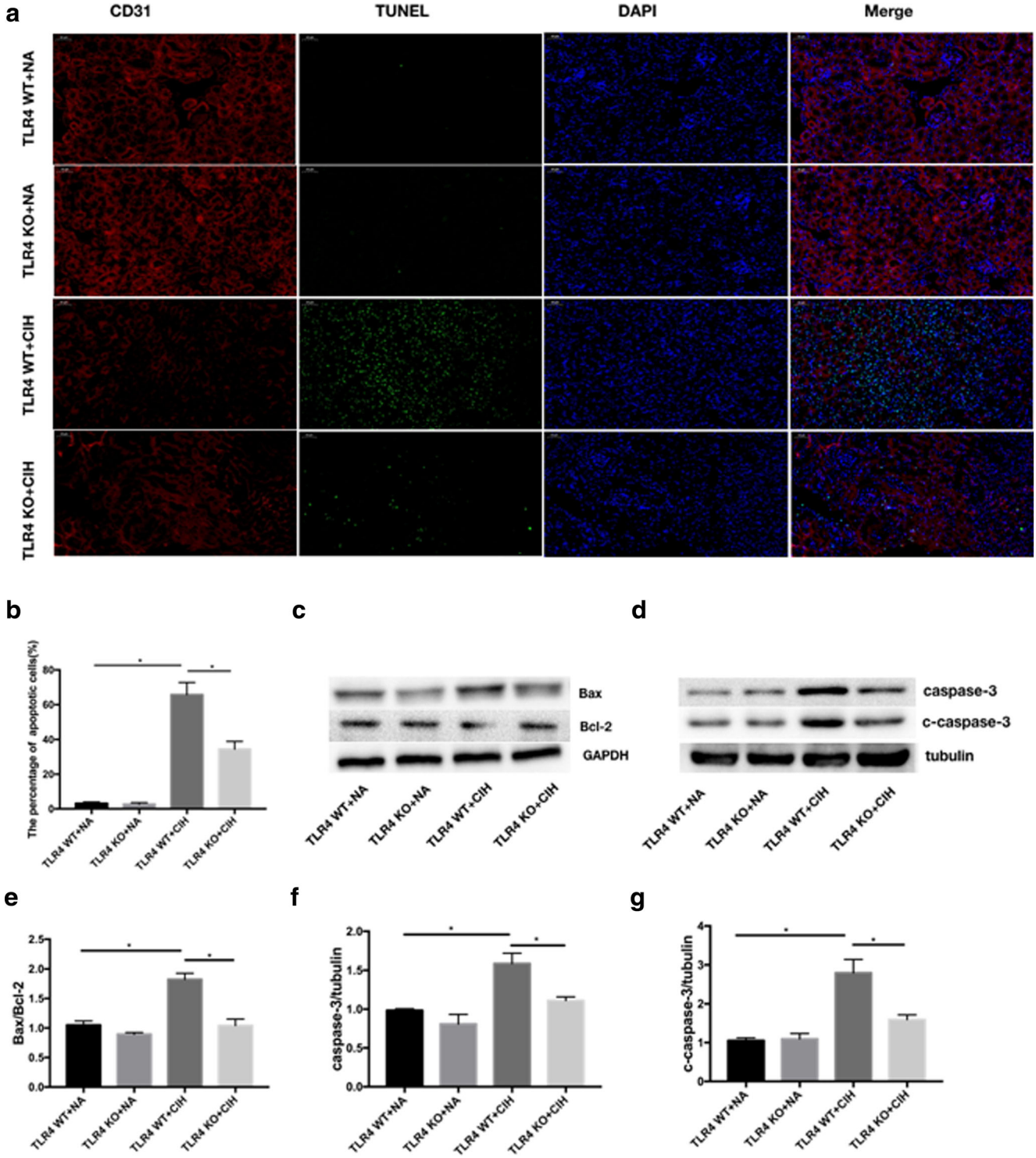

Fig 2 TLR4 deficiency alleviates CIH-induced renal tubular endothelial cell apoptosis. Representative immunofluorescence staining for CD31 (red), TUNEL (green), DAPI (blue), and the merged pictures from kidney tissues of four groups (**a**, magnification × 200, scale bars = 50 μm). Quantitative assessment of the percentage of apoptosis by counting the TUNEL+/DAPI+ cells in ten random fields (× 200) for each section (**b**). Western blot analysis of Bax and Bcl-2 protein expression in comparison with a loading control of GAPDH (**c**). Western blot analysis of caspase-3 and c-caspase-3 protein expression with the control of tubulin (**d**). Representative bar diagram showing quantitative relative ratio of Bax and Bcl-2 expression (**e**). Representative bar diagram showing quantitative relative levels of caspase-3 and c-caspase-3 in each group (**f**–**g**). Data are presented as the mean ± SEM, $*p < 0.05$, $n = 10$

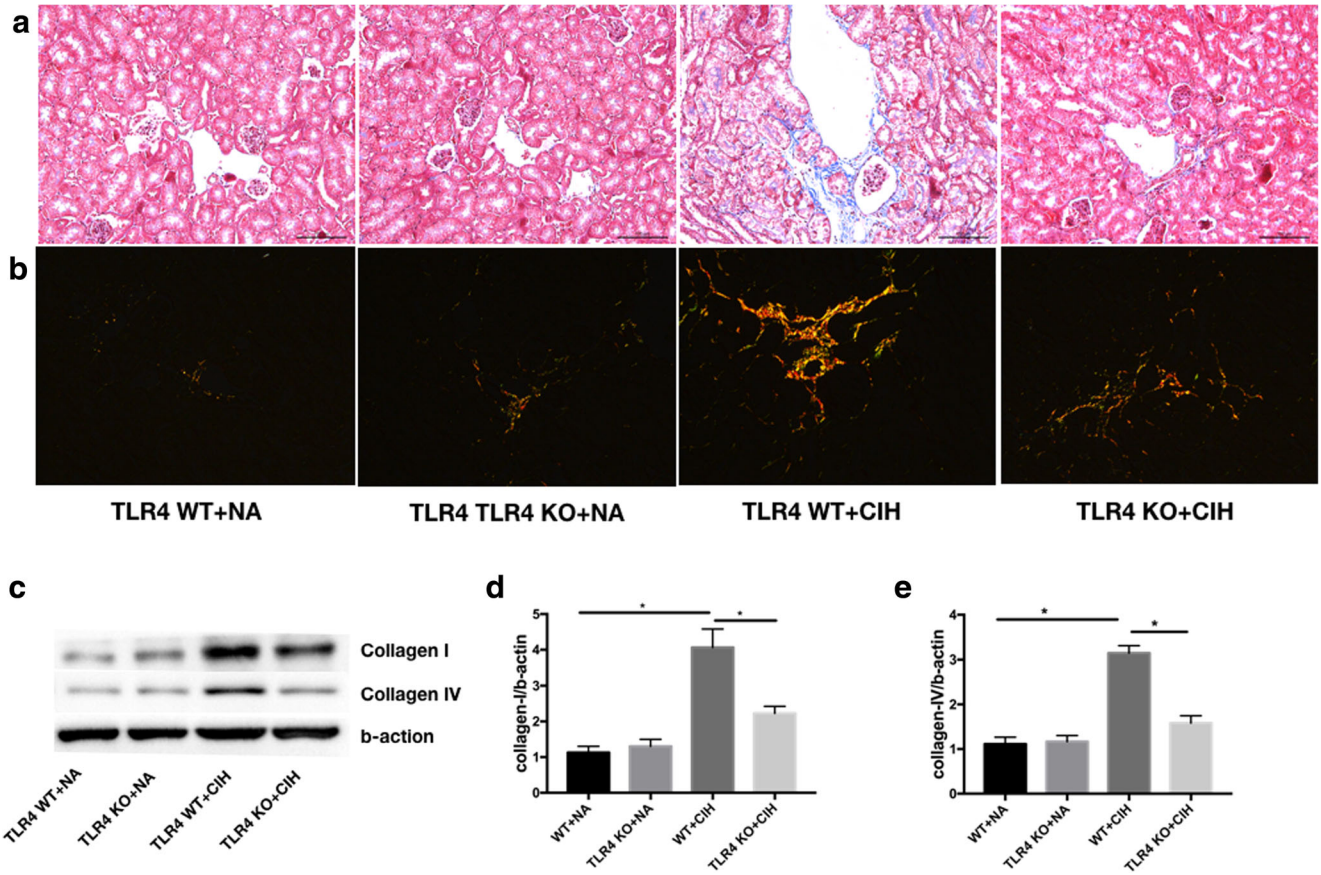

Fig 3 TLR4 deficiency attenuates CIH-induced renal fibrosis. Representative photomicrographs of Masson's staining of kidney sections from each group. Fibrosis and fibrotic areas are depicted in the renal cortical sections (**a**, magnification, × 200, scale bar = 100 μm). Representative images for Sirius red staining illuminated with polarized light to illustrate areas of fibrosis in kidneys of chronic intermittent hypoxia (**b**, magnification, × 200). Representative immunoblot images for collagen I and collagen IV and quantified with ImageJ software (**c**–**e**). Data are presented as the mean ± SEM, $*p < 0.05$, $n = 10$

indicative of cells responsible for extracellular matrix (ECM) protein accumulation. There was no difference in the expression of α-SMA and CD68 in wide mice and TLR4 deficiency mice with normal air. In contrast, mice with wild TLR4 receiving CIH treatment showed increased CD68 and α-SMA in the tubular areas near renal glomeruli and vascular predominantly. However, compared to WT mice, TLR4 deficiency mice treated with CIH significantly decreased these expression of CD68 and α-SMA (Fig. 4a). CD68 and α-SMA expression were also confirmed by western blot (Fig. 4b–d). Furthermore, we also investigated the production of some pro-inflammatory chemokines and cytokines, which were associated with classical activation of pro-inflammatory M1 phenotype. Macrophages can release several inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, and MCP-1. The enhanced expression of these cytokines and chemokines was observed in renal tissues and serum during CIH and alleviated by TLR4 deficiency (Fig. 5). All these results showed that CIH significantly increased inflammatory macrophages infiltration in the kidney, which might result in renal inflammatory responses and interstitial fibrosis.

TLR4 deficiency protects CIH-induced renal damage through inhibiting MyD88 and NF-kB p65 activation

In order to investigate the potential mechanism of CIH-induced inflammatory response and macrophages activation, we measured the classical TLR4-mediated MyD88/NF-kB activation by immunohistochemistry and found that CIH significantly increased MyD88 and NF-kB p65 expression in the injured kidney and TLR4 deficiency decreased their expression after CIH treatment (Fig. 6).

Discussion

In this study, we illustrated the following: chronic intermittent hypoxia causes renal injury, inflammation, and fibrosis; TLR4 plays a vital role during the process of CIH induced renal chronic damage and TLR4 deficient mice attenuates the proceeding renal dysfunction. Furthermore, it is the first evidence that TLR4 deficiency demonstrated its anti-inflammatory and

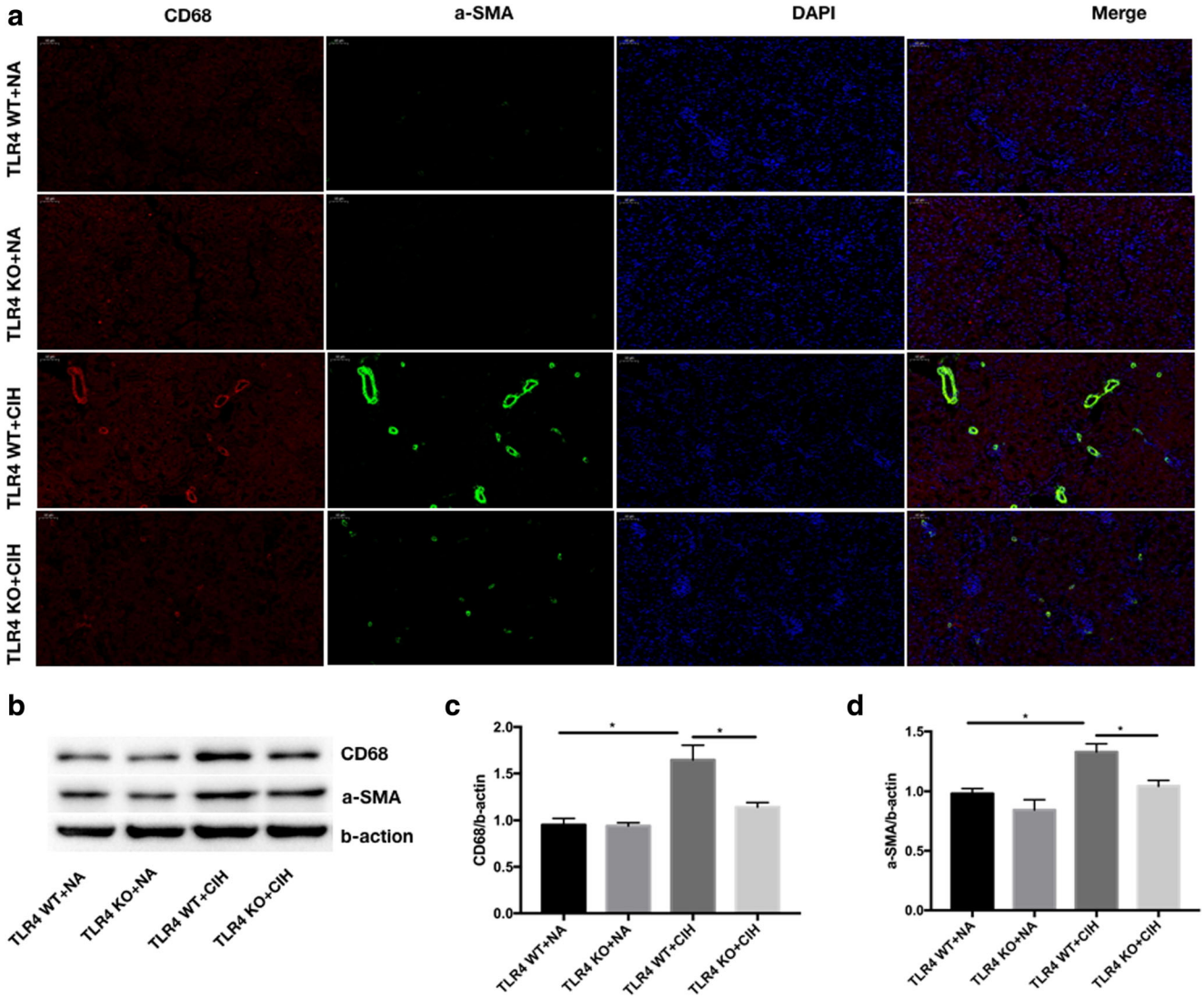

Fig 4 TLR4 deficiency decreases CIH-induced macrophages and fibroblasts recruitment. Immunofluorescence image for CD68 (red), a marker for inflammatory macrophage and α-SMA (green), a fibroblast marker (**a**, magnification × 200, scale bar 50 μm). Representative western blot images for CD68 and α-SMA and quantified with ImageJ software (**b**–**d**). Data are presented as the means ± SEM, $*p < 0.05$, $n = 10$

anti-fibrosis effects by altering macrophages and fibroblasts accumulation in CIH mouse model.

CKD develops significantly more often in patients with OSA. CKD frequency also increases with the severity of OSA and eliminating OSA will improve the prognosis of CKD patients [22–24]. Accumulating evidences suggest that OSA might contribute to renal disease through vascular dysfunction and inflammation, oxidative stress, and TLR4 signaling may mediate both oxidative stress and inflammation [5, 25]. Studies have also demonstrated that TLR4 deficiency protects chronic kidney injury by reducing kidney dysfunction, inflammation, and fibrosis [12–14], thus potentially representing a novel molecular target for OSA-associated chronic kidney disease. Therefore, to test the hypothesis of this study, we successfully established a mouse model mimicking chronic intermittent hypoxia process in OSA patients. We chose mice with wild TLR4 and deficient TLR4 to study the role of TLR4 in CIH-induced renal injury and remodeling. The histological examination and renal dysfunction confirmed that our CIH protocol was sufficient to trigger renal damage, and TLR4 deficiency mice showed blunted response to CIH-induced renal injury compared to mice with normal TLR4. It is well known that TLR4 control these innate responses through a conserved downstream signaling pathway, starting with the translocation of MyD88 (myeloid differentiation factor 88) that ultimately leads to the subsequent downstream activation of NF-κB (p65), an important nuclear transcription factor, regulates the expression of a large number of pro-inflammatory genes [26]. Amount of evidences demonstrate that TLR4-dependent MyD88/NF-κB (p65) activation plays a vital role in pathogenesis of I/R-induced damage [27–29]. CIH is somewhat analogous to the process of I/R. We also

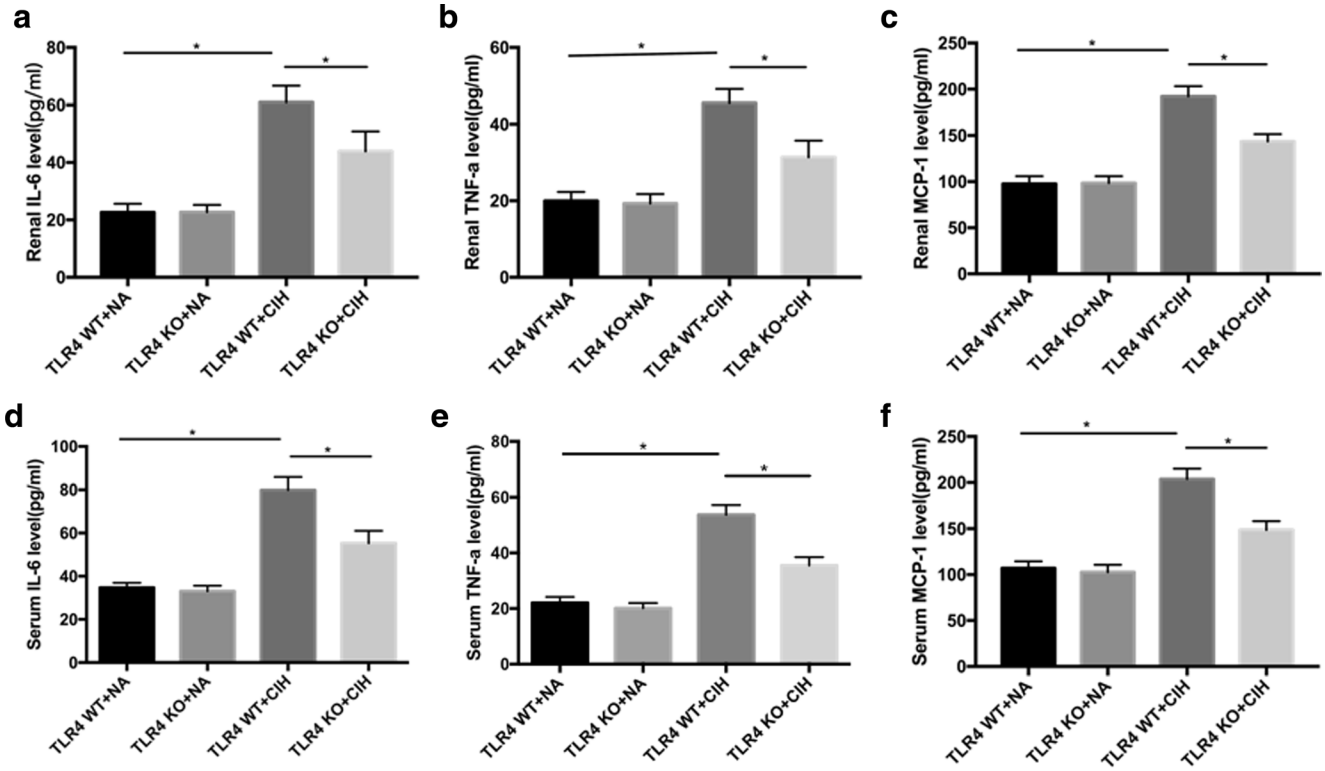

Fig 5 Effect of TLR4 knockdown on the secretion of inflammatory cytokines in CIH mice. The concentrations of IL-6 (**a**, **d**), TNF-α (**b**, **e**), and MCP-1 (**c**, **f**) in kidney tissues and serum were measured by ELISA analysis. The data was expressed as mean ± SEM, $n = 10$, $^*p < 0.05$, $n = 10$

observed MyD88 and NF-kB (p65) expression in our CIH animal model by immunohistochemistry and found that CIH significantly increased MyD88 and NF-kB (p65) expression in injured kidney and TLR4 deficiency decreased their expression after CIH treatment. Previous studies showed that intermittent hypoxia can promote the expression of TLR4 in the mice heart and hypothalamus, and atorvastatin can attenuate myocardial remodeling and hippocampal neuronal damage caused by chronic intermittent hypoxia by inhibiting TLR4-mediated MyD88 and NF-kB pathway [10, 11]. In addition, obesity, hypertension, and diabetes are common disorders concomitant with OSA, which may contribute to the process of OSA concomitant chronic kidney disease. Amount of studies have demonstrated that CIH-induced oxidative stress play

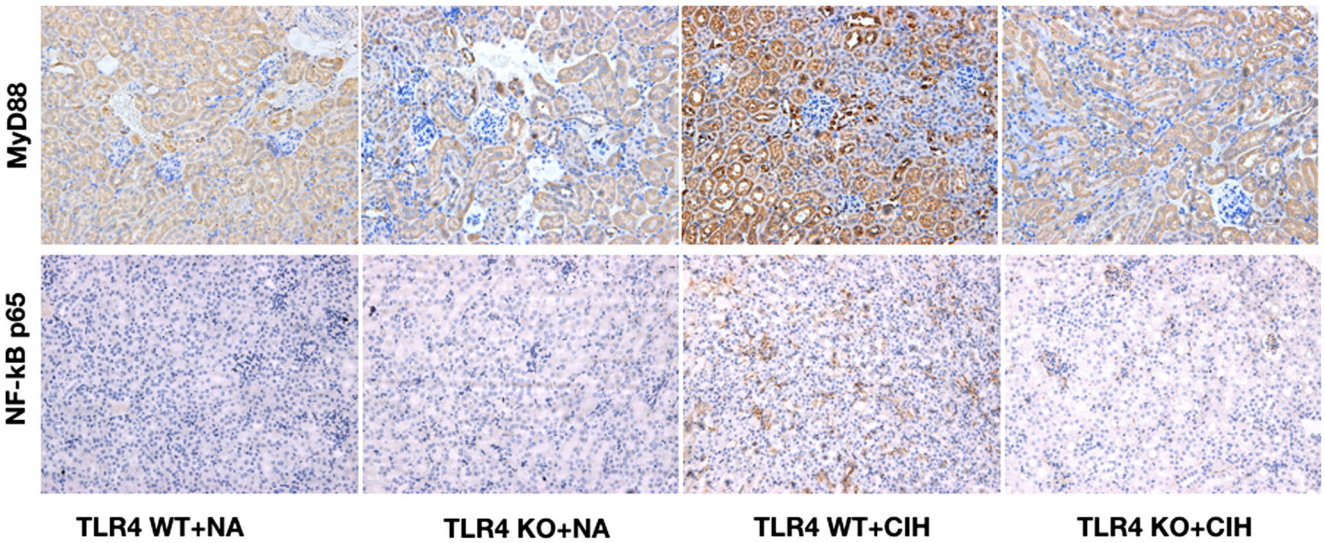

Fig 6 Representative immunohistochemical images of MyD88 and NF-kB p65 in renal tissue. MyD88 dominantly expressed in tubular epithelial cells, and NF-kB p65 dominantly expressed in glomerular epithelial cells and tubular epithelial cells (magnification × 200)

a pivotal role in process of CIH-induced damage, including renal injury [30–32], and oxidative stress and reactive oxygen species (ROS) generation are also central link in the pathogenesis of those chronic diseases like hypertension and diabetes [14, 33]. TLR4 can also mediate the process of ROS generation and oxidative stress-mediated injury in these kinds of chronic models [14, 34]. In reverse, chronic intermittent always exacerbates endothelial dysfunction, insulin resistance, and fibrosis in mice with obesity, diabetes, or hypertension [35–37]. Therefore, we firmly believe that obesity, diabetes, hypertension, or other common disorders concomitant with OSA have negligible impacts on each other.

Presently, the renal tubular endothelial cell injury has been recognized as a critical factor in the pathogenesis of several chronic kidney diseases (CKD). To demonstrate the role of TLR4 in the effect of CIH on renal tubular endothelial cells, we therefore used CD31/TUNEL colocalization immunofluorescent staining to observe the role of TLR4 in CIH-induced apoptosis of renal tubular epithelial cells and revealed that CIH accelerated tubular endothelial apoptosis and TLR4 deficiency abrogated the apoptotic response, which also further confirmed by increased pro-apoptotic caspase-3 and c-caspase-3 expression and Bax/Bcl-2 protein ratio. Furthermore, in our study, one of the most striking findings is that CIH induced renal macrophages recruitment, which might be associated with renal inflammation and fibrosis. Macrophages accumulation is correlated with the degree of renal dysfunction and/or severity of renal fibrosis in several animal models of kidney diseases including glomerulonephritis, renal injury of I/R, and diabetic nephropathy [38–41]. Macrophages-mediated inflammation has been demonstrated in chronic kidney damage in various models and its potential pathway may involve TLR4 activation [42, 43]. Macrophages are an extremely heterogeneous population, displaying a combination of inflammatory and anti-inflammatory functions. Resident kidney macrophages are thought to be derived from both yolk sac and hematopoietic progenitors. The expanding knowledge of the diversity of tissue macrophages in vivo has also highlighted the need to redefine macrophage activation states. The two extremes in the spectrum of macrophage function are represented by the classically activated (or M1) and the alternatively activated (or M2) phenotypes. M1 macrophages upregulate the expression of genes involved with drive inflammation in response to injury. In contrast, M2 macrophages upregulate the expression of genes involved with wound healing, clearance of dead and dying cells and tissues, and are involved in anti-inflammatory response [44]. Our study was the first to show that CIH induced renal pro-inflammatory macrophages (M1) recruitment, which was associated with pro-inflammatory chemokines and cytokines (IL-6, TNF-α, MCP-1) release, and TLR4 deficiency decreased macrophages recruitment. We used CD68 and α-SMA colocalization immunofluorescent staining to find that pro-inflammatory macrophages recruitment may be associated with fibroblasts recruitment, which subsequently lead to extracellular matrix (ECM) protein accumulation and fibrosis. This process was attenuated in TLR4 deficient mice. Pushpakumar et al. also confirmed that TLR4 deficiency reduced M1 macrophages recruitment in a model of Ang-II-induced hypertension, which subsequently reduced renal injury and fibrosis [14]. Different macrophage subsets may play different role for renal diseases. Future studies should identify the role of renal macrophages in CIH-induced renal damage and its potential mechanisms, and refine techniques by targeting specific macrophages for treatment of CIH-induced renal damage or other renal diseases.

However, several limitations should be noticed in this study. First of all, although our studies provide evidence that knockout of TLR4 leads to obvious renal benefits, targeting all TLR4 would be of little therapeutic value because of the systemic immunosuppressive effects. TLR inhibition can be achieved by two major strategies: (1) blocking the binding of TLR ligands to the receptor and (2) interfering the intracellular signaling pathways to stop the signal transduction. Despite many efforts that have been put in developing (bio)molecular inhibitors targeting TLR signaling pathways, unfortunately, very few compounds are currently available for clinical uses [45]. There exists a huge gap between TLR4 inhibition and its practical use in OSA patients. Therefore, it is very important to search for novel TLR selective inhibitors that can target its pathological pathway. Moreover, this is the preliminary study to demonstrate that TLR4 plays a crucial role in the CIH-induced renal damage but the concrete mechanisms, such as how TLR4 modulates macrophages recruitment, are unclear. Further studies are required to identify TLR4-mediated signaling pathways involved in macrophages activation and delineate the crosstalk between macrophages and renal damage to elucidate potential therapeutic targets to reduce CIH-induced renal damage.

Conclusion

Taken together, our study demonstrates that TLR4 deficiency mice are protected from CIH-induced renal injury by a robust anti-inflammatory mechanism. The TLR4 deficiency decreases CIH-induced pro-inflammatory macrophages and fibroblasts recruitment, subsequently attenuates CIH-induced inflammatory response and fibrosis. Further studies are required to identify TLR4-mediated signaling mechanisms involved in macrophages activation and renal damage and more potential TLR4 selective inhibitors should be explored to elucidate therapeutic function in clinical.

Funding The present study was supported by grants from the Central South University Innovation Foundation For Postgraduates (2017zzts209). The sponsor had no role in design or conduct of this research.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

Ethical approval The animal protocol was approved by the Animal Care Committee of Xiangya Hospital, Central South University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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