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Original Article

Interleukin-10 deficiency increases renal inflammation and fibrosis in a mouse ischemia-reperfusion injury model

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Abstract: Ischemia-reperfusion injury is a main cause of acute kidney injury. Typical pathologic changes of acute kidney injury are tubular necrosis and interstitial inflammatory cell infiltration. Inflammation is an important factor that is involved in renal fibrosis. As a general immunosuppressive cytokine. Interleukin-10 functions also negatively regulates inflammatory responses. Recent studies suggested that IL-10 may also play an important role in inhibiting fibrosis in various diseased models. To investigate the effects of IL-10 in the transition from acute kidney injury (AKI) to chronic kidney disease (CKD), we created the ischemia-reperfusion (IR) injury model in IL-10 knockout (IL-10$^{-/-}$) mice. We performed sham or IR surgery in IL-10$^{-/-}$ male mice and wild type (WT) mice, 8 to 12 weeks old. We found IL-10 deficiency resulted in more severe tubular injury and tubulointerstitial fibrosis and higher expression of profibrotic factor TGF-β. In our results, we also found IL-10$^{-/-}$ mice developed more severe renal inflammation, which was significant increase in inflammatory cells infiltration and upregulation of inflammatory chemokines Interleukin-6. In summary, our study provides the evidence that IL-10 has a key role in protecting against development of renal inflammation and fibrosis in the process of AKI to CKD. Enhancement of IL-10 expression could be a potential therapy for patients with AKI.

Keywords: Interleukin-10, ischemia-reperfusion injury, inflammation, fibrosis

Introduction

The incidence of acute kidney injury (AKI) is increasing. A 10 year follow-up study found that 19%-31% patients with acute kidney injury (AKI) eventually progressed into chronic kidney disease (CKD) or end-stage renal disease (ESRD), 12.5% of patients need to rely on dialysis for the rest of life [1]. In response to AKI, a large number of inflammatory cells infiltrate to the ischemia site and activate the inflammatory response [2], even though inflammatory mechanisms resulting from ischemic acute kidney injury and leading to chronic kidney disease have not yet been fully defined, but it still may provide opportunities for developing novel therapeutic approaches [3, 4]. Therefore, to find an early intervention is a key point to stop AKI from transmit to CKD.

Interleukin-10 (IL-10), produced by regulatory T cells, B cells as well as microglial/monocytic cells, is a key immunosuppressive cytokine with potent inhibitory effects on innate as well as adaptive immune cells [5, 6]. IL-10 also has key roles in suppressing inflammatory processes, mainly through blocking activation of inflammatory pathways and inhibiting the secretion of pro-inflammatory mediators, like IL-6, IL-8, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), chemokine monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1, and IL-8 [7]. In addition, IL-10 has been shown to inhibit fibrosis in various animal models. Clinical studies also showed that IL-10 was able to reverse liver fibrosis without increasing viral titers in patients with chronic hepatitis C infection [8].

Our previous study showed that in the early phase of renal I/R injury IL-10 deficiency aggravated early inflammatory infiltration and delayed the kidney repair [9]. This study is to further

explore the role of IL-10 in the progression of AKI to CKD and provides a new theoretical basis for its prevention and treatment.

Materials and methods

Mice

IL-10 gene knockout mice (IL-10$^{-/-}$ mice), 8 to 12 weeks old and 200 g to 240 g in weight, were from Jackson Laboratory (Bar Harbor, Maine, USA) and underwent rederivation to achieve pathogen-free status in the Model Animal Research Center of Nanjing University (Nanjing, China). Male wild-type (WT) control mice (C57BL/6) aged 10 weeks were from the Model Animal Research Center of Nanjing University. All animal procedures were performed in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Surgical procedures of the renal ischemic model

Mice were anesthetized with chloral hydrate (10%, 0.40 ml/10 g). After abdominal midline incision, the left renal pedicles were clamped for 45 min. 45 min after the clamps, blood flow was re-established and the kidneys returning to original color, then the wounds were sutured. Sham-operated animals received similar surgical procedures with the exception of clamping the renal pedicles. Mice were sacrificed with cervical dislocation at day 14 or 28 following reperfusion respectively. Both post-ischemic kidneys and contralateral (right side, not clamped) kidneys were harvested, snapped frozen in liquid nitrogen, and stored at -80°C for further analysis.

Mice were divided into 4 groups (n = 6, each): (1) WT: sham group (WT sham), (2) IL-10$^{-/-}$: sham group (KO sham), (3) WT: IR and recovery (WT IR), and (4) IL-10$^{-/-}$: IR and recovery (KO IR).

Assessment of renal injury

Formalin-fixed kidney sections of 4 μm paraffin sections were stained with haematoxylin and eosin (H&E). Briefly, stained tissue sections were scored using a previously described semi-quantitative scale designed to evaluate the degree of tubular necrosis in a blind manner. Tubulointerstitial injury was defined as tubular necrosis, tubular dilatation and/or atrophy, inflammatory cell infiltrate, or cellular edema, graded on a scale from 0 to 5, where 0 = none, 1≤10%, 2 = 11% to 25%, 3 = 26 to 50%, 4 = 51 to 75% and 5>75%.

Assessment of renal fibrosis

Masson's trichrome staining, the tubulointerstitial injury was evaluated by calculating the percentage of the affected area fraction (the relative blue area of renal fibrosis/total area of the vision ×100%), each section selected 15 or more different vision (×400), using Image Proplu 6.0 Image analysis software to analysis, calculation the relative area of renal fibrosis.

Immunohistochemistry

For immunohistochemistry, renal tissues were fixed in 4% (wt/vol) buffered paraformaldehyde. 3 μm renal sections were deparaffinized and rehydrated in graded alcohol. The antigen retrieval procedure (citrate buffer, pH 6.0) was performed by heating in an electrical pressure cooker for 5 min. After that, sections were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase, and incubated overnight at 4°C with rabbit anti-mouse ki67 (1:2,000, Abcam) and rabbit anti-mouse IL-6 antibody (1:500, Abcam, USA), both of which were primary antibodies. The next day, the slide was incubated with anti-rabbit secondary antibody for 1 h at room temperature in a humidified box. Then, 3,3-diaminobenzidine tetrahydrochloride (DAB, Beijing Zhongshan Biotechnology Co., Beijing, China) was applied to the slides to develop a brown color. Counter staining was performed with hematoxylin, and photomicrographs were taken with an Olympus camera (Olympus BX51, Japan).

Western blotting analysis

Total protein samples (50 μg) were extracted from the total length of the kidney from mice of each group and separated by SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Mass., USA), which were washed twice in Trisbuffered saline (TBS) with Tween® diluted 1:1,000 (TBST; Promega, Madison, Wisc., USA), for 15 min each time, and blocked with TBS containing 5% nonfat milk powder for 1 h. The mem-

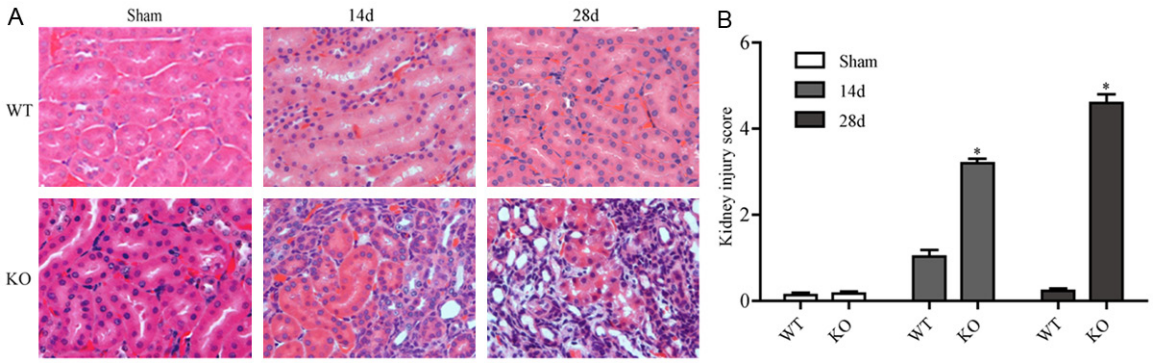

Figure 1. Histology changes of kidney after IR injury. A. Kidney tissue injury over time following 45 min of unilateral renal IR injury, Mice were sacrificed at various times and kidney samples were collected. Representative photomicrographs of hematoxylin and eosin-stained paraffin-embedded kidney sections (×400) are presented. B. Histological damage was scored in a blind manner by the skilled staff in the field. The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT. All fields were chosen form cortex and outer medulla.

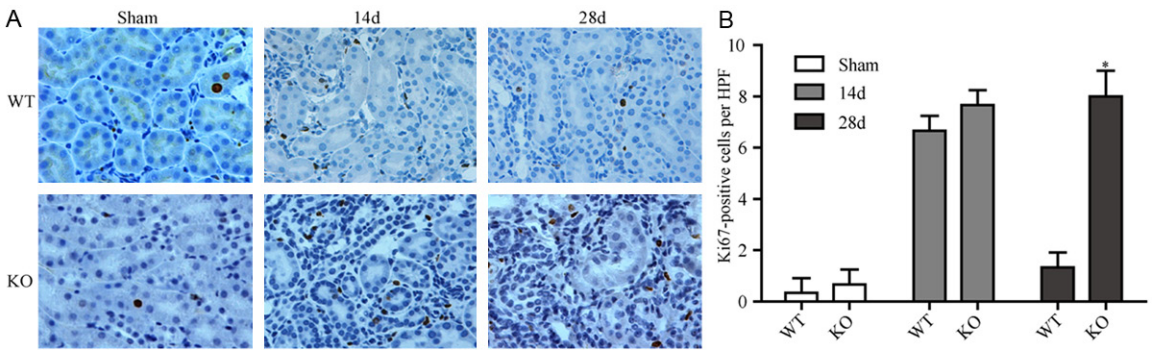

Figure 2. Proliferation of kidney after IR injury. A. Immunohistochemistry staining of ki67 in the kidney. B. Number of ki67-positive cells per HPF (×400) in the kidney. The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT. All fields were chosen form cortex and outer medulla.

branes were probed with the following primary antibodies in TBS with Tween plus 5% milk overnight at 4°C: anti-TGF-β (1:500, BIOS, China), anti-IL-6 (1:1000, BIOS, China), anti-β-actin antibody (1:500, BIOS). The next day, polyvinylidene fluoride membranes were washed with TBS with Tween 4 times, for 15 min each time. Subsequently, the polyvinylidene fluoride membranes were incubated with appropriately diluted peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5,000; Beijing Zhongshan Biotechnology Co.) at room temperature for 1 h. Specific proteins were detected using an ECL reagent (GE Healthcare, Piscataway, N.J., USA) and captured on Hyperfilm (Amersham, GE Healthcare). The results were then analyzed through the Image J software for semiquantitation of the mean gray value of each blot. Thereafter, SPSS statistical software was used to perform one-way ANOVA to detect the differences among the groups. All presented results are representative of at least 3 independent experiments.

Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed using ANOVA, followed by the least significant difference method. The differences were evaluated with SPSS 18.0 software (SPSS, Chicago, IL, USA); a *P* value < 0.01 was considered significant.

Results

Inflammatory cells infiltration and severity of tubular injury in IR kidneys

As shown in **Figure 1A**, interstitial inflammatory cell infiltration, partial renal tubular expansion, loose tissue arrangement and vacuolar degeneration in the epithelial cells were observed on the 14th day after IR in KO mice. Furthermore,

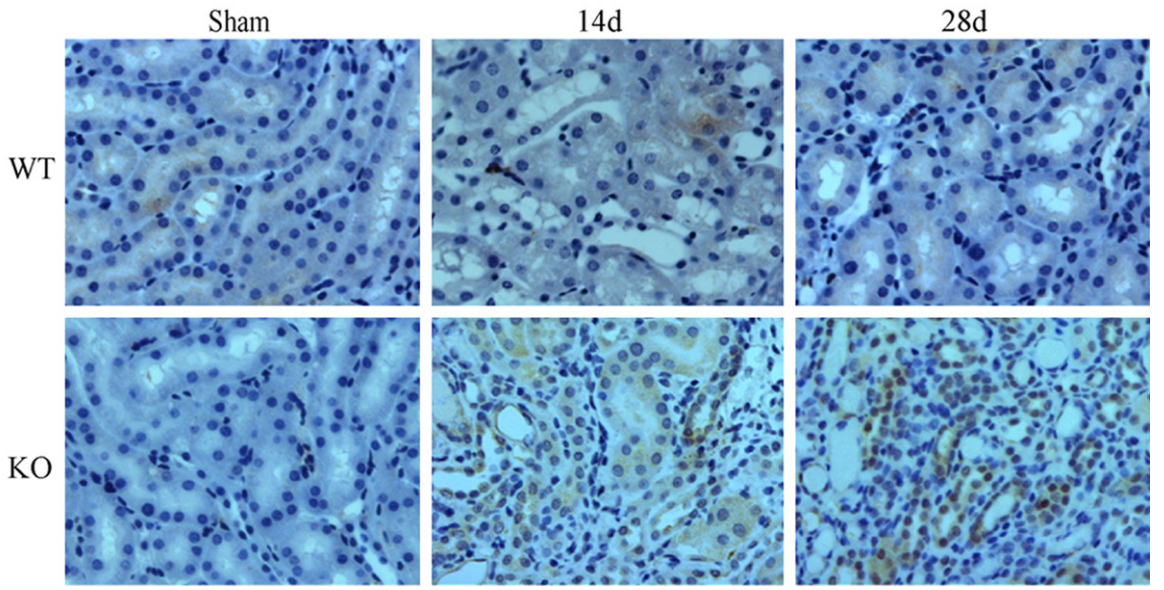

Figure 3. Expression of IL-6 after renal IR injury. Immunohistochemical staining for IL-6 was performed by anti-IL-6 antibody. Positive staining for IL-6 was distributed in the renal TE cells and tubulointerstitium (×400). The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT. All fields were chosen form cortex and outer medulla.

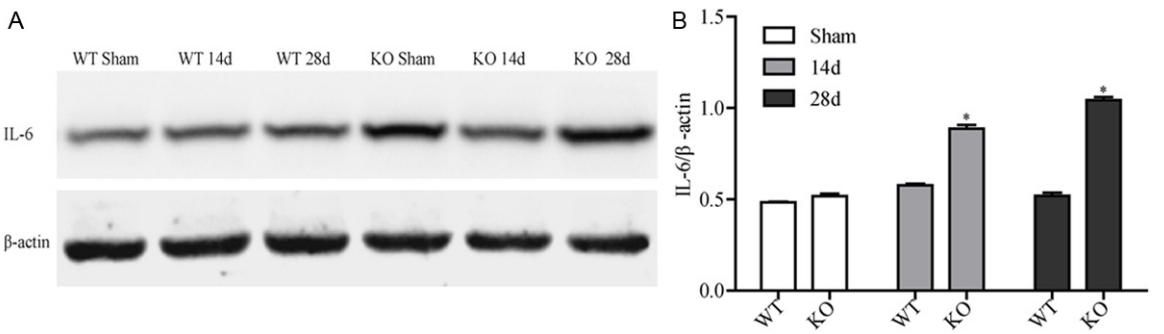

Figure 4. Western blot analysis. A. Expression level of IL-6 was quantified by densitometry and normalized with β-actin. B. The histogram is the analysis result of IL-6 protein expression in the kidneys. The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT.

28 days after the operation, the changes above were even more significant, we observed partial epithelial cell exfoliation and partial renal tubular atrophy. Compared with the KO IR group, Sham group and WT 28 d showed normal renal tubular epithelial cells and glomerular basal membrane, WT 14 d kidney structure had only a small amount of inflammatory cell infiltrated, kidney injury score of the mice in the KO IR group were significantly higher compared with those in the Sham group and WT IR group (P < 0.01) (**Figure 1B**).

Renal tubular epithelial cells' proliferation

To explore the proliferation of the kidneys, immunohistochemistry of ki67 were observed in IR kidneys respectively. As shown in **Figure 2**, ki67 in Sham group showed no significant expression, WT IR group also gradually approached to normal. In contrast, Ki67 expression of KO IR group were significant, however, they were mainly expressed in inflammatory cells.

Inflammatory cytokine IL-6 expression in the mice

To study the potential antifibrotic effects of IL-10 in the IR model, we examined inflammatory cytokine IL-6 in both IR and control mice. As shown in **Figure 3**, only a few of IL-6 expressed in the Sham group and WT IR group. At day 14 and day 28 after IR, expression of IL-6 pro-

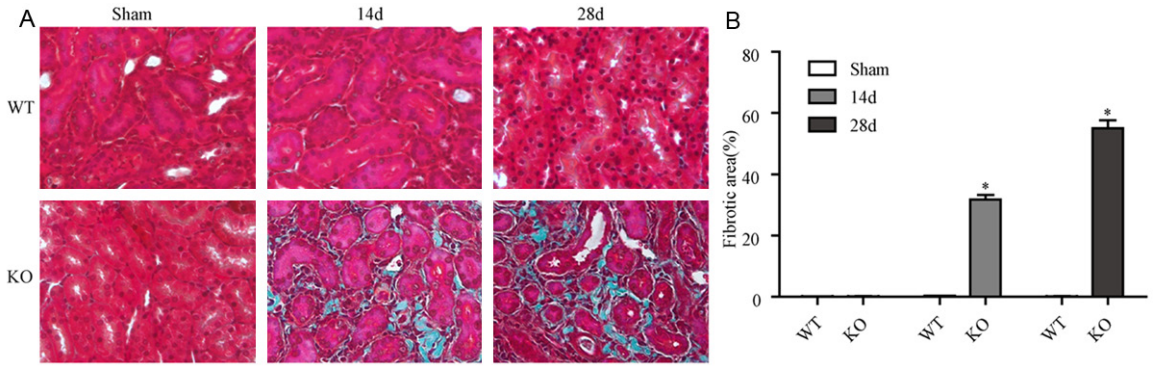

Figure 5. IL-10 deficiency increases renal tubulointerstitial collagen deposition after IR. A. The graphs show representative images of collagen deposition stained with blue (×400). B. The histogram is the semiquantitative analysis result of tubulointerstitial fibrosis area in the kidneys. The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT. All fields were chosen form cortex and outer medulla.

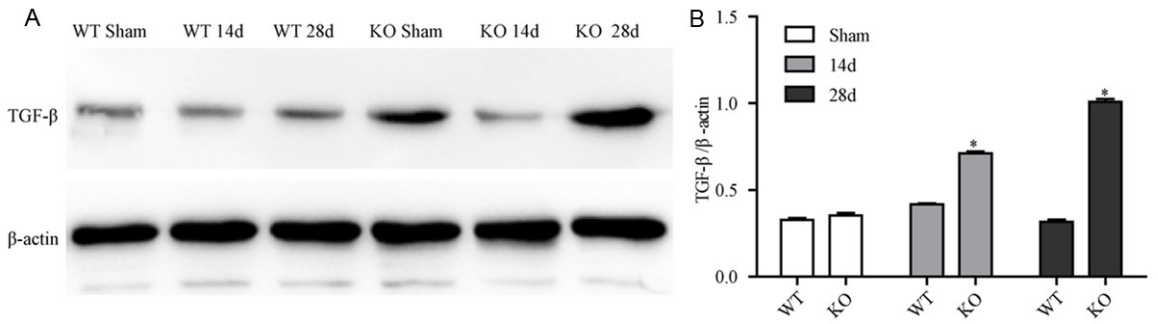

Figure 6. Western blot analysis. A. Expression level of TGF-β was quantified by densitometry and normalized with β-actin. B. The histogram is the analysis result of TGF-β protein expression in the kidneys. The data shown were the mean ± SD. n = 6 per group. *P < 0.01, vs WT.

gressively increased in the IR kidneys of KO mice. Correspondingly, western blot also showed a dramatic increase of IL-6 protein in KO IR group mice kidneys (**Figure 4**).

Renal fibrosis and pro-fibrotic factor expression in IR mice

By Masson's trichrome staining, no interstitial fibrosis was observed in the Sham group and WT IR group at both observation time points. In contrast, KO IR lead to a significant increase of fibrosis (blue area) and the collagen mainly accumulated in the renal interstitium (**Figure 5A**). As shown in **Figure 5B**, the percentage of the area of renal interstitial fibrosis in the WT IR group was significantly higher than that in the Sham group and WT IR group (P < 0.01). At the same time, according to the results of Western blot, TGF-β expression were significantly increased in the IR kidneys of KO mice compared with WT mice at each time point (**Figure 6**).

Discussions

AKI could greatly increase the risk of CKD and ESRD, despite fairly rapid recovery of renal function, the prevalence of chronic kidney disease (CKD) was still twice higher than the patients never suffer from AKI [10]. As the final common pathway leading to end-stage renal disease (ESRD), renal tubulointerstitial fibrosis exist in various types of renal diseases and its severity correlates strongly with the inflammatory status. Exogenous IL-10 decreases death from endotoxin sepsis, and prevents tissue injury in a wide variety of animal models [11]. Studies in other organs also showed IL-10 could suppress inflammatory response and inhibit matrix remodeling and fibrosis process, even if fibrosis had developed [12, 13]. However, the relationship between IL-10 and AKI transmit to CKD is not fully understood. Our study was to examine the role of IL-10 in the process from AKI to CKD using the IR model, which has been widely used as an animal model of AKI [14, 15].

Studies confirmed that IL-10 administration could significantly reduce renal inflammatory cells infiltration in the interstitial areas of cortex, suppress chemokines (MCP-1, RANTES) and cytokines (IFN-γ, IL-2, iNOS, TNF-α) expression, resulting in a less fibrotic response in models of chronic renal diseases [16, 17]. In our study we found the pathological changes of the KO IR mice model include tubular injuries and renal fibrosis was more sever. We also found IL-10 deficiency lead to more severe inflammatory cell infiltration and higher expression of pro-inflammatory cytokines IL-6, which is an important pro-inflammatory mediator in kidney [7]. In addition, compared to the WT IR and sham group mice, Ki67, a proliferation marker whose function has previously been reported as a useful and accurate tool to assess renal tubular proliferation [18], were mostly expressed on chronic inflammation cells instead of renal tubular epithelial cells in KO IR kidneys. These results are also consistent with our previous studies suggesting IL-10 plays an important role in the improvement of renal IR injury. Furthermore, TGF-β, which is a well-characterized factor that promotes fibrosis in many diseases and organs, including the kidney [19, 20], also showed significant expression. These findings indicated that IL-10 has a critical role in protecting against development of renal inflammation and fibrosis.

In summary, this study suggested that IL-10 deficiency may play an important role in the pathophysiology of renal fibrosis, where IL-10 may be an important effector to prevent acute kidney injury from progressing. The mechanism may be closely related to IL-10 deficiency promote TGF-β expression and pro-inflammatory and anti-inflammatory disorders, but the exact mechanism still needs further study. Our study open a new path for early intervention and prevention of AKI transmit to CKD. Therefore, further investigation of the role of IL-10 and its molecular mechanisms has important clinical significance.

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Disclosure of conflict of interest

None.

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