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Original Article

Knockout of Bruton's tyrosine kinase in macrophages attenuates diabetic nephropathy in streptozotocin-induced mice

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Abstract: As a cytoplasmic tyrosine kinase in the Tec family, Bruton's tyrosine kinase (Btk) participates in various biological processes, including cell growth, differentiation, and apoptosis. Although recent studies have indicated that Btk is involved in pro-inflammatory cytokine production, the underlying impact of Btk on the development and pathogenesis of diabetic nephropathy (DN) has not been elucidated. The aim of this study was to determine whether Btk knockout (KO) could reduce inflammation and kidney injury in DN. First, diabetic mice models were established via an intraperitoneal injection of streptozotocin. Thereafter, the underlying mechanism was explored by comparing *Btk* $^{flox/flox}$ Lyz-*Cre* mice to wild-type (C57BL/6N) mice. Albuminuria was significantly reduced, and kidney injuries were attenuated in Btk conditional deletion diabetic mice. More importantly, these changes were demonstrated to be associated with decreased levels of pro-inflammatory cytokines owing to the downregulation of the MAPK and NF-κB signaling pathways. Collectively, these findings indicate that Btk plays a critical role in the regulation of kidney inflammation and provides a prospective therapeutic strategy for the treatment of DN.

Keywords: Diabetic nephropathy, Bruton's tyrosine kinases, inflammation, macrophage

Introduction

Diabetic nephropathy (DN) is not only the most common chronic kidney disease, but also a major microvascular complication of patients with diabetes [1]. With the increased prevalence of DN, a huge socio-economic burden is placed on the global population. The pathogenesis and progression of DN are associated with several factors, such as oxidative stress, inflammation, glycoxidation, peroxidation, and other pathological factors [2]. Accumulating evidence suggests that the infiltration and activation of macrophage may play vital roles in DN by secreting pro-inflammatory cytokines and nuclear factor-κB (NF-κB) [3]. Moreover, according to a clinical study, macrophage infiltration in the kidney of diabetic patients is higher than that in healthy people [4]. This may occur because the activation of tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) could promote macrophage migration and activation in the hyperglycemia environment, followed by kidney injury [5].

Being a component of the Tec kinase, Bruton's tyrosine kinase (Btk) has occupied a key position in primary studies as part of adaptive immunity [6]. In fact, several studies have suggested that Btk is involved in autoimmunity and inflammatory diseases through various signaling pathways, including the toll-like receptors pathway [7, 8]. There has been evidence suggesting that macrophages from X-linked immunodeficiency (*xid*) mice produced fewer pro-inflammatory cytokines and impaired the transactivation of NF-κB [9-11]. For instance, Ni Gabhann et al. [12] found that in Btk-deficient mice, the recruitment of M1 macrophages was significantly reduced after the addition of LPS into the abdominal cavity. Moreover, *in vitro* analysis showed that $Btk^{-/-}$ macrophages were polarized to the M1 macrophage subset, and the function of NF-kB p65, iNOS and Akt was suppressed. Similarly, liver ischemia-reperfusion injury was proven to be related to Btk-mediated neutrophils and macrophage activation, as the Btk inhibitor, Btkb66, could strongly inhibit LPS-mediated activation of bone mar-

row-derived neutrophils and macrophages, eventually ameliorating hepatocellular injury [13, 14]. Besides, the selective inhibition of Btk prevented immune-mediated glomerulonephritis, including immunoglobulin A (IgA) nephropathy [19] and lupus nephritis [6, 15].

Although the above advances have been achieved, whether Btk is involved in the occurrence and progression of DN has not been revealed. Our previous study suggested that PCI-32765, a potent Btk inhibitor, attenuated pro-inflammatory cytokine expression in bone marrow-derived macrophages treated with high glucose [16]. In this study, we attempted to verify and further explore the modulatory roles of Btk in STZ-induced DN mice by using *Btk* $^{flox/flox}$ Lyz-*Cre* mice and wild type (WT; C57BL/6N) mice.

Materials and methods

Animals

The research protocol was approval by the Animal Science Committee of Anhui Medical University (No: LLSC20200064). All animals, including the Lyz-*Cre* mice, were purchased from the Experimental Animal Center of Nanjing University (Nanjing, China). Frozen embryos of Btk-floxed mice were bought from The European Mutant Mouse Archive (EMMA), and resuscitated with the help of the Experimental Animal Center of Nanjing University. Mice were administered STZ on a daily basis at a body weight of 50 mg/kg for 5 days. Thereafter, mice were randomly divided into four groups: (1) WT (C57BL/6N, n=7), (2) diabetic (C57BL/6N+STZ, n=7), (3) *Btk* $^{flox/flox}$ Lyz-*Cre* (n=7), and (4) *Btk* $^{flox/flox}$ Lyz-*Cre* diabetic (*Btk* $^{flox/flox}$ Lyz-*Cre* +STZ, n=7). Mice were anesthetized by inhalation of 5% isoflurane and blood was collected under the fasting state. All experimental subjects were sacrificed after anesthesia.

Antibodies and reagents

The antibodies specific for Btk, phospho-Btk, phospho-P38mapk, phospho-ERK, phospho-JNK, JNK, p38MAPK, ERK, IκB, phospho-IκB, NF-κB p65, and phosphor-NF-κB p65 were obtained from CST (Beverly, MA, USA). The primary antibodies for CD68, iNOS, WT-1, and nephrin were procured from Abcam Biotechnology (Cambridge, UK). The antibodies for MCP-1 and TNF-α and secondary antibodies (Alexa Fluor 594-anti-mouse, Alexa Fluor 488-anti-rabbit) were purchased from Wuhan Sanying Biotechnology Inc. Streptozotocin (STZ) was bought from Sigma-Aldrich (St. Louis, MO, USA), and mouse urinary albumin ELISA was bought from Abcam (Cambridge, UK).

Biochemical analyses

The 12 h fasting blood glucose levels in mice were measured every 2 weeks according to the Accu-Chek glucose meters (Roche Diagnostic, Inc.) recommended by the Diabetes Complications Animal Model Consortium. At the end of 12 weeks, 24 h urine was collected from all mice using a metabolic cage, and an ELISA kit was used to detect the urinary albumin concentration. We collected mice blood samples in the fasted state via heart punctures.

Renal histology analysis

For renal histology analysis, after being cut into stick slices and fixed with 4% paraformaldehyde, paraffin-embedded renal tissues were stained with PAS (Periodic Acid-Schiffstain), reagent to discern the structures of kidney. In terms of the expansion of glomerular mesangial and the injury index of tubulointerstitial, their evaluation and grading were carried out by Image J [22]. The tissues were fixed in 2% glutaraldehyde, then postfixed in 1% osmium tetroxide embed in resin, cut, stained, and observed under an electron microscope.

Renal immunohistochemistry

Immunohistochemistry, which was used to detect WT-1 and nephrin as podocyte markers, was carried out using a heat-induced antigen retrieval system for 10 mins in an oven. The sections were immersed with the anti-nephrin and anti-WT-1 primary antibodies at 4°C overnight. After washing with PBS, the sections were incubated in secondary antibody for 20 min and developed with DAB peroxidase substrates. Podocyte was measured by identifying WT-1-positive podocyte nuclei and nephrin-positive glomerular cells through immunohistochemical staining of 10 glomeruli.

Immunofluorescence staining

The renal tissue slices were deparaffinized and rehydrated in graded ethanol concentrations to

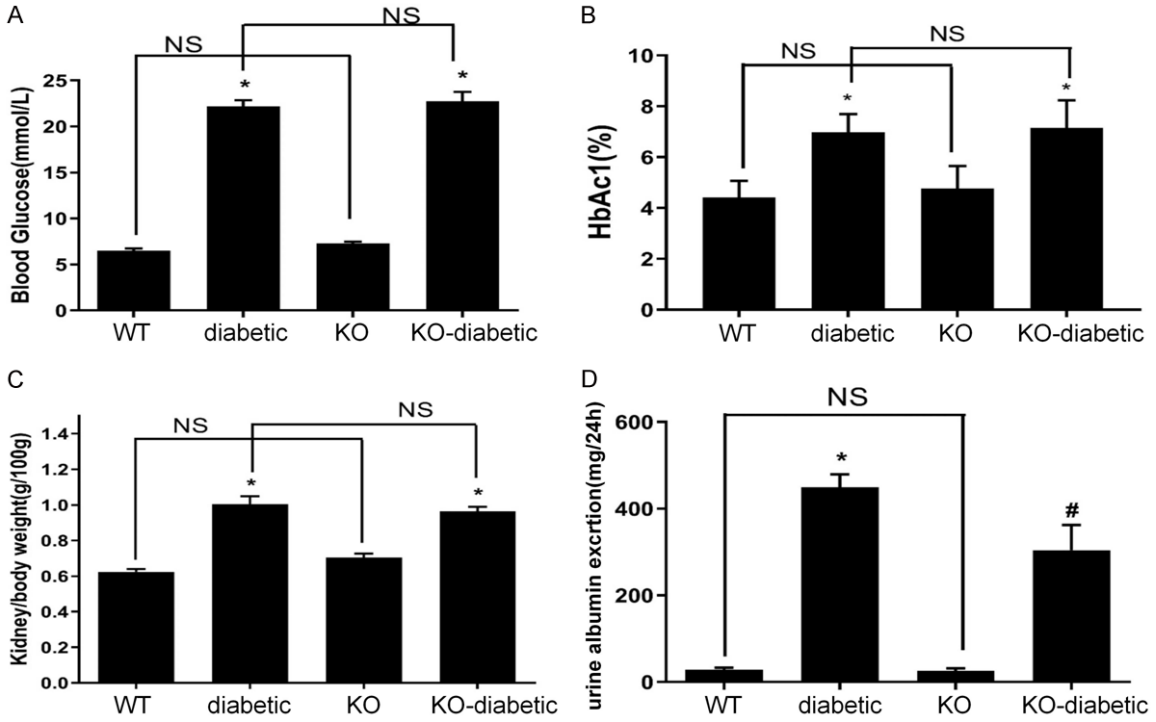

Figure 1. Effect of Btk on the general parameters of the four groups (n=7 per group): WT, diabetic, KO, diabetic-KO. A. Blood glucose levels in the four mice groups (mmol/L). B. The levels of HbAc1 (%) for the four groups. C. The kidney/body weights (g/100 g) of the four mice groups. D. The levels of urinary albumin excretion ratio in the four groups (mg/24 h). Data represent the mean ± SEM of 6-8 mice. $^{*}P<0.05$ vs. WT, $^{\#}P<0.05$ vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

the distilled water. Thereafter, the tissue slices were blocked with goat serum at 37°C for 30 min, incubated with anti-CD68 (1:200) and anti-Btk (1:200) at 4°C overnight, and washed with PBS. Later, the sections were incubated with immunofluorescence secondary antibody for 2 h in the dark, and stained with 4',6-diamidino-a-phenylindole (DAPI). Finally, slices were observed under a confocal microscope (LSM880, Germany).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Under the guidance of the manufacturer's instructions, renal tissues were harvested in TRIzol reagent; thereafter, total RNA extraction and RNA reverse transcription were carried out. The primers used for PCR were previously reported [17].

Western blot analysis

The total proteins were extracted from the renal tissues and the concentration of proteins was measured with the bicinchoninic acid assay (BCA). Thereafter, the total proteins were separated and electro-transferred to a polyvinylidene difluoride membrane blocked with 5% skimmed milk for 2 h and probed with primary antibodies at 4°C overnight. After washing, the membranes were incubated with appropriate secondary antibodies at room temperature for 45 min. After washing three times with PBST, the membrane was detected using an enhanced chemiluminescence system. Finally, the optical densities of the captured bands and the relative ratios of protein expression levels were analyzed by Image J.

Statistical analysis

All experiments were independently conducted in triplicate. Data are expressed as mean ± SEM and difference between groups was considered statistically significant at $P<0.05$. Statistical significance between groups was determined by the unpaired t test or ANOVA followed by Tukey's post hoc analysis using SPSS software ver.16.0 (IBM, Armonk, NY, USA). $P<0.05$ indicated significance in the statistics.

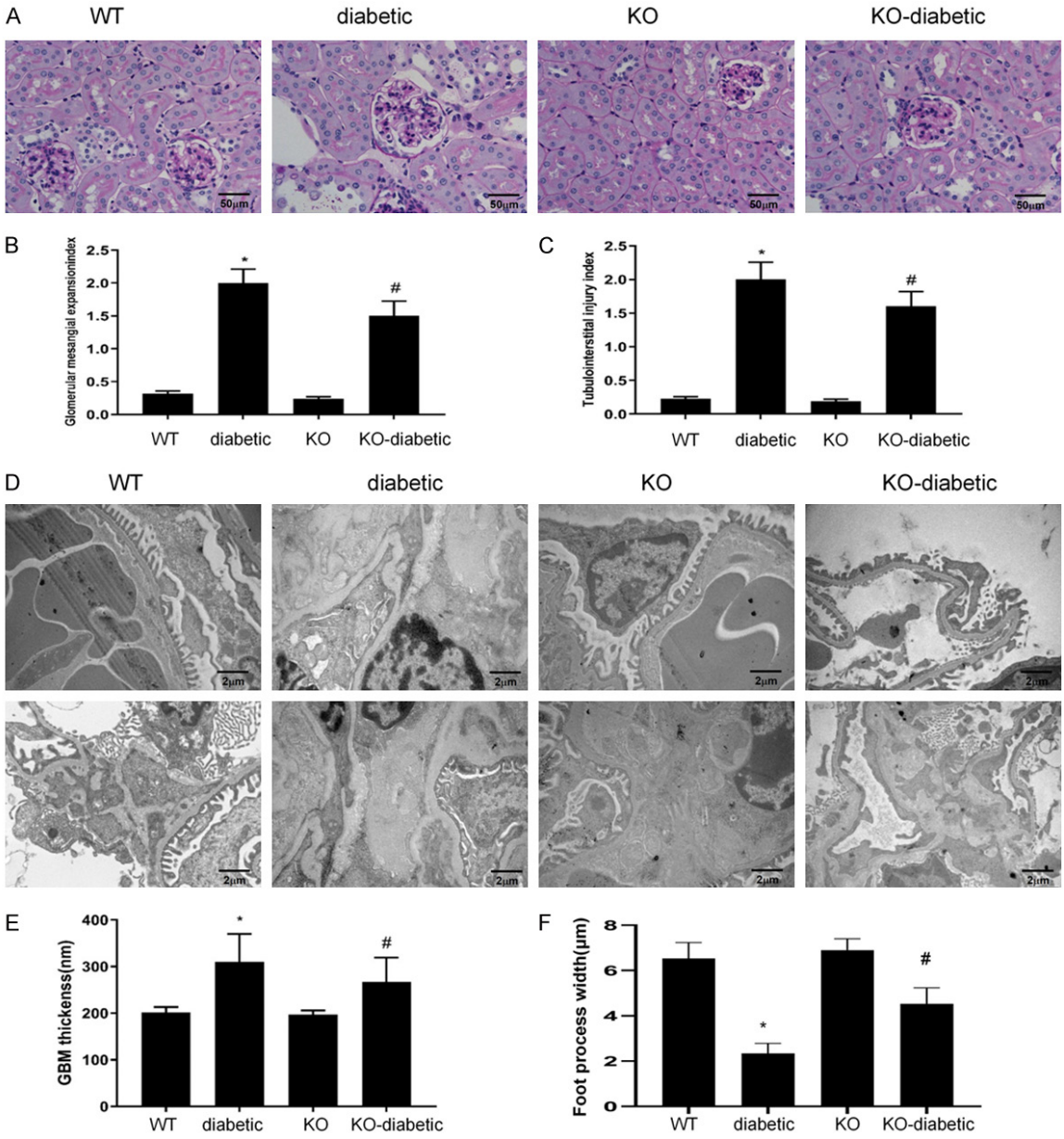

Figure 2. Renal histopathologic changes in the four mice groups (n=7 per group): WT, diabetic, KO, diabetic-KO. A. The renal tissues obtained from the four groups were stained with PAS Scale bar =50 μm. B. The glomerular mesangial expansion index of the four groups. C. The tubulointerstitial injury index of the four groups. D. Electron micrographs of the glomeruli from the four mice groups. Scale bar =2 μm. E. The analyses of GBM thickness in the four groups. F. The analyses of podocyte foot process density in the four groups. Data represent the mean ± SEM of 6-8 mice. $^{*}P<0.05$ vs. WT, $^{\#}P<0.05$ vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

Results

Deletion of Btk decreased clinical and metabolic parameters in diabetic mice

After 12 weeks, the general and metabolic parameters of mice were measured. As shown in **Figure 1**, there were significant changes in the blood glucose levels, HbAc1, and the kidney/weight of body of diabetic mice ($^{*}P<0.05$ vs. WT) compared to WT mice; however, no obvious changes were found in Btk KO mice (*Btk* $^{flox/flox}$ Lyz-*Cre mice*). Further, the 24 h urinary albumin excretion rate (UAER) of diabetic mice was more than that of WT mice ($^{*}P<0.05$ vs. WT). However, the UAER was significantly decreased in Btk KO diabetic mice ($^{\#}P<0.05$ vs. diabetic), which suggested that the knockout of

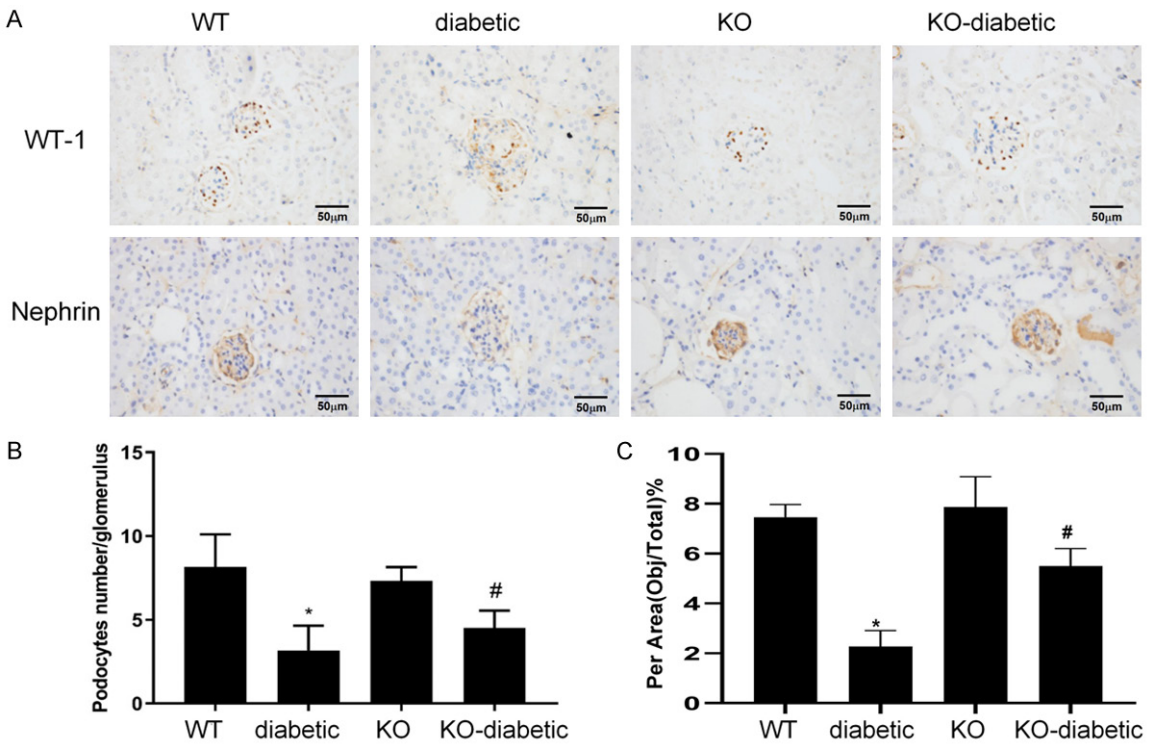

Figure 3. The expression of WT-1 and nephrin in the kidneys of *Btk* $^{flox/flox}$ Lyz-*Cre* diabetic mice (n=7 per group). A. WT-1 and nephrin in the glomeruli were stained in the four mice groups. Scale bar =50 μm. B. The number of podocytes per glomerulus in the four mice groups. C. Semiquantitative analyses of immunostaining for nephrin in the four mice groups. Data represent the mean ± SEM of 6-8 mice. **P*<0.05 vs. WT, $^{\#}$*P*<0.05 vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

Btk could protect against STZ-induced renal injury.

Deletion of Btk prevents renal histopathological injury in diabetic mice

Renal histopathologic alterations in the four mice groups were evaluated. Based on PAS staining, renal injury was more severe in diabetic mice. Further, the pathological lesion index of Btk KO diabetic mice was found to be remarkably decreased (**Figure 2A-C**, *P< 0.05 vs. WT, $^{\#}$P<0.05 vs. diabetic). Glomerular damage was also observed using an electron microscope. Diabetic kidneys displayed thickened glomerular basement membrane and podocyte injury, which were not observed in control mice (**Figure 2D-F**, *P<0.05 vs. WT, $^{\#}$P<0.05 vs. diabetic). Furthermore, the Btk KO diabetic group presented less thick basement membrane and decreased podocyte injury. Consistently, in Btk KO diabetic mice, the mesangial expansion matrix was significantly decreased.

Deletion of Btk prevents renal podocyte injury in diabetic mice

To evaluate the podocyte number in the glomeruli of diabetic mice, WT-1 and nephrin stainings were performed. In diabetic mice, the podocyte number and expression of nephrin were significantly decreased (**P*<0.05 vs. WT). Conversely, in Btk KO diabetic mice, the number of podocytes was increased compared to that found in diabetic mice (**Figure 3A**, **3B**, $^{\#}$*P*<0.05 vs. diabetic). Nephrin expression was reduced in STZ-induced diabetic kidneys but increased in Btk KO diabetic kidneys (**Figure 3C**, $^{\#}$*P*<0.05 vs. diabetic).

Deletion of Btk attenuates TGF-β1-induced collagen matrix expression in diabetic mice

Consistent with the above findings, the levels of TGF-β1, Col-IV, and Fn were significantly higher in the kidneys of diabetic mice than those in the kidneys of WT mice (**P*<0.05 vs. WT). However, Btk knockout could inhibit these protein levels (**Figure 4**, $^{\#}$*P*<0.05 vs. diabetic).

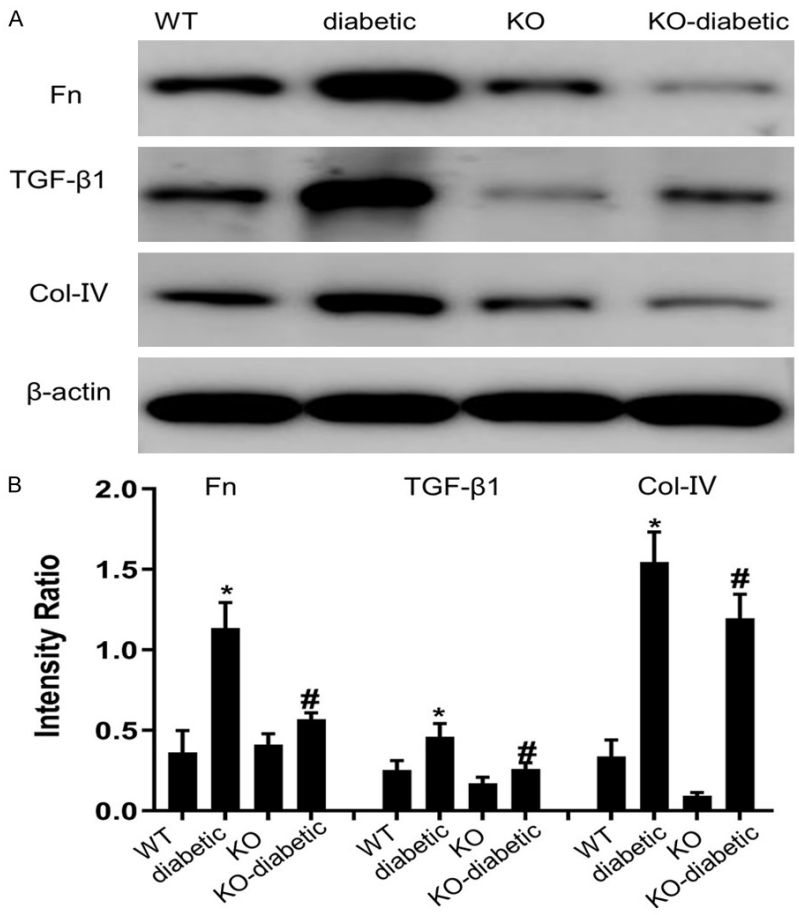

Figure 4. TGF-β1, Col-IV, and Fn expression in the kidneys of *Btk* $^{flox/flox}$ Lyz-*Cre* diabetic mice (n=7 per group). A. The expression levels of TGF-β1, Col-IV, and Fn were determined in the four mice groups by western blot analysis. B. Intensity ratio of TGF-β1, Col-IV, and Fn in the four mice groups. Data represent the mean ± SEM of 6-8 mice. $^{*}P$<0.05 vs. WT, $^{\#}P$<0.05 vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout; Fn, fibronectin.

Deletion of Btk attenuates macrophage infiltration and activation in diabetic mice

To determine the effects of Btk on inflammation in DN, CD68, a specific macrophage marker, was detected by immunofluorescence staining. As shown in **Figure 5**, there was an accumulation of CD68-positive macrophages in diabetic mice compared to that in control mice; however, in Btk knockout diabetic mice, the corresponding positive rate was significantly decreased.

Deletion of Btk attenuates inflammatory cytokine levels in diabetic mice

The levels of inflammatory factors in mice were detected using RT-PCR. Compared with WT mice, the expressions of IL-β, TNF-α, and MCP-1 were significantly increased in diabetic mice (*P<0.05 vs. WT). However, Btk knockout decreased the levels of these factors in Btk $^{flox/flox}$ Lyz-Cre diabetic mice (**Figure 6A**, $^{\#}$P<0.05 vs. diabetic). In addition to RT-PCR, the iNOS, IL-1β, TNF-α and p-Btk levels were further analyzed. Similarly, the expression of these proteins was significantly higher in the diabetic mice kidneys (*P<0.05 vs. WT), while these protein levels were significantly downregulated after Btk knockout (**Figure 6B**, $^{\#}$P< 0.05 vs. diabetic).

Deletion of Btk suppress of MAPK signaling pathway in diabetic mice

To further explore the underlying mechanism, the expression of MAPK signaling pathway in the kidneys were detected. Compared with WT mice kidneys, those in diabetic mice kidneys were higher as showed in **Figure 7** (*P< 0.05 vs. WT). As expected, Btk knockout decreased these proteins expression ($^{\#}$P<0.05 vs. diabetic), which suggested that Btk is a perspective intervention component in the obstruction of the DN progression.

Deletion of Btk suppress of NF-κB p65 in diabetic mice

NF-κB was found to be activated in diabetic kidney and played a vital role in inflammatory responses. The expression of NF-κB p65 was significantly increased in the kidneys of diabetic mice (**Figure 8**, $^{*}P$<0.05 vs. WT). However, the expression levels of p-IκB and p-p65 were downregulated in the kidneys of Btk diabetic mice ($^{\#}P$<0.05 vs. diabetic), indicating that Btk could modulate NF-κB p65 in diabetic mice.

Discussion

DN is a major life-threatening complication of diabetes [18]. Although the prevalence of DN

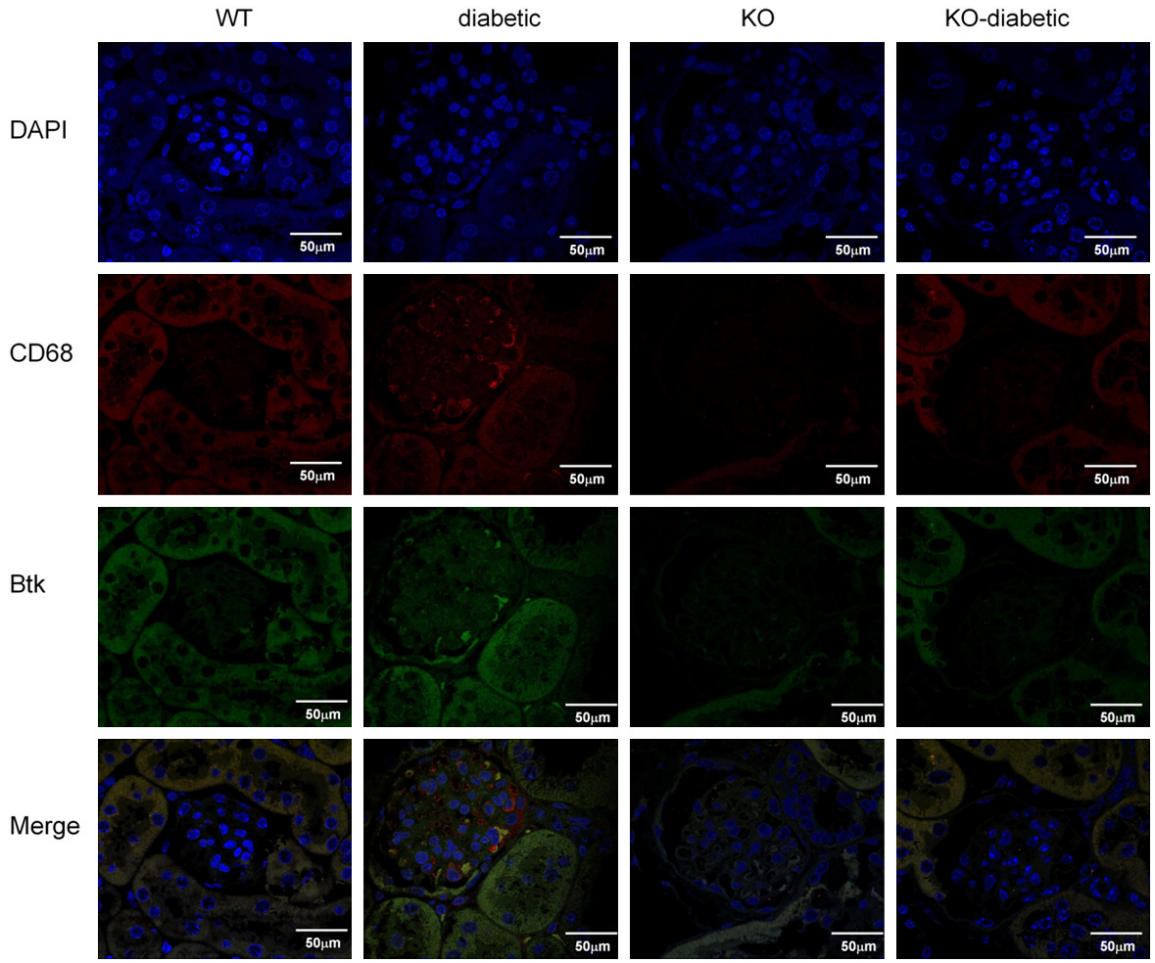

Figure 5. Immunofluorescence of CD68 and Btk expression in mice kidney samples. Scale bar =50 μm. Abbreviations: WT, wild type; KO, knockout.

has been increasing over the past few decades, effective prevention or intervention of DN progression remains a challenge [19]. As the earliest detectable biomarker of glomerular injury, microalbuminuria has been shown to be a major risk element for DN [20-22]. In this study, we observed that Btk deficiency in WT mice did not induce albuminuria. More importantly, after the development of kidney injury in diabetic mice, mesangial matrix accumulation, glomerular basement membrane thickening and inflammatory infiltration were attenuated through Btk knockout.

Btk, a member of the Tec family, interacts with various signaling pathways in hematopoietic cells [23]. In recent years, the role of Btk in innate immunity has gradually been revealed [24, 25]. For instance, the inhibition of Btk leads to the impaired expression of pro-inflammatory factors in various types of cells (such as MCP-1, IL-1β, and TNF-α) [14], which has also been observed in high glucose-induced bone marrow derive macrophages in our previous study [26, 27]. In addition, it has been suggested that Btk may interact with TLR4, 6, 8, 9 and their adaptors [28]. Btk has been proven to be an indispensable part of NF-κB [29]. Consistently, we found that a close link exists between Btk inhibition and NF-κB pathway suppression.

NF-κB is a vital transcription factor in macrophages that can promote the transcription of pro-inflammatory cytokines [30]. NF-κB activation in rat models has been reported. The findings of this study indicated that Btk participates in the NF-κB signaling pathway in diabetic mice, and Btk-deficient mice displayed more effective protection against DN [31]. Studies have

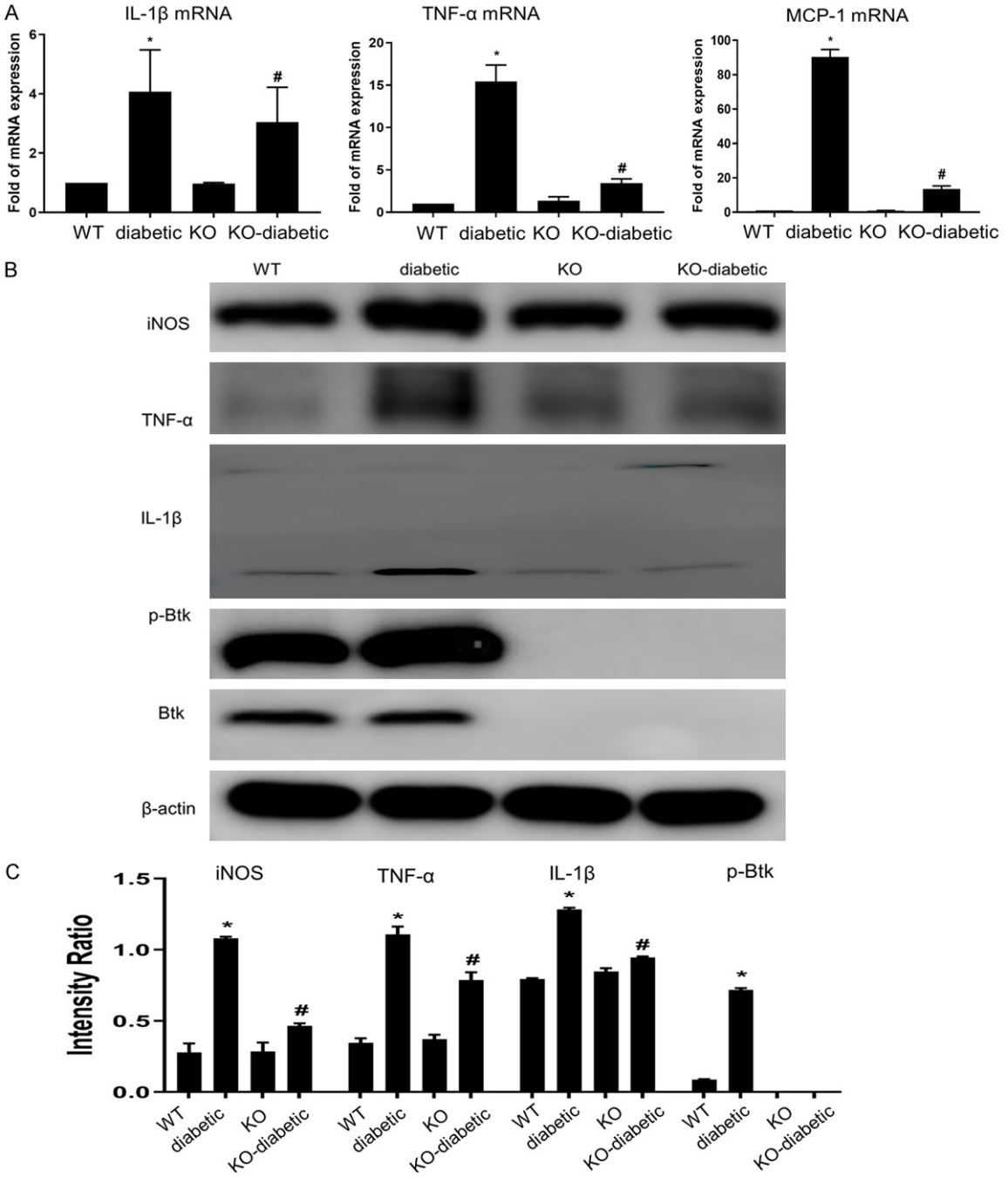

Figure 6. A. Real-time PCR analysis of the inflammation (TNF-α, MCP-1, IL-1β) indices in mice kidney samples (n=7 per group). B. The expression levels of iNOS, IL-1β, TNF-α, and p-Btk in the four mice groups were determined by western blot analyses. C. Intensity ratio of iNOS, IL-1β, TNF-α, and p-Btk in the four groups. Data represent the mean ± SEM of 6-8 mice. $^{*}P<0.05$ vs. WT, $^{\#}P<0.05$ vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

suggested that the phosphorylation of p38 and ERK was associated with the progression of DN [32]. Herein, the levels of these proteins were higher in diabetic mice and significantly decreased after Btk knockout.

Currently, numerous studies have found that renal inflammation is indispensable in the progression of DN in animal models or even clinical patients [4]. In hyperglycemia, the infiltration and activation of macrophages increases

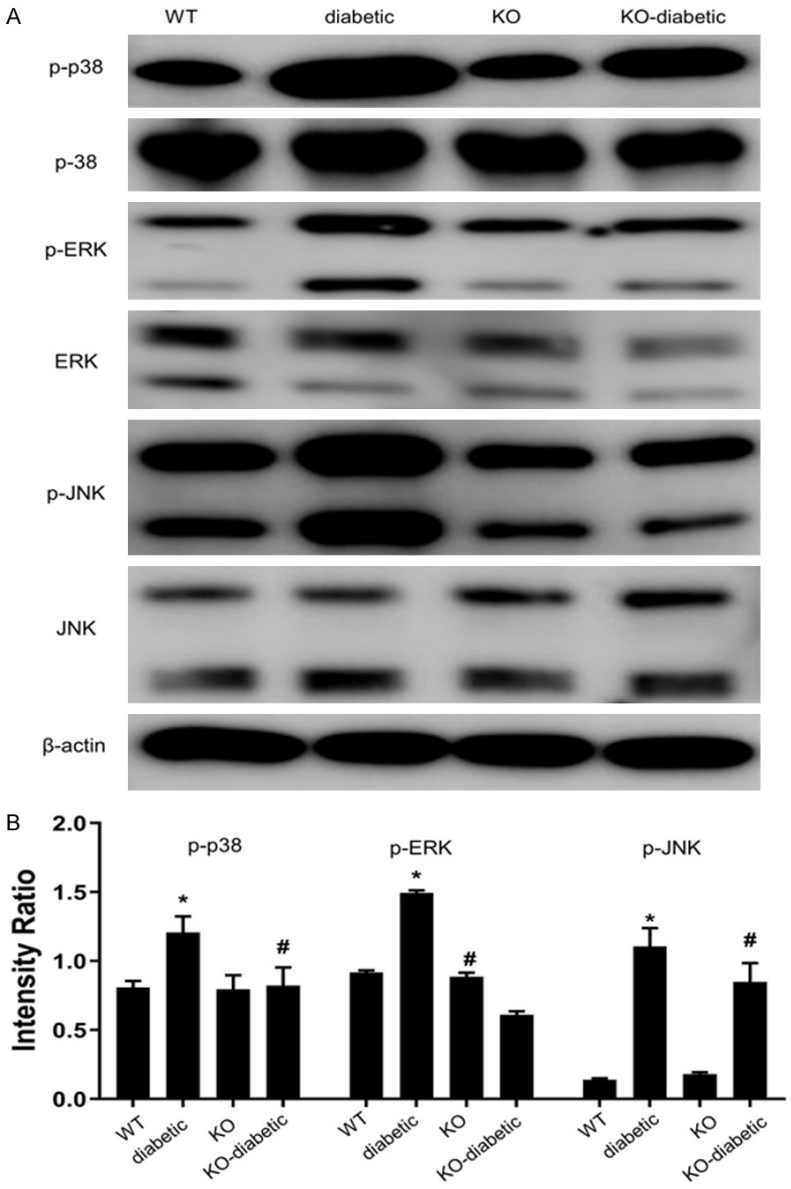

Figure 7. MAPK signaling pathway suppression in the kidneys (n=7 per group). A. The levels of p-p38, p-ERK, and p-JNK in the four mice groups were determined by western blot analyses. B. Intensity ratio of p-p38, p-ERK, and p-JNK in the four groups. Data represent the mean ± SEM of 6-8 mice. **P*<0.05 vs. WT, #*P*<0.05 vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

pro-inflammatory cytokine production, which correlates with albuminuria in DN [5, 33]. Two types of macrophages are associated with renal damage: M1 macrophages cause inflammation and tissue damage, while M2 is involved in repairing and protecting the renal tissue [34]. Inducible nitric oxide synthase (iNOS) is considered to be a specific marker of M1 macrophages [35]. In our study, CD68-positive macrophages were found to infiltrate the kidneys of diabetic mice. Further, the level of iNOS was higher in the kidney of diabetic mice than that in the kidney of WT mice. However, the infiltration of macrophages and higher expression of iNOS were partially reversed in Btk$^{-/-}$ diabetic mice [36]. These results indicate that the knockout of Btk could protect the kidneys from inflammation caused by M1 macrophages.

Hyperglycemia can stimulate macrophages to release TNF-α and MCP-1 [37]. Subsequently, macrophages are recruited by MCP-1 into the kidneys and TNF-α might induce cellular apoptosis, leading to the progression of DN [38]. In our investigation, there were large numbers of inflammatory cytokines in the kidneys of diabetic mice, and knockout of Btk could downregulate the levels of these pro-inflammatory factors.

Generally, loss of podocytes is a distinguishing feature of DN and is also found in STZ-induced diabetic mice [34, 39, 40]. In our study, the expression levels of WT-1 and nephrin were decreased in diabetic mice; however, Btk blockade restored the number of WT-1 and nephrin, which are specific markers of podocytes. Based on our results, Btk knockout may be a promising treatment strategy for DN by protecting the podocytes.

In the present study, we not only observed the infiltration and activation of macrophages and pro-inflammatory cytokine secretion, but also revealed that the NF-κB and MAPK signaling pathways were activated in diabetic mice. Furthermore, we provided direct convincing evi-

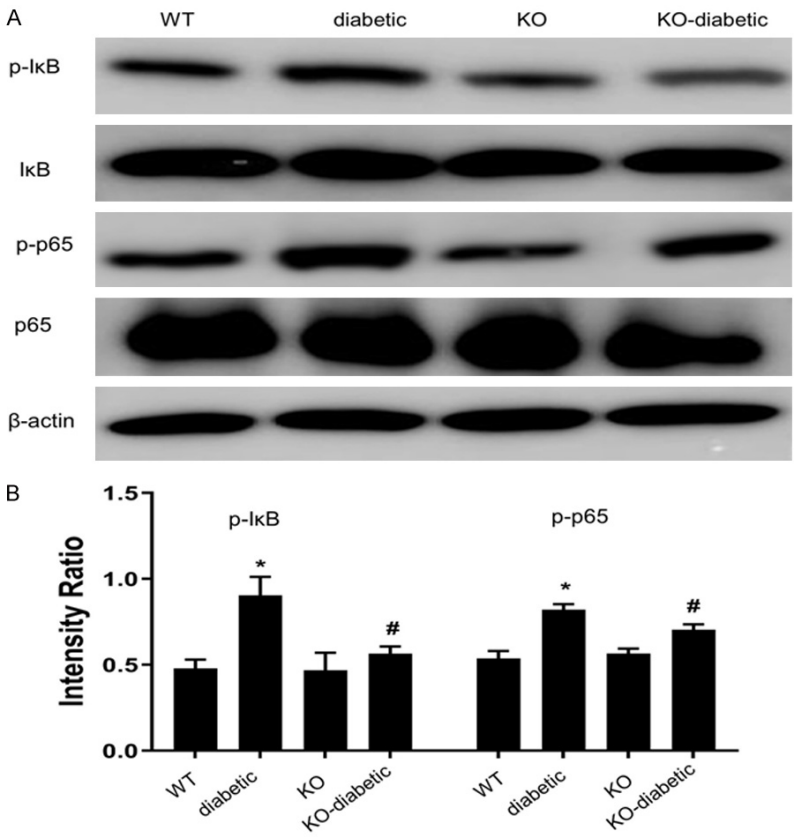

Figure 8. NF-κB p65 suppression in the kidneys (n=7 per group). A. The levels of p-p65 and p-IκB in the four mice groups were determined using western blot analyses. B. Intensity ratio of p-p65 and p-IκB in the four groups. Data represent the mean ± SEM of 6-8 mice. $^{*}P<0.05$ vs. WT, $^{\#}P<0.05$ vs. diabetic based on One-way ANOVA. Abbreviations: WT, wild type; KO, knockout.

dence that the knockout of Btk alleviates renal lesions by inhibiting the infiltration and activation of macrophages and suppressing the expression of pro-inflammatory factors through the NF-κB and MAPK signaling pathways. In summary, our study shows that Btk may provide a promising treatment strategy for halting the progression of DN.

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Disclosure of conflict of interest

None.

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