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Mannan-binding lectin ameliorates renal fibrosis by suppressing macrophage-to-myofibroblast transition

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ABSTRACT

Mannan-binding lectin (MBL) is a pattern-recognition molecule that plays a crucial role in innate immunity. MBL deficiency correlates with an increased risk of chronic kidney disease (CKD). However, the molecular mechanisms are not fully defined. Here, we established a CKD model in wild-type (WT) and MBL-deficient ($MBL^{-/-}$) mice via unilateral ureteral obstruction (UUO). The result showed that MBL deficiency aggravated the pathogenesis of renal fibrosis in CKD mice. Strikingly, the *in vivo* macrophage depletion investigation revealed that macrophages play an essential role in the MBL-mediated suppression of renal fibrosis. We found that MBL limited the progression of macrophage-to-myofibroblast transition (MMT) in kidney tissues of UUO mice. Further *in vitro* study showed that $MBL^{-/-}$ macrophages exhibited significantly increased levels of fibrotic-related molecules compared with WT cells upon transforming growth factor beta (TGF-β) stimulation. We demonstrated that MBL inhibited the MMT process by suppressing the production of matrix metalloproteinase 9 (MMP-9) and activation of Akt signaling. In summary, our study revealed an expected role of MBL on macrophage transition during renal fibrosis, thus offering new insight into the potential of MBL as a therapeutic target for CKD.

1. Introduction

Renal fibrosis, which results in end-stage renal disease together with tubular shrinkage and excessive extracellular matrix

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deposition, is thought to be the last characteristic that unites all chronic kidney diseases (CKD) [1,2]. Myofibroblasts are activated fibroblasts characterized by the expression of α-smooth muscle actin (α-SMA) and pathogenic collagen production [3]. At the later stage of CKD, macrophages contribute to eliminating fibrous tissue and mediating the resolution phase of collagen remodeling [4]. Indeed, cells co-expressing macrophage and myofibroblast markers were observed and participated in tissue fibrosis [5]. A previous study showed that the macrophage-myofibroblast transition (MMT) allows macrophages to develop into α-SMA-positive myofibroblasts in the damaged kidney [6]. The role of MMT in tissue scarring was manifested *in vivo*, and the macrophage-lineage myofibroblasts were found in the fibrotic kidney tissue of CKD mice [7]. Thus, it is crucial to broaden our understanding of the molecular mechanisms that control the MMT process during the pathogenesis of CKD.

The innate immune responses are the first line of inflammatory cells infiltrated into the injured kidney, which amplifies the inflammatory response to renal repair [8]. Pattern recognition receptors (PRRs) family is essential for controlling inflammatory cell activity and the innate immune response. Accumulating evidence indicates the importance of different PRRs in developing inflammation. MBL is a soluble PRR that is primarily produced in the liver and is presented in circulation [9,10]. Seyfarth [11] et al., demonstrated mbl2 gene expression in differentiated and lipopolysaccharide-treated THP-1 cells, a human monocyte cell line. We also observed the expression of MBL in the mouse BMDMs, and TGF-β stimulation significantly increased the MBL levels in the BMDMs (Supplementary Fig. 1). It is now evident that MBL deficiency seems to be a risk factor for several infectious and autoimmune diseases [12]. Increased serum MBL level correlates with the development of diabetic nephropathy [13]. Østergaard et al., demonstrated that MBL deficiency diminishes renal changes in the streptozotocin-induced mice model of type 1 diabetes [14]. MBL deficiency and MBL excess both have harmful effects on IgA nephropathy (IgAN) progression, indicating multiple roles of MBL in developing IgAN pathogenesis [15]. Notably, we previously found that MBL at high concentrations might inhibit the conversion of monocytes to dendritic cells (DCs) in the culture condition for DC differentiation [16]. Additionally, MBL interfered with the transition of monocytes into TRAP-positive osteoclasts, which contributed to the pathogenesis of inflammatory arthritis [17]. Upon lipopolysaccharide (LPS) stimulation, MBL can bind to monocytes and alters the expression of inflammatory cytokines [18,19]. However, it is unknown whether MBL affects the differentiation of monocytes into fibroblasts in kidney fibrotic progression, which needs thorough investigation.

The UUO method is the most widely used model of renal fibrosis in mice [20,21]. In this study, we used $MBL^{-/-}$ mice to generate a UUO model for evaluating the effect of MBL on the process of renal fibrosis, especially the function of macrophage in the MMT process. The data exhibited that MBL deficiency significantly aggravated UUO-induced renal fibrosis. MBL limited the MMT process in the kidney tissues of UUO mice and the TGF-β1-induced MMT process *in vitro*. Mechanistically, we demonstrated that MBL suppressed the Akt signaling and decreased matrix metalloproteinase 9 (MMP-9) expression, thereby inhibiting the MMT process. In summary, our study reveals that MBL might regulate the MMT process, which provides new insight into the disease mechanisms of CKD or other fibrotic-related diseases, especially in patients with MBL deficiency.

2. Materials and methods

2.1. Animals

Male MBL-deficient ($MBL^{-/-}$) C57BL/6J mice (8 weeks old, 20–23 g) were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in an animal facility at Southern Medical University (Guangzhou, China). Male C57BL/6J mice (8 weeks old, 20–23 g) were obtained from the Animal Institute of Southern Medical University. The 12-h light-dark cycle, a specified pathogen-free environment, and unlimited access to food and water were all provided for the mice. All animal experiments in this study were approved by the welfare and ethical committee for experimental animal care of Southern Medical University (Approval No: L2023077).

2.2. UUO-induced CKD

WT and $MBL^{-/-}$ mice were given general anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg body weight). A left flank incision was used to expose the left ureter, which was then cut between the two places of ligation using 4-0 silk. There was no ligation in the Sham-operated group. On days 7 and 14 following surgery, the mice were euthanized with 4 % isoflurane inhalation, and the left kidneys were taken.

2.3. Macrophage depletion

For the purpose of depleting macrophages *in vivo*, the mice were administered intraperitoneally with 2 mg of liposomes containing clodronate (88416-50-6, Sigma-Aldrich, St. Louis, MO, USA) or a control substance three days before UUO induction.

2.4. Pathological analyses

According to routine protocols, the histological sections of the kidney (5 μm) were dyed with hematoxylin-eosin (H&E) to observe morphological changes. Following the manufacturer's directions, periodic acid-schiff, sirius red, and masson's trichrome staining were used to identify extracellular matrix buildup in the kidney. The histopathologically altered H&E stained slides were scored by a quantitative percentage of the damaged area, as follows: 0, 0%–5%; 1, 5%–10 %; 2, 11%–25 %; 3, 26%–45 %; 4, 46%–75 %; and 5, >76 %. The three fields analyzed in each section were selected at random. To evaluate tubulointerstitial collagen deposition, three

randomly selected fields in each section stained with masson's trichrome. The area stained in light blue in the interstitium was semiquantitatively calculated using ImageJ analysis software.

2.5. Western blotting analysis

Protease and phosphatase inhibitor cocktail (P1046, Beyotime, Shanghai, China) were added to RIPA buffer to prevent proteolysis and maintain phosphorylation of proteins. Protein lysates were denatured, then resolved by SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were first treated with the primary antibodies (alpha-smooth muscle actin polyclonal antibody, 14395-1-AP; fibronectin polyclonal antibody, 15613-1-AP; collagen type I polyclonal antibody, 14695-1-AP; MMP9 polyclonal antibody, 10375-2-AP; β-actin polyclonal antibody, 20536-1-AP; proteintech, Chicago, IL, USA) and then secondary antibody (HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L), SA00001-2; proteintech) before being blocked in phosphate-buffered saline (PBS) that containing 0.1 % Tween20 and 2 % BSA. Finally, Novex™ ECL Chemiluminescent Substrate Reagent Kit (WP20005, Thermo Fisher, Carlsbad, CA, USA) was used to detect the target protein in accordance with the manufacturer's instructions. The protein bands were quantitatively analyzed using ImageJ software 1.52a.

2.6. Quantitative RT-PCR analysis

TRIzol reagent (15596026CN, Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cultivated cells. A PrimeScript RT-PCR Kit was utilized to create first-strand cDNA. Real-time PCR was used to measure the amounts of mRNA encoding fibronectin, collagen I, α-SMA, and MMP-9. The results were standardized to the expression of GAPDH. The relative changes in expression were computed using the $2^{-\Delta\Delta Ct}$ change-in-cycling-threshold approach. The sequences of the primers used for PCR are listed in Table 1.

2.7. Immunofluorescence staining

Paraffin-embedded mice kidney tissue sections were dewaxed in Histo-Clear (National Diagnostics, Atlanta, GA, USA) followed by graded ethanol solutions. The tissue slides were placed in an antigen retrieval unit with pH 8.0 EDTA, then incubated in 10 % normal goat serum for 1 h. Kidney tissue sections were incubated with primary antibody (α-SMA polyclonal antibody, 14395-1-AP, proteintech) at 4 °C for overnight and then incubated with secondary antibody (CoraLite488-conjugated Goat Anti-Rabbit IgG(H + L), SA00013-2; proteintech).

Cells were fixed with 4 % paraformaldehyde in PBS on ice for 10 min. After fixation, cells were incubated with PBS containing 0.2 % Triton X-100 for 5 min. Subsequently, the cells were then exposed to α-SMA antibodies (14395-1-AP, proteintech) for 1 h at room temperature. For confocal imaging, secondary antibodies conjugated with Alexa-488 were employed at 1/500 (Invitrogen, A-11011). DAPI was used as a counterstained for nuclei. Images All measurements were carried out conditionally blind.

2.8. Flow cytometry

The kidney tissues were decapsulated, diced, and incubated with Liberase (0.5 mg/ml; Roche, 5401119001) and DNase (100U/ml; Roche, 10104159001) in HBSS at 37 °C for 30 min. The leukocytes in kidney were enriched by a discontinuous percoll gradient (33 % and 66 % in PBS) followed by centrifugation for 20 min as previously described [22]. The isolated cells were incubated with 0.5 μg of purified CD16/CD32 monoclonal antibody (MA5-18012, Invitrogen) per million cells for 20 min on ice prior to FACS staining. Subsequently, the cells were surface-stained with PE-labeled F4/80 monoclonal antibody (12-4801-82, Invitrogen), fixed, permeabilized, and stained with Alexa Fluor™ 488-labeled α-SMA monoclonal antibody, (53-9760-82, Invitrogen). 7-Aminoactinomycin D (7-AAD) (A1310, Invitrogen) was used to stain and exclude dead cells in the flow cytometry analysis. The stained cells were examined using the BD FACSDivaTM software and the FACS LSRFortessaTM flow cytometer (version 8.0.1; BD Biosciences).

2.9. Preparation and stimulation of bone marrow-derived macrophages

Bone marrow cells were isolated from WT or $MBL^{-/-}$ mice by washing the femur and tibia with Dulbecco's modified Eagle medium (DMEM)/F12 medium as described previously [23]. To prevent fibroblast contamination, cell suspensions were moved into culture flasks the following day. After 5 days of stimulation in DMEM/F12 containing 10 % fetal bovine serum (FBS) and 10 ng/mL macrophage colony-stimulating factor, cell suspensions were differentiated into macrophages. After that, TGF-β1 (5 ng/mL)

Table 1
List primer sequences used for RT-PCR analysis in this study.

	Forward primer (5′-3′)	Reverse primer (5′-3′)
Fibronectin	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
Collagen I	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
α-SMA	TCAGGGAGTAATGGTTGGAATG	CCAGAGTCCAGCACAATACCAG
MMP9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
GAPDH	GGCATCCTCACCCTGAAGTA	GGGGTGTTGAAGGTCTCAAA

stimulation in DMEM/F12 containing 1 % FBS stimulated MMT on the second passage of bone marrow-derived macrophages (BMDMs). To eliminate the effect of the Akt, 10 μM of MK2206 (S1078, Selleck, Houston, TX, USA) was added simultaneously with TGF-β1 (5 ng/mL). On day 5 following TGF-β1 stimulation, MMT-derived myofibroblasts were recognized by flow cytometry based on the co-expression of macrophage (F4/80) and myofibroblast (α-SMA) markers.

2.10. Isolation of kidney macrophages

Kidney was minced into small fragments and incubated in collagenase solution (collagenase type I [45 U/ml], collagenase type II [15 U/ml], collagenase type III [45 U/ml], collagenase type IV [45 U/ml]), elastase [0.08 U/ml], hyaluronidase [30 U/ml], and DNAse type I [25 U/ml] in RPMI-1640) on a shaker at 37 °C for 40 min. Dissociated cells were passed through a 70 μm cell strainer and washed twice in PBS. A MagniSort mouse F4/80 positive selection kit (8802-6863-74, Thermo Fisher) was then used for the separation of $F4/80^{+}$ cells in single-cell suspensions by positive selection.

2.11. Statistical analysis

The statistical evaluations were performed using GraphPad Prism 8.0.1. The mean and standard deviation (SD) of the data are displayed. Two unpaired groups' differences were compared using an unpaired Student's *t*-test. A statistically significant difference was defined as $p < 0.05$. Triplicates of each experiment were independently performed again.

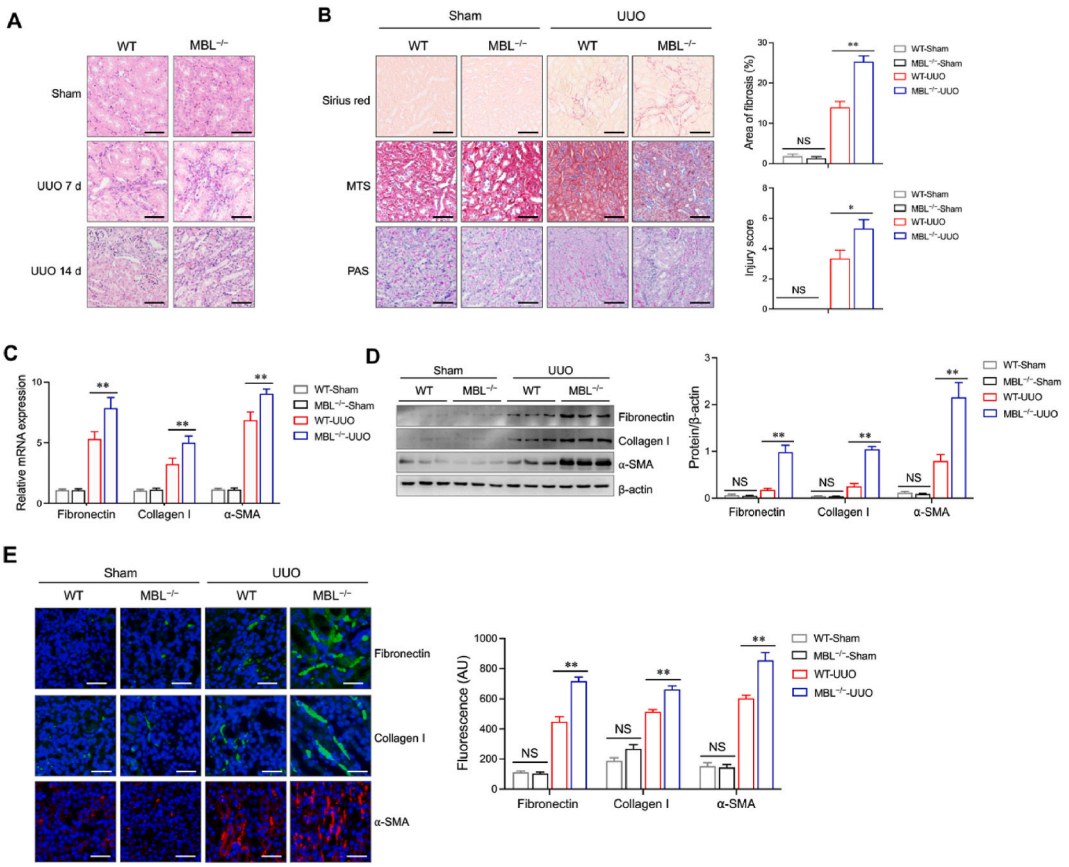

Fig. 1. MBL deficiency aggravated UUO-induced renal fibrosis in mice
WT and $MBL^{-/-}$ mice (n = 6) chronic kidney disease was induced by UUO. (A) Histopathological evaluation of renal fibrosis was performed with H&E staining. Scale bars = 100 μm. (B) Deposition of collagen in the kidney from WT and $MBL^{-/-}$ mice were determined by sirius red staining and Masson's trichrome staining after 2 weeks with UUO. Accumulation of glycogen or polysaccharides was assessed by periodic acid-schiff (PAS) staining. Scale bar = 100 μm. (C) The mRNA level of fibrosis-related genes, α-SMA, collagen I, and fibronectin, was detected by quantitative q-PCR analysis and expressed as a ratio to GAPDH. The protein level of α-SMA, collagen I, and fibronectin was evaluated by immunoblotting analysis (D) and immunofluorescence analysis (E), respectively. Scale bar = 50 μm**p < 0.01. Data are representative of three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

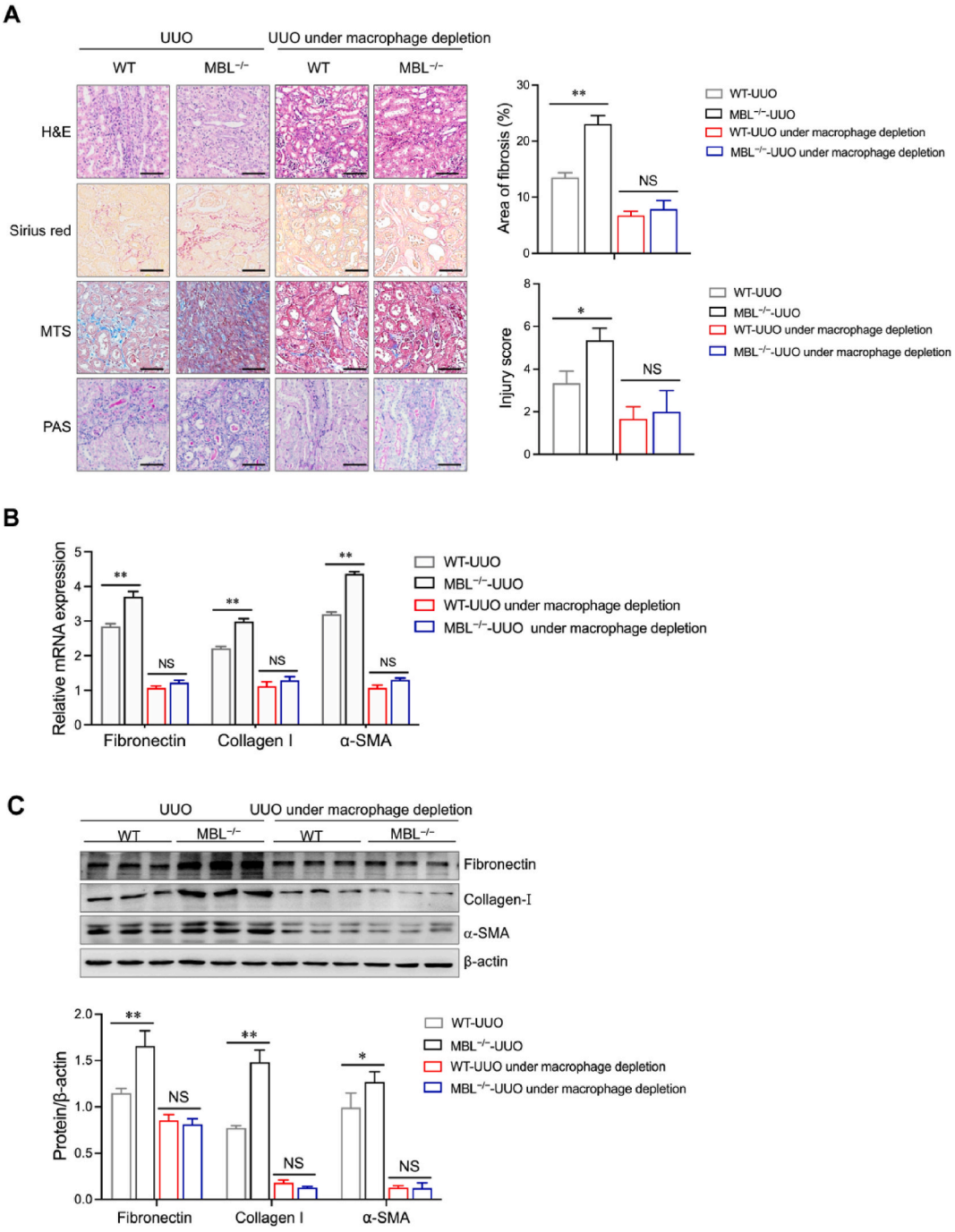

Fig. 2. Macrophages are involved in MBL inhibiting renal fibrosis
The WT and MBL$^{-/-}$ mice (male, n = 6, per group) were treated with 2 mg clodronate-containing or control liposomes 3 days before UUO. (A) Histopathological evaluation of the renal fibrosis was performed with H&E, sirius red, MTS, and PAS staining. (B) The mRNA level of α-SMA, collagen I, and fibronectin in kidney was detected by q-PCR analysis and expressed as a ratio to GAPDH. (C) The protein level of α-SMA, collagen I, and fibronectin was evaluated by immunoblotting analysis. **p < 0.01. NS, not significant. Data are representative of three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. MBL deficiency enhances renal fibrosis in UUO mice

To elucidate the function of MBL in renal fibrosis, we investigated whether MBL deficiency would affect the pathogenesis of UUO-induced renal fibrosis in mice. After 14 days, H&E and Masson's trichrome staining showed that MBL deficiency significantly increased collagen deposition and fibrotic lesions (Fig. 1A and B). We assessed the fibrotic-related molecules and found that the mRNA expression of collagen I, fibronectin, and α-SMA was significantly enhanced in the UUO-treated MBL$^{-/-}$ mice compared to those in the WT controls (Fig. 1C). Immunoblotting and immunostaining analyses also confirmed the accumulation of interstitial matrix proteins (Fig. 1D and E). Overall, these results indicated that MBL deficiency aggravates the pathogenesis of renal fibrosis.

3.2. Macrophages are critical mediators in the MBL-mediated inhibition of renal fibrosis

Macrophages are the master regulators in kidney diseases and are related to the development of renal fibrosis in CKD [24]. To further clarify the effect of macrophages on MBL-mediated regulation of renal fibrosis, mice were injected with clodronate liposome to deplete the macrophage population *in vivo*. The data showed that renal fibrosis was comparable in WT and MBL$^{-/-}$ CKD mice after macrophage depletion (Fig. 2A). Notably, the levels of fibrotic-related molecules were similar in the kidney of WT and MBL$^{-/-}$ CKD

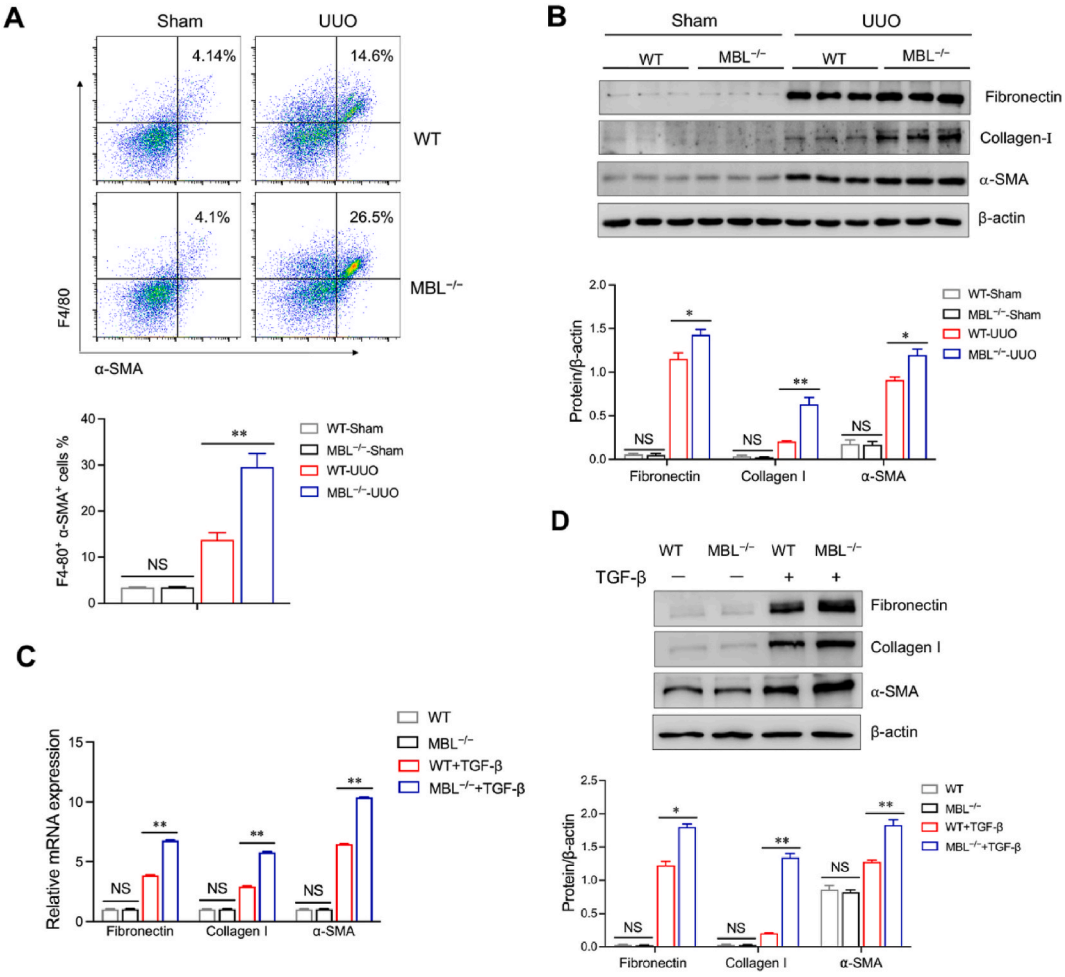

Fig. 3. The MMT process is increased in MBL-deficiency CKD mice
WT and MBL$^{-/-}$ mice CKD were induced by UUO for 14 days. (A) The frequency of F4/80^{+} α-SMA^{+} in the kidney was analyzed by flow cytometry at 2 weeks after UUO. (B) Macrophages in the kidney were sorted by mouse F4/80 positive selection kit. The protein level of α-SMA, collagen I, and fibronectin of macrophages in the kidney from WT and MBL$^{-/-}$ mice was evaluated by immunoblotting analysis. (C, D) Primary BMDMs derived from WT and MBL$^{-/-}$ mice were cultured with TGF-β1 (5 ng/mL) for 5 days. (C) The mRNA expression of α-SMA, collagen I, and fibronectin in myofibroblast differentiation was analyzed by qRT-PCR. (D) The protein level of α-SMA, collagen I, and fibronectin was evaluated by immunoblotting analysis. **$p < 0.01$. Data are representative of three independent experiments with similar results.

mice after macrophage depletion (Fig. 2B and C). Immunofluorescence staining also showed that fibrotic protein expression was similar between WT and MBL$^{-/-}$ mice upon macrophage depletion (Fig. 2D). These findings indicated that MBL regulated macrophage function during CKD pathogenesis.

3.3. MBL deficiency facilitates the MMT process in the CKD mice

Accumulating evidence pointed out that macrophages have essential roles in the maintenance of renal homeostasis [24]. It should be noted that macrophages were found to transform into myofibroblasts in the kidneys of renal fibrosis patients and UUO-induced renal fibrosis mice [6,24]. To determine whether MBL participates in the MMT process, F4/80 and α-SMA double staining was performed on the mononuclear cells of the kidney isolated from UUO-treated MBL$^{-/-}$ and WT mice. Flow cytometry analysis showed that the percentage of F4/80^{+}α-SMA^{+} myofibroblasts in the kidney from UUO-treated MBL$^{-/-}$ mice was higher than that from WT counterparts (Fig. 3A). Immunoblotting analysis confirmed the increased levels of fibrotic proteins in the renal F4/80^{+} macrophages from MBL$^{-/-}$ CKD mice (Fig. 3B). Next, we examined whether MBL affected the MMT process *in vitro*. Primary bone marrow cells were cultured with M-CSF in the presence of TGF-β, which is known to induce myofibroblast formation [20]. Upon the stimulation, MBL$^{-/-}$ macrophages showed significantly increased mRNA expression levels of several fibrotic-related genes, including fibronectin, collagen-I, and α-SMA, compared with WT cells (Fig. 3C). The immunoblotting analysis further confirmed the increased protein level of fibrotic in MBL$^{-/-}$ mice compared to WT mice (Fig. 3D). These data indicated that MBL might inhibit the MMT process during renal fibrosis.

3.4. MBL affects the MMT process by regulating MMP-9 expression

MMPs are the multifunctional proteases that have been traditionally linked to the remodeling and degradation of the ECM [25]. Among them, MMP-9 is one of the major components which is crucial to the development of renal fibrosis [26]. We observed an increased expression of MMP-9 in renal isolated from MBL$^{-/-}$ UUO mice compared with those from WT controls (Fig. 4A and B). Intriguingly, the MMP-9 level was strongly reduced in CKD mice and was comparable between MMT cells isolated from the kidney of WT and MBL$^{-/-}$ CKD mice upon macrophage depletion (Fig. 4C). MBL suppressed the MMP-9 production during the TGF-β-mediated differentiation of bone marrow-derived macrophages into myofibroblasts, according to *in vitro* experiments as well (Fig. 4D and E).

To assess the effect of MMP-9 on MBL-regulated MMT process, macrophages were pre-treated with an MMP-9 inhibitor, MMP-9-IN-1, before TGF-β stimulation. The levels of myofibroblast markers were significantly decreased and comparable between TGF-β-induced myofibroblast differentiation in WT and MBL$^{-/-}$ cells upon MMP-9 blockade (Fig. 5A–C). These results suggest that MBL-mediated regulation of myofibroblast differentiation depends on the MMP-9 expression.

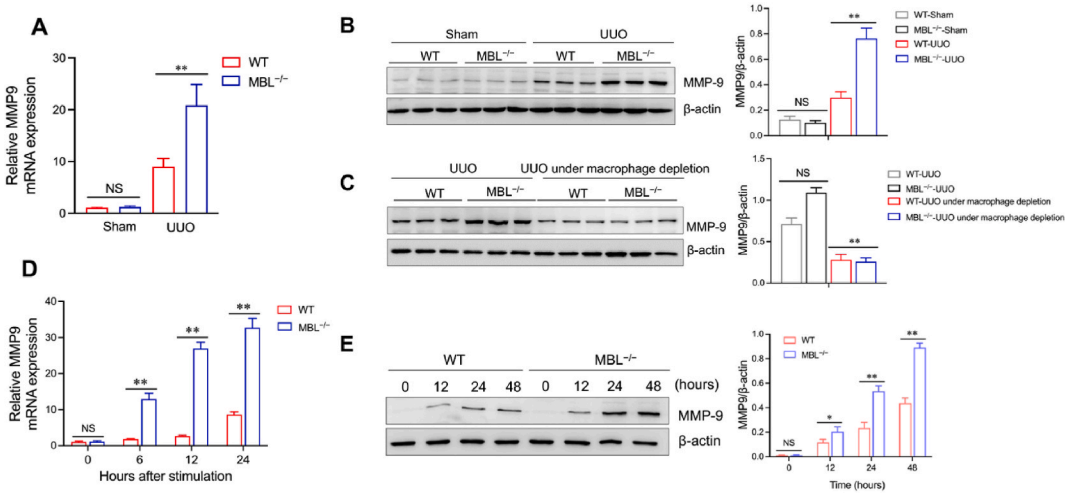

Fig. 4. MBL inhibits MMP-9 expression during the MMT process (A, B) WT and MBL$^{-/-}$ mice (n = 6) chronic kidney disease was induced by UUO for 14 days. F4/80^{+} macrophages of kidney tissue were isolated on the last day of the experiment. The mRNA level of MMP-9 in F4/80^{+} macrophages from mice kidney was examined by qRT-PCR analysis (A), and the protein level was determined by Western blot analysis (B). (C) Western blot analysis determined the MMP-9 protein level in kidney tissue from UUO mice after macrophage depletion. (D, E) Primary BMDMs from WT and MBL$^{-/-}$ mice were cultured with TGF-β1 (5 ng/mL) for 12, 24, 48 h. The mRNA level of MMP-9 in cells was assessed by qRT-PCR analysis (D), and the protein level of MMP-9 was determined by Western blot analysis (E). **p < 0.01. Data are representative of three independent experiments with similar results.

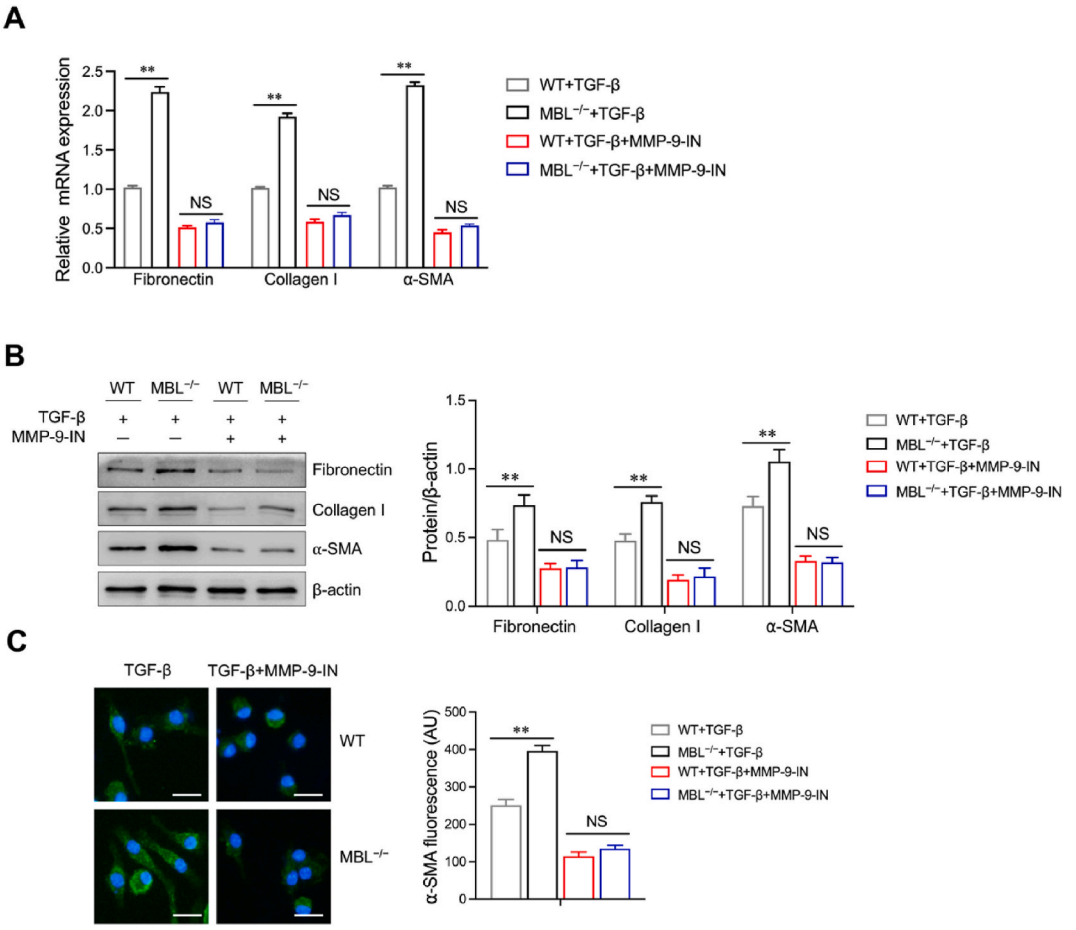

Fig. 5. MBL inhibits MMT via downregulating MMP-9 expression
Primary BMDMs from WT and MBL$^{-/-}$ mice were cultured with TGF-β1 (5 ng/mL) for 5 days with or without MMP-9 inhibitor, MMP-9-IN. (A) The mRNA expression of α-SMA, collagen I, and fibronectin in myofibroblast differentiation was analyzed by qRT-PCR. (B) The protein level of α-SMA, collagen I, and fibronectin in MMT was analyzed by immunoblotting assay. (C) α-SMA level in the cells was analyzed by immunofluorescence. **p $<$ 0.01. NS, not significant. Data are representative of three independent experiments with similar results.

3.5. Akt activation is involved in MBL-enhanced MMP-9 expression and further MMT process

PI3K/Akt is an upstream component of the JNK1/2 pathway for MMP-9 expression [27]. To provide insights into the molecular mechanisms underlying the modulation of MMP-9 expression by MBL, we investigated Akt pathway activation in WT and MBL$^{-/-}$ macrophages cultured with TGF-β. The phosphorylation of Akt was increased in MBL$^{-/-}$ macrophages stimulated with TGF-β compared with WT controls (Fig. 6A and B). The inhibition of Akt signaling by MK2206 decreased the MMP-9 expression in macrophages, and the MMP-9 level was similar between TGF-β-treated WT and MBL$^{-/-}$ macrophages in the presence of MK2206 (Fig. 6C). The levels of myofibroblast differentiation-related markers were reduced and comparable between TGF-β-induced myofibroblast differentiation in WT and MBL$^{-/-}$ macrophages upon Akt signaling blockade (Fig. 6D and E). These findings imply that MBL-mediated regulation of MMP-9 production and the MMT process depends on the Akt signaling pathway (see Fig. 7).

4. Discussion

MBL is a plasma collectin that has a collagen-like domain, which is thought to be a crucial component of innate immunity [10]. In the current study, we observed that MBL deficiency aggravated UUO-induced CKD in mice, which was associated with an enhanced MMT process. Our findings showed that MBL suppressed the MMT progression by inhibiting Akt pathway activation and suppressing MMP-9 production.

The last stage of chronic kidney disease is renal fibrosis, which is characterized by tubulointerstitial fibrosis and glomerulosclerosis [1]. The Unilateral Ureteral Obstruction (UUO) model is used to cause renal fibrosis, where the primary feature of UUO is tubular injury as a result of obstructed urine flow. The UUO-obstructed kidney is characterized by tubular dilation, interstitial expansion, loss of proximal tubular mass, hypertrophy, hydronephrosis, infiltration of leukocytes, tubular epithelial cell death, and presence of

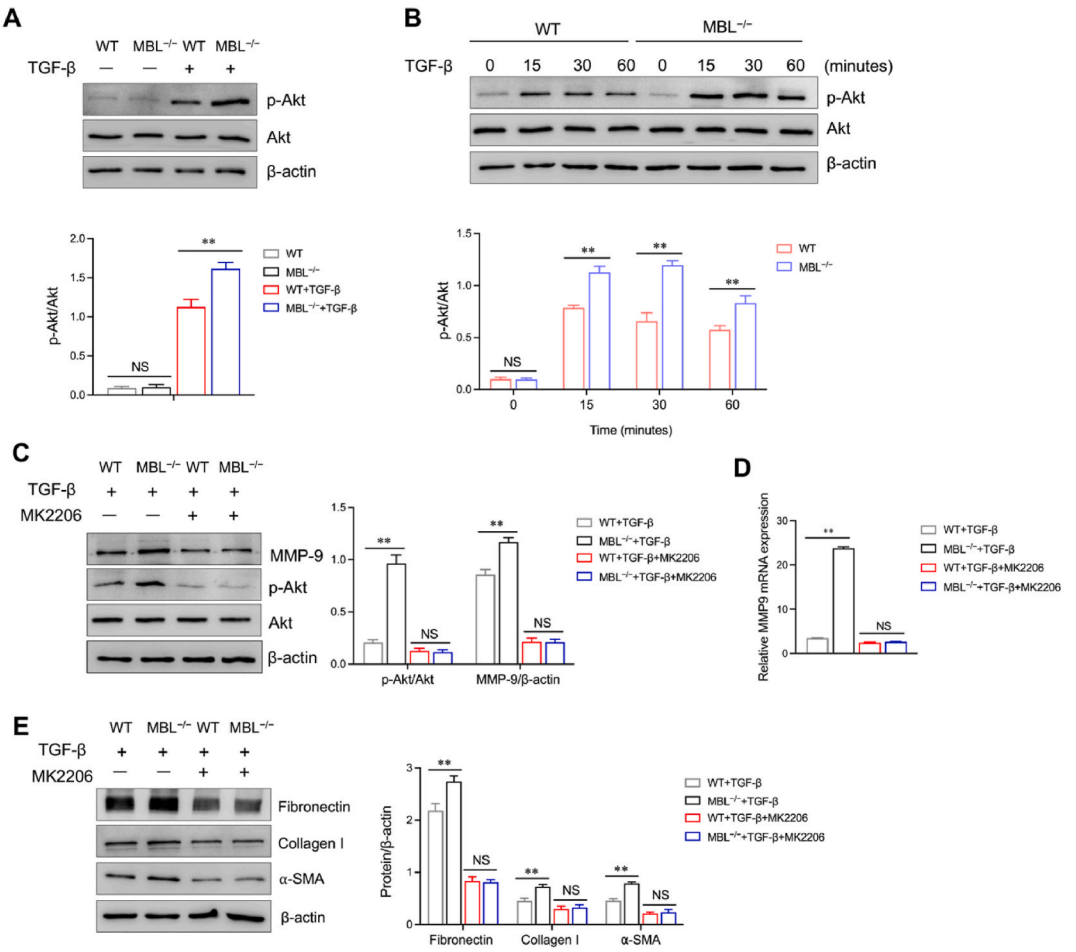

Fig. 6. MBL suppresses *p*-Akt/MMP 9 activation during the MMT process
Primary BMDMs from WT and MBL$^{-/-}$ mice were cultured with TGF-β1 (5 ng/mL) for 30 min. (A) The protein level of *p*-Akt and Akt in macrophages was analyzed by immunoblotting. (B) The protein level of *p*-Akt and Akt in macrophages was analyzed by immunoblotting after being treated with TGF-β1 (5 ng/mL) for indicated time points. (D–E) Primary BMDMs from WT and MBL$^{-/-}$ mice were cultured with TGF-β1 (5 ng/mL) for 5 days in the presence of MK2206 (10 μM). (C) The protein level of *p*-Akt, Akt, and MMP-9 in macrophages was analyzed by immunoblotting. The mRNA expression (D) and the protein level (E) of α-SMA, collagen I, and fibronectin in myofibroblast differentiation were analyzed by q-PCR and western blotting, respectively. **p < 0.01. NS, not significant. Data are representative of three independent experiments with similar results.

fibroblasts [28]. Activated myofibroblasts are a crucial kind of matrix-secreting cell type that is important in the development of renal fibrosis [3,29]. Myofibroblasts are a diverse population originating from various sources, such as local fibroblast or pericyte and epithelia through epithelial-to-mesenchymal transition and local fibroblast or pericyte proliferation [30]. Monocytes and macrophages are now known to play a crucial part in developing renal fibrosis [24], and the MMT process has been identified as another extrarenal origin for myofibroblasts [31]. In addition to MBL, other complement molecule C3 is commonly found in the afflicted glomeruli and is associated with poor renal outcomes in focal segmental glomerulosclerosis [32]. In UUO mice, C3 deficiency greatly decreased tubular atrophy and interstitial fibrosis. C3 deficiency reduced an epithelial-to-mesenchymal transition within the kidney [33]. Herein, we found that MBL$^{-/-}$ UUO mice exhibited severe renal fibrosis accompanied by increased MMT compared with WT counterparts, implying that MBL suppresses the etiology and progression of CKD. We speculate that complement (including C3a and MBL) affects the transition of epithelial cells and macrophages to myofibroblasts through different mechanisms, which warrants further investigation. It is well known that the MBL-initiated lectin pathway is an essential part of the complement system [10]. We, therefore, assumed that MBL might be critical in regulating macrophage differentiation through the MBL-dependent complement lectin pathway in the pathogenesis of CKD. On the other hand, the interaction of MBL with meprins, which are known to be some of the major matrix-degrading metalloproteases in the kidney, resulted in significant decreases in the proteolytic activity and matrix-degrading ability of meprins [34].

Our observations indicate that MBL has potent activity in suppressing the expression of myofibroblast markers, such as α-SMA, collagen I, and fibronectin, in TGF-β treated macrophages. It is noteworthy to mention that CD68^{+} macrophages and a-SMA^{+} myofibroblasts colocalize in areas of active interstitial fibrosis in renal allografts [35]. Indeed, both F4/80 and CD68 can be used as markers

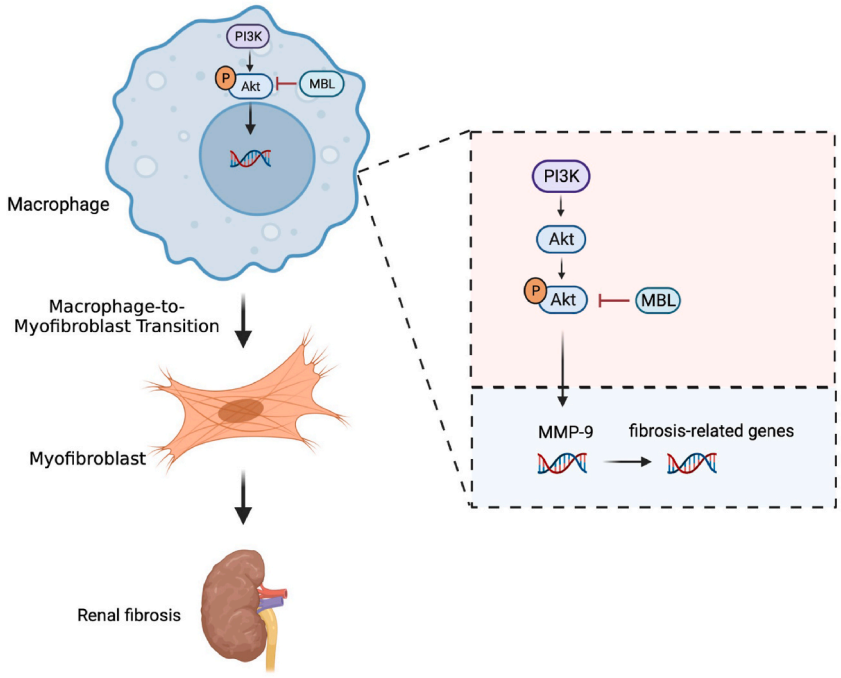

Fig. 7. A model of the proposed mechanism by MBL ameliorates renal fibrosis
The proposed scheme shows that MBL downregulates the phosphorylation of Akt and subsequently reduces the expression of MMP-9, leading to reduction of macrophage-to-myofibroblast transition and remission of renal fibrosis.

of macrophages (including myofibroblasts transformed from macrophages). Macrophages-series of antibodies are overlapping while F4/80 best distinguishes macrophages from FSP1^{+} fibroblasts, and is the preferred lineage marker for macrophages [36]. Although proinflammatory and profibrotic cytokines and growth factors produced from macrophages may indirectly drive the process of renal fibrosis, a direct connection between inflammatory macrophages and myofibroblast formation during chronic allograft rejection has not yet been elucidated [37]. In patients with prolonged active allograft rejection, CD68^{+} macrophages were associated with interstitial fibrosis development and loss of allograft function [5]. MBL promotes M1 macrophage polarization by activating the NF-κB pathway in diabetic nephropathy [13]. Guo et al., reported that MBL deficiency is a risk factor for poor renal outcomes in IgAN [15]. In this study, we provided evidence that MBL might limit the TGF-β-mediated MMT process, which significantly expanded our knowledge of the pathophysiology of renal fibrosis in patients with low MBL levels.

MMPs are known to play essential roles in the condition of inflammation and are implicated in the initiation and progression of renal fibrosis [38]. Patients with CKD have been found to have higher levels of MMP2 and MMP-9 in serum, and these elevated MMPs levels may act as biomarkers for these patients [39]. As one of the significant sources of MMPs, macrophage has been related to the excessive accumulation of myofibroblasts in CKD patients [40]. MMP-9 has been reported to be secreted by activated macrophages, leading to renal fibrosis [38]. Our previous study demonstrated that MBL suppressed MMP-9 expression in differentiated human osteoclasts, impairing osteoclast differentiation *in vitro* [17]. The present study showed that MBL inhibited the MMT process in kidney by downregulating MMP-9 expression. We postulated that MBL/MMP-9 axis plays a critical role in the macrophage transition and/or differentiation during inflammatory responses.

Several classical signaling pathways are involved in renal fibrosis, including TGF-β/Smad, JAK/STAT, NF-κB, Notch, Wnt, Hedgehog, PI3K/AKT, etc. Some of these signal pathways participate in the progression of renal fibrosis by controlling the MMT procedure [6]. In mouse embryonic fibroblasts and fibroblasts, Akt mediated TGF-β-induced α-SMA synthesis through the contractile gene transcription factors myocardin and serum response factor (SRF), independent of mammalian target of rapamycin (mTOR) [41]. Tumor necrosis factor (TNF)-induced endothelial-to-mesenchymal transition in renal cancer cells is related to MMP-9 activation in the PI3K/Akt signaling pathway [42]. In head and neck squamous cell carcinoma, PI3K/Akt activation boosts MMP-9 expression and encourages cell invasion and migration [43]. Lin et al., discovered that MMP-9 released by tumor-associated macrophages promotes the EMT process via PI3K/Akt-dependent pathway in gastric cancer [44]. Herein, we have also shown that MBL suppresses the MMT process by inhibiting the activation of the Akt signaling pathway. In conclusion, our study revealed an unexpected function of MBL in the MMT process, thus providing new insight into CKD and other macrophage-related diseases in patients with MBL deficiency.

Data availability statement

Data will be made available on request.

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Author contributions

Li Xu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Honglian Jiang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Jingwen Xie: Performed the experiments; Analyzed and interpreted the data. Qishan Xu: Performed the experiments; Analyzed and interpreted the data. Jia Zhou: Performed the experiments. Xiao Lu: Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data. Mingyong Wang: Contributed reagents, materials, analysis tools or data. Lijun Dong: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper. Daming Zuo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ethics approval and consent to participate

No application.

Consent for publication

We all agree to publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21882.

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