Mannose-binding lectin activates the nuclear factor-κB and renal inflammation in the progression of diabetic nephropathy

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Abstract
Increased serum mannose-binding lectin (MBL) level has been proven to correlate with the development of diabetic nephropathy (DN). Here, we aim to find the role and mechanism of MBL involved in the progression of DN. Patients with DN were recruited and divided into two groups according to different rs1800450 genotypes of the MBL2 gene, and inflammatory profiles in monocytes/macrophages were compared between the two groups. MBL was given to treat macrophages, HK2, and HMC, and a co-culture transwell system was then employed. Renal inflammation and fibrosis parameters were measured after knocking down or overexpressing MBL genes in mice. Proinflammatory profile, manifesting as enhanced IL-1β production and M1 polarization, was found in monocytes/macrophages from DN with a rs1800450 GG genotype who had higher MBL level, compared with those with a rs1800450 GA genotype. In mechanism, MBL directly induced inflammatory responses in macrophages, which promoted inflammatory and fibrotic markers in HK2 and HMCs during co-culture. Further experiments showed that MBL can promote macrophages transforming to the M1 subset mainly by activating the nuclear factor-κB pathway. After downregulation of MBL, the blood glucose, triglyceride, urine protein, injuries of glomerulus and tubules, and the degree of renal inflammation and fibrosis were ameliorated in db/db mice treated with AAV-MBL1/2-shRNA. Overexpression of MBL promoted macrophage infiltration in the kidney. In conclusion, MBL is a crucial mediator in the progression of DN via activating the nuclear factor-κB pathway in
macrophages. This will serve as a genetic base for some patients with DN who have poor outcomes and provide a direction for the screening.

**KEYWORDS**
diabetic nephropathy, macrophage, mannose-binding lectin, nuclear factor-κB

## 1 | INTRODUCTION

As one of the major complications of diabetic mellitus, diabetic nephropathy (DN) is the leading cause of chronic kidney disease (CKD) and imposes a huge burden on the global economy and health care.1 Currently, renin-angiotensin system blockade in addition to strict glycemic and blood pressure control has been shown to slow down the progression of DN. Sodium-glucose cotransporter 2 (SGLT2) inhibitors are recent new target treatments for maintaining glucose homeostasis in diabetic patients, and renoprotection by SGLT2 inhibitors has been documented in type 2 diabetes patients with a high cardiovascular risk.2 However, a substantial proportion of patients still progress to end-stage renal disease (ESRD),3 and further understandings on the pathogenesis, as well as novel intervention, are still urgently needed for treating DN.

Accumulated evidence indicates a critical involvement of chronic inflammation in the development of DN.4,5 Activation of innate immunity, conjoined with activated intrinsic renal cells, produces profibrotic cytokines and growth factors, which eventually leads to fibrosis. As a key soluble pattern recognition molecule in the innate immune system and the upstream of the lectin pathway, mannose-binding lectin (MBL) has been implicated in the development of DN.6 As reported in an early study, polymorphisms in the MBL gene and serum MBL level were associated with vascular complications in type 1 diabetic patients.7 A study by Li et al. showed that serum and urine MBL levels were higher in DN patients than diabetic patients without kidney damage, and urine MBL level correlated with urine protein level.8 Our group performed a follow-up study on DN patients and found augmented serum MBL level as an independent risk factor for DN progression to ESRD.9 In addition, we identified single nucleotide polymorphism (SNP) rs1800450 GA genotype of MBL2 gene as an independent protective factor for ESRD since patients with rs1800450 GA genotype had lower serum MBL level than patients with rs1800450 GG homozygotes.9 Nevertheless, the exact role of MBL in the progression of DN and the underlying mechanism needs further investigation.

Macrophage has been proven as the major inflammatory cell type in DN, and renal infiltration of these cells is associated with DN progression to ESRD.10,11 In animal models, depleting macrophages in the kidney prevents renal inflammation, alleviate albuminuria and the severity of DN.12 The function of macrophage is regulated by a palette of cytokines and chemokines, such as interleukin (IL)-6, tumor necrosis factor (TNF), and C–C motif chemokine ligand 2 (CCL2).13 Targeting C-C chemokine receptor type 2 (CCR2) with an antagonist has been proven as reno-protective on top of the standard care of patients with type 2 diabetes and nephropathy.14 Growing evidence suggests that macrophages are a spectrum of heterogenous cells, and renal inflammation and fibrosis are also very complex processes, all of which necessitate further investigation in the inflammatory mechanisms in DN.15,16

In the current study, we hypothesized that MBL promotes renal inflammation and fibrosis during DN progression. We compared the inflammatory profiles of monocytes/macrophages from DN patients who carried SNPs rs1800450 GA or GG phenotypes of the MBL2 gene. We further performed MBL gene knockdown and overexpression in murine models as well as in vitro experiments on cell lines including U937 macrophages, human tubular epithelial cells (HK2), human mesangial cells (HMCs). This study may provide new insights into the understanding of DN progression.

## 2 | MATERIALS AND METHODS

### 2.1 | Human subjects

To measure the downstream molecules of the lectin pathway, the serum samples from the patient cohort as described in our previous study were further studied.9 The diagnosis of DN was based on the criteria proposed by the renal pathology society in 2010.17 The clinical characteristics, laboratory examination, and pathological parameters were collected from the hospital information system. Serum samples were collected on the same day of renal biopsy. To perform RNA sequencing on monocyte-derived macrophages, 3 healthy donors were recruited, and 10 ml of blood were collected. For analyzing the profile of monocytes/macrophages from different rs1800450 SNPs, 4–6 ml peripheral blood from DN patients was collected. Blood samples from 11 patients were used to analyze
the inflammatory status of monocytes using sorting, and blood samples from other 8 patients were collected to measure the iNOS expression of macrophages after culturing with M-CSF. The MBL gene rs1800450 SNP genotype of all 19 patients was identified as described in our previous study, and the influence from different rs1800450 phenotypes on monocytes/macrophages inflammatory profiles were determined. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (reference number 2021106), and informed consent was obtained from all human subjects.

2.2 | Cell culture

HK2 cell line was purchased from the American Type Culture Collection and cultured in RPMI 1640 medium (Sigma, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (Gibco America) and 1% penicillin/streptomycin (Gibco America). The cell line human mesangial cells (HMCs) were purchased from the American Type Culture Collection and cultured in Dulbecco’s Modified Eagle’s Medium (Gibco America) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. U937 cell line was provided by Z. Chen (Zhejiang University School of Medicine, Hangzhou, China) and was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. U937 cells were differentiated into macrophages by stimulation with 100 ng/ml phorbol 12-myristate 13-acetate for 24 h before use. All cells were cultured in a 37°C incubator containing 5% CO₂. Cells were plated into either 6-well or 12-well plates induced by 10 μg/ml recombinant MBL (R&D systems, Minneapolis, MN, USA; this reagent was derived from HEK293 cells, with endotoxin level <0.1 EU/μg protein measured by the Limulus amebocyte lysate method). For transfection of siRNA-p65 (GenePharma, Shanghai, China), Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer’s instructions.

2.3 | Cell co-culture

A transwell chamber with a membrane pore size of 0.4 μm (Corning, NY, USA) was used to co-culture macrophages with HK2 cells or HMCs. The macrophages were seeded in the upper culture chamber, and HK2 or HMCs were seeded in the lower culture plate. HK2 and HMC were collected for further analysis after co-culturing.

2.4 | Culture of primary macrophages

PBMCs were isolated from healthy human donors through Ficoll gradient centrifugation. Monocytes were then enriched with Stemcell easysep human monocyte isolation kit (Stemcell, Vancouver, BC, Canada), according to the protocols supplied by the manufacturer. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. M-CSF (50 ng/ml) were used to induce the differentiation of monocytes into macrophages for 7 days, and then flow cytometry was performed to validate (Figure S2).

2.5 | mRNA sequencing

The total RNA was extracted from primary macrophages with rMBL treatment using the TRIZol method. mRNA sequencing was performed using an Illumina HiSeq™ 2500. Subsequently, bioinformatics analyses including raw data process, DEG, BP, and KEGG analysis were performed.

2.6 | Quantitative PCR

The total RNA was extracted from cells by using TRIZol reagents (Invitrogen), first-strand cDNA was prepared from the total 2-μg RNA using the Reverse Transcripase kit (Tiangen, Beijing, China) and 1-μl cDNA was amplified in triplicate using SYBR Green qPCR Supermix (YiSheng, Shanghai, China) on CFX96™ Real-Time PCR detection systems (Bio-Rad, CA, USA). The primers for specific genes were designed via Primer-Blast (Table S1). Data were normalized to β-actin and presented as fold increase compared with RNA isolated from the control group using the 2^−ΔΔCT method.

2.7 | Mouse models

Male 4-week-old C57BL/6J (wild type, WT) and C57BLKS-Lepr db (db/db) mice were obtained from the Shanghai SLAC Laboratory Animal CO. LTD (Shanghai, China) and were kept under specific pathogen-free (SPF) conditions. For knockdown experiments, db/db mice were randomly divided into two groups: db/db+AAV-U6-shRNA group (n = 6, 2.2 × 1011 vg AAV9-U6-shRNA at 4, 12, and 20 weeks of age, via tail vein injection) and db/db+AAV-MBL1/2-shRNA group (n = 6, 1.1 × 1011 vg AAV9-MBL1-shRNA, and 1.1 × 1011 vg AAV9-MBL2-shRNA at 4, 12, and 20 weeks of age, via tail vein injection). A group of WT mice (n = 6) were studied as healthy controls. Animals
were euthanized at 24 weeks of age, and serum, urine, and kidney samples were collected for analyses. For MBL overexpression, WT mice were randomly divided into two groups: AAV-control (C57, n = 5, 1.1 × 10^{11} vg AAV-control at 8w, via tail vein injection), and AAV-MBL1/2 (C57, n = 5, 1.1 × 10^{11} vg AAV-MBL1 in combination with 1.1 × 10^{11} vg AAV-MBL2 at 8w, via tail vein injection). After 4 weeks, serum, urine, and kidney samples were collected for further analyses. All mice were euthanized by isoflurane overdose. The animal study was approved by the Animal Experimental Ethics Inspection of the First Affiliated Hospital of Zhejiang University School of Medicine (reference number 2021542).

### 2.8 | Metabolic measurements

Body weight of each mouse was recorded monthly. Blood glucose was measured using a Lifescan One Touch Glucometer (Roche, Basel, Switzerland) with blood collected from the mouse tails at designated time points. We used the REB triglyceride test kit (Purebio biotechnology company, Ningbo, China) for triglyceride measurements according to the manufacturer’s instructions. Urine albumin was measured using an ELISA kit (ab108792, Abcam), and urine creatinine was measured using a colorimetric assay kit (KGE005, R&D).

### 2.9 | Kidney histology

Mice were euthanized at 24 weeks under isoflurane, and kidneys were harvested and fixed with 4% paraformaldehyde 16 h at 4°C. Then, kidney tissue samples were embedded in parafin, then sliced into sections, and stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) for histopathological analysis. Subsequently, renal sections were evaluated under 200× magnification, ten images were randomly obtained. Positive PAS- or HE-stained areas in each image were quantified with ImageJ software (NIH, Bethesda, MA, USA). HE-stained kidney specimens were used to evaluate the tubular injury, protein cast, tubular dilation, tubular detachment, and brush border damage. The above changes were quantitated in 10 randomly selected fields (original magnification, ×200). The tubular injury index was evaluated by the percentage of areas per the whole section presented.\(^\text{18}\)

### 2.10 | Electron microscopy

Kidney samples were fixed in 5% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.4) for 24 h at 4°C. The mesangial matrix, the thickness of glomerular basement membrane, and tubular mitochondria were measured using an electron microscope (EM), and we randomly examined three mice from each group.

#### 2.11 | Immunohistochemistry and immunofluorescence study

Immunohistochemistry staining by antibodies targeting fibronectin (FN, 1:50, ab2413, Abcam) and α-smooth muscle actin (α-SMA, 1:100, ab5694, Abcam) was performed to assess the renal fibrosis. Positively-stained areas were quantified with ImageJ software and the percentages of positively-stained areas in the whole image were calculated. Frozen sections of the kidney were incubated with anti-F4/80 antibody (1:50, sc-37009, Santa Cruz Biotechnology) overnight at 4°C. At least eight random fields of each sample were observed under a fluorescent microscope and the average number of positive cells was calculated. For cell immunofluorescence, cells were incubated with anti-nuclear factor (NF)-κB (1:400, 8242s, Cell Signaling Technology) antibody overnight at 4°C and were observed under a confocal microscope (Leica, Wetzlar, Germany).

#### 2.12 | Western blot analysis

Renal tissues and cells were lysed in protein lysis buffer containing protease and phosphatase inhibitors. Immunoblotted proteins were detected using specific antibodies targeting FN (1:500, ab2413, Abcam), α-SMA (1:500, ab5694, Abcam), IκBα (1:1000, 4814, CST), p-IκBα (1:1000, 2859, CST), NF-κB (1:1000, 8242s, CST), p-NF-κB (1:1000, 3033s, CST), or GAPDH (Proteintech, Rocky Hill, NJ, USA). Then, we visualized target proteins with an enhanced chemiluminescence substrate. Semiquantitative western blot analysis was performed using ImageJ software.

#### 2.13 | Flow cytometry analysis and sorting

Kidneys of mice were excised, homogenized with a gentleMACSTM dissociator and C tubes (Miltenyi Biotech) in 5 ml digestion buffer containing 1.5 mg/ml collagenase IV and 1000 units/ml DNase I, and then incubated for 30 min at 37°C under agitation. Tissues were triturated and cells filtered through a 70 mm nylon mesh. Then, cells were stained at 4°C in FACS buffer. Anti-mouse CD16/32 (BD Pharmingen, San Diego, CA, USA) antibody was used for blocking the non-specific binding of the immunoglobulin to the Fc receptors.
Anti-mouse CD45 (Biolegend, San Diego, CA, USA) and anti-mouse F4/80 (Biolegend) were used to stain cell surface antigens, then anti-mouse iNOS (eBioscience, San Diego, CA, USA) was used to detect intracellular antigen after fixation and permeabilization. Cells passed through the cytometer were gated by granularity and size, and singlets were selected in order to exclude cell clumps. The mean fluorescence intensity of iNOS in CD45+ and anti-human CD45 (Biolegend) and anti-human CD68 (Biolegend) antibodies to validate the macrophages. PBMCs were isolated from DN patients and were planted into 12-well plates. After treatment with M-CSF (50 ng/ml) for 7 days, cells were harvested to detect the iNOS expression of macrophages using anti-human CD45 (Biolegend), anti-human CD68 (Biolegend), and anti-human iNOS antibodies. Peripheral blood from DN patients was incubated in the lysing buffer for 15 min until most red cells were lysed. After staining with live/dead (zombie blue), anti-human CD45 (Biolegend), anti-human CD11b (Biolegend), anti-human CD14 (Biolegend), and anti-human CD16 (Biolegend) antibodies, CD14+CD16+, CD14+CD16+, and CD14++CD16− cells were sorted using MoFlo Astrios sorter for mRNA extraction.

2.14 | Statistical analysis

All data were analyzed using SPSS MAC, version 23.0 (SPSS Inc., Chicago, IL, USA). The figures were constructed by GraphPad Prism 8. The normally distributed continuous variables were expressed as means ± SE. Data from two groups were analyzed using Student’s t-test. Data from multiple groups were compared via one-way ANOVA followed by Tukey’s post hoc test. Non-normally distributed continuous variables were expressed as median values (interquartile ranges, IQR) and categorical variables were expressed as frequencies and percentages. Comparisons of non-normally distributed continuous variables were performed using Mann-Whitney U-test. The Chi-square test was used for categorical variables. p < .05 was considered statistically significant.

3 | RESULTS

3.1 | The monocytes/macrophages isolated from DN patients with MBL SNP rs1800450 GG genotype are more proinflammatory than those with GA genotype

Since MBL functions as an opsonin and an important soluble pattern recognition molecule in the lectin complement pathway, we initially evaluated the downstream molecules of the lectin pathway (C3, C4, and C5b-9 levels) between two groups of DN patients, as defined by whether they were prone to progress to ESRD (as shown in Figure S1). However, no difference was observed between the two groups. Besides, no significant differences in renal function and pathological parameters existed between patients with or without glomerular C3/C4 deposition, indicating that the progression of DN was independent of lectin pathway activation (Table S2).

We, therefore, hypothesized that MBL enhances proinflammatory phenotypes in macrophages. Based on our previous findings, DN patients with MBL SNP rs1800450 GG had higher serum MBL levels than those with rs1800450 GA. Therefore, monocytes/macrophages from patients with SNP rs1800450 GG would be more pro-inflammatory than those from patients with SNP rs1800450 GA according to our hypothesis. To test this, we recruited a total of 11 patients with DN for peripheral blood collection. These patients included two different rs1800450 genotypes as mentioned above, and had matched clinical profiles when divided into two groups according to the rs1800450 genotypes (Table S3). All patients had not received glucocorticoids or immunosuppressants. There were no differences in age, median duration of diabetes history, percentage of angiotensin-converting enzyme inhibitor (ACEI)/angiotensin II receptor blocker (ARB) usage, percentage of calcium channel blockers (CCB) usage between the two groups. With the gating strategy shown in Figure 1A, CD14−CD16+, CD14−CD16+, and CD14+CD16− monocyte populations were sorted from the peripheral blood using fluorescence-activated cell sorting (FACS). The proportion of three monocytes subsets was not significantly different between the rs1800450 GG and GA groups, but the mRNA expression of IL-1β and TNF-α in CD14+CD16+ cells was significantly higher in GG patients (Figure 1B,C). To further study the effects of MBL on the monocyte-derived macrophage, we recruited another eight patients according to the same protocol, and the clinical profiles shown in Table S4. Peripheral mononuclear cells (PBMCs) were isolated from peripheral blood, and monocytes were differentiated into macrophages in vitro by treating PBMCs with 50 ng/ml M-CSF for 7 days. As evaluated by flow cytometry, iNOS expression in the monocyte-derived macrophages in the GG group was substantially higher than that in the GA group (Figure 1D,E).

3.2 | MBL promotes inflammatory responses in macrophages and indirectly promotes inflammatory and fibrotic responses in renal parenchymal cells

We tested the hypothesis that MBL promotes DN development through direct effects on macrophages and determined
**FIGURE 1** Single nucleotide polymorphism rs1800450 GG genotype is associated with proinflammatory property in monocytes/macrophages of patients with DN. (A) The representation gating strategy of cell sorting. (B) the cell percentage of CD14+CD16−, CD14+CD16+, and CD14++CD16− cells. (C) Relative mRNA expression of IL-1β and TNF-α. (D) Gating strategy of macrophages by flow cytometry after primary culture. (E) Expression of iNOS in macrophages was determined by flow cytometry. Data in panels B and C were analyzed with two-way ANOVA followed by multiple comparisons, and data in panel E were analyzed by Student’s t-test. *p < .05; **p < .01.

DN, diabetic nephropathy; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α

**FIGURE 2** Direct effects of MBL on macrophages and its indirect effects on renal parenchymal cells cocultured with macrophages. For (A–G), differentiated U937 macrophages, HK2 cells and human mesangial cells (HMCs) were treated with 10 μg/ml recombinant MBL, and cells were collected after 12 h for mRNA extraction and immunofluorescence, or were collected after 24 h for western blot. (A) IL-1β and TNF-α mRNA levels as determined by qPCR. (B) Representative western blots and quantification of p-IκBα levels, p-NF-κB levels, and NLRP3 levels normalized to total IκBα, NF-κB, and GAPDH, respectively. (C) Immunofluorescence images of p-NF-κB accumulation in the nucleus after 12 h with rMBL treatment, ×1800. (D) Quantitative PCR analysis of IL-1β, TNF-α, FN, and ACTA1 expression in HK2 cells. (E) Representative western blots and quantification of p-NF-κB, FN, and α-SMA levels in HK2 cells. (F) Quantitative PCR analysis of IL-1β, TNF-α, FN, and ACTA1 expression in HMCs. (G) Representative western blots and quantification of p-NF-κB, FN, and α-SMA levels in HMCs. *p < .001, **p < .01, versus control group, as analyzed by Student’s t-test. For (H–K), U937 macrophage was added in the upper insert and HK2 or HMC plated in the lower chamber respectively in a coculture Transwell system and stimulated with 10 μg/ml recombinant MBL for 24 h. (H) Representative western blot results of HK2 cells co-cultured with macrophages. (I) Quantification of p-NF-κB, NLRP3, FN, and α-SMA. (J) Representative Western blot results of HMCs co-cultured with macrophages. (K) Quantification of p-NF-κB, NLRP3, FN, and α-SMA. Data were analyzed with one-way ANOVA followed by multiple comparisons, *p < .05, versus group of no U937 or rMBL; **p < .05, versus group of U937 without rMBL. FN, fibronectin; IL-1β, interleukin-1β; MBL, mannose-binding lectin; TNF-α, tumor necrosis factor-α; α-SMA, α-smooth muscle actin
the effects of MBL on macrophages as well as renal parenchymal cells in vitro. Recombinant MBL (rMBL) was given to treat human macrophages and renal parenchymal cells, including U937, HK2, and HMCs. In fully differentiated U937 macrophages, IL-1β and TNF-α expression was up-regulated after 12-h treatment of 10 µg/ml rMBL, as determined by qPCR (Figure 2A). Western blot analysis indicated enhanced phosphorylation of IкBα, phosphorylation of
NF-κB p65 subunit, and increased expression of NLRP3 (Figure 2B). Consistently, confocal microscopy highlighted the translocation of p65 into the nucleus after 12 h of rMBL treatment (Figure 2C). On the contrary, the same rMBL treatment did not alter mRNA levels of IL-1β, TNF-α, FN, and ACTA1, and protein levels of phosphorylated p65, FN, and α-SMA in HK2 or HMCs (Figure 2D–G).

To further explore whether MBL promotes renal inflammation and fibrosis via crosstalk between macrophages and renal parenchymal cells, we performed a transwell coculture experiment with macrophages added in the upper insert and HK2 or HMCs plated in the lower chamber. As shown in Figure 2H–K, when comparing to respectively untreated control groups, macrophages alone did not alter NF-κB p65 phosphorylation or the expression of NLRP3, FN, and α-SMA in HK2 or HMC. Interestingly, these proinflammatory or profibrotic indices of both HK2 and HMCs were all augmented by MBL with macrophages presenting in the system.

### 3.3 MBL promotes M1 macrophage polarization by activating the NF-κB pathway

To further study the mechanism involved in the effects above, we performed RNA sequencing on isolated macrophages treated with rMBL from healthy human subjects. As shown in the volcano plot, 264 upregulated genes and 246 downregulated genes were detected in rMBL-treated macrophages in comparison with the untreated control macrophages (Figure 3A). The top 30 upregulated differentially expressed genes (DEGs) are shown in Figure 3B, and the top 4 key genes (TNF-α, CXCL1, CXCL8, IL-1β) were confirmed by qPCR (Figure 3C). We further performed BP and KEGG analysis, and identified that the most upregulated genes upon rMBL treatment were related to the NF-κB pathway and Toll-like receptor signaling pathway (Figure 3D,E).

To determine if the NF-κB pathway was downstream of MBL in macrophages, we inhibited NF-κB activity in U937 macrophages using siRNA-p65. The mRNA level of the p65 subunit was reduced by 51% two days after transfection with siRNA-p65 (Figure 4A). Upon rMBL treatment, IL-1β, TNF-α, and iNOS expressions were augmented as determined by qPCR. Notably, siRNA-p65 completely abrogated the rMBL-induced upregulation of IL-1β, TNF-α, and iNOS (Figure 4B). IL-1β and TNF-α secretion from macrophages were confirmed by ELISA, which showed consistent changes (Figure 4C). In the previous coculture experiment, HK2 or HMC were cocultured with macrophages that had been transfected with siRNA-p65. In consistent with the above changes in macrophages, the expression of p-NF-κB, FN, and α-SMA in HK2 and HMC were normalized after inhibiting NF-κB in macrophages (Figure 4D,E).

### 3.4 Downregulation of MBL1/2 alleviates DN progression in db/db mice

To further evaluate the in vivo role of MBL in the development of DN, we manipulated MBL gene expression in db/db mice. Different from humans with only one gene encoding MBL (namely, MBL2), mice together with lower primates and other mammals have two functional MBL genes (MBL-A and MBL-C, also known as MBL1 and MBL2) which encode MBL1 and MBL2 proteins. In initial studies, we acquired adeno-associated virus (AAV) vectors encoding a short-hairpin RNA (shRNA) to knockdown MBL gene expression in diabetic db/db mice. This was achieved via repeated tail-vein injection into mice at 4, 12, and 20 weeks of age, and the animals were sacrificed at 24 weeks. As shown in Figure 5A, the serum MBL1/2 levels of db/db mice that received control AAV-U6-shRNA vectors were significantly elevated compared with WT mice, and MBL1/2 levels were significantly attenuated in mice that received AAV-MBL1/2-shRNA.

DN progression in the above two groups of db/db mice was assessed during the treatments and after euthanasia. Blood glucose was markedly elevated in all db/db mice at 4 weeks of age and stayed at high levels until 24 weeks when the animals were sacrificed (Figure 5B). Triglyceride was also increased in db/db mice at 24 weeks, but ameliorated by knocking down MBL (Figure 5C). The db/db mice receiving AAV-MBL1/2-shRNA had more weight gain and delayed weight loss compared to their counterparts that received control AAV vectors (Figure 5D). Proteinuria appeared in db/db mice at 4 weeks of age, and was more severe at 24 weeks while knocking down MBL significantly decreased urine albumin creatinine ratio (UACR) level at 24 weeks (Figure 5E). At week 24, histological analysis of PAS, and HE staining confirmed mesangial matrix expansion and tubulointerstitial injury in db/db mice, which were also ameliorated in db/db mice that received AAV-MBL1/2-shRNA (Figure 5F,G). EM further confirmed the thickened glomerular basement membrane in db/db mice (WT group vs. db/db+AAV-U6-shRNA group, 110.0 ± 13.2 nm vs. 298.3 ± 2.9 nm, p < .001), and knocking down MBL1/2 attenuated the thickening (db/db+AAV-U6-shRNA group vs. db/db+AAV-MBL1/2-shRNA group, 298.3 ± 2.9 nm vs. 160.0 ± 26.5 nm, p < .001) (Figure 5H,I).

### 3.5 Modulating MBL1/2 influences renal macrophage infiltration and fibrosis

To determine the effects of MBL1/2 on renal infiltration of macrophage, kidney samples from the three groups of mice were analyzed by immunofluorescence and flow cytometry. As shown in Figure 6A, renal macrophage counts in
db/db mice receiving AAV-MBL1/2-shRNA were significantly lower than those receiving AAV-U6-shRNA. When we gated macrophages by the CD45+F4/80+ population under flow cytometry, macrophage iNOS expression, the major marker of M1 inflammatory profile, was enhanced in kidney single-cell suspension from control db/db mice;
FIGURE 4 Involvement of NF-κB pathway in the pro-inflammatory effects of MBL. (A) Confirmation of NF-κB (p65) gene knockdown by siRNA in macrophages, n = 3 per group. (B) The mRNA expression of IL-1β, TNF-α, and iNOS in macrophages after MBL stimulation. (C) IL-1β and TNF-α levels in the supernatants as measured by ELISA. (D) The representative western blot images of HK2 co-cultured with macrophages after NF-κB inhibition. Quantification of p-NF-κB, FN, and α-SMA. (E) The representative western blot images of HMC co-cultured with macrophages after inhibiting NF-κB and its quantification. Data were analyzed with two-way ANOVA followed by multiple comparisons, *p < .05, **p < .01, ***p < .001. FN, fibronectin; IL-1β, interleukin-1β; MBL, mannose-binding lectin; TNF-α, tumor necrosis factor-α; α-SMA, α-smooth muscle actin
FIGURE 5  Effects of knocking down MBL1/2 on the progression of diabetic nephropathy in db/db mice. (A) Expression of MBL1 and MBL2 in wild-type (WT) and db/db mice after control AAV-U6-shRNA vector or AAV-MBL1/2-shRNA injection. (B) Blood glucose, (C) triglycerides, (D) body weight, and (E) urine albumin to creatinine ratio (UACR) of the three groups of mice were measured over 4 to 24 weeks of age. (F) Representative images of PAS, HE stained kidney at 24 weeks of age. ×200, scale bar = 100 µm. (G) Tubulointerstitial injury index at 24 weeks of age. (H) Representative EM images of glomerulus and mitochondria at 24 weeks of age. (I) Thickness of GBM quantified by EM at 24 weeks of age. EM1, ×2850, scale bar = 5 µm; EM2, ×9700, scale bar = 2 µm. All mice were euthanized at 24 weeks. n = 3 per group for panel I, n = 6 per group for other panels, *p < .05, **p < .01, ***p < .001, versus WT group; #p < .05, ##p < .01, ###p < .001, versus db/db+AAV-U6-shRNA group. AAV, adeno-associated virus; EM, electron microscope; GBM, glomerular basement membrane; HE, hematoxylin-eosin; MBL, mannose-binding lectin; PAS, periodic acid-Schiff stain; TII, tubulointerstitial injury index; UACR, urine albumin creatinine ratio.
this upregulation was attenuated by MBL1/2 gene knockdown (Figure 6B). In contrast, levels of CD206, the marker of alternatively activated macrophages (M2), were similar among the three groups (Figure 6C). Besides, renal inflammatory markers, such as IL-1β and CCL2, were ameliorated after knocking down MBL1/2 (Figure 6D). Also, knocking down of MBL1/2 attenuated FN and α-SMA levels in the renal cortex as shown by immunohistochemistry, and the attenuation was confirmed by western blots (Figure 6E,F).

We went on and determined whether overexpression of MBL alone would induce at least a part of the above pathological processes. Four weeks after tail vein injections of AAV vectors encoding MBL1/2 genes (AAV-MBL1/2) or AAV-control in C57BL/6J mice, the serum levels of MBL1 and MBL2 in the AAV-MBL1/2 group were significantly increased compared with those in the AAV-control group (Figure 6G). No changes were observed in body weight, blood glucose, UACR, or IL-1β levels with MBL1/2 overexpression (Figure 6G,H). However, CCL2 level and renal macrophage infiltration were increased and iNOS expression of macrophages tended to increase by MBL1/2 overexpression (Figure 6H–J).

4 | DISCUSSION

In this study, DN patients with MBL2 SNP rs1800450 GG genotype are associated with enhanced IL-1β production and M1 polarization in monocyte/macrophage. The current results provide a mechanistic insight into our previous findings that high serum MBL level and GG genotype of MBL2 SNP rs1800450 are independent risk factors for the outcome of ESRD in DN patients.10 We showed macrophage is the pivotal target of MBL mediating renal inflammation and fibrosis during the progression of DN. These effects are directly caused by MBL via NF-κB activation and are independent of the complement lectin pathway. More interestingly, despite the lack of a direct effect, MBL induces fibrotic changes in renal parenchymal cell lines through crosstalk with macrophages. Knocking-down MBL with shRNA ameliorates proteinuria and associated renal macrophage infiltration and fibrosis in db/db mice, while overexpression of MBL promotes M1 phenotype polarization of macrophages.

Consistent with our results, extensive studies uncovered circulating MBL level as a robust predictor of DN in both type 1 and type 2 diabetes.20,21 Results from gene knockout mice indicate that MBL level is augmented in streptozotocin-induced diabetes and contributes to glomerular injury and renal fibrosis.22,23 This may provide a genetic base for some DN patients with poor outcomes and a method to screen these patients.

We show that monocytes/macrophages isolated from rs1800450 GG genotype have a higher proinflammatory profile compared with the GA genotype. It has been reported that six polymorphisms of the MBL2 gene including three variants positioned in the promoter 1 and three variants in the exon 1 are related to change in quantity and/or function of MBL in serum, and patients with rs1800450 GG genotype and rs11003125 CC genotype have elevated serum MBL levels.24,25 Subjects with GA genotype of rs1800450 have lower MBL levels and rs1800450 GA genotype is an independent protective factor for progressing to ESRD.9 In the current study, we further found differential productions of IL-1β and TNF-α in the CD14+CD16+ monocyte subset between rs1800450 GA and GG genotypes. CD14+CD16+ monocyte subset has been shown to be proinflammatory and the main producer of TNF-α.26 CD14+CD16+ monocytes have also been shown to be associated with increased body mass index and subclinical atherosclerosis in humans.27 Therefore, results from cell sorting and those from RNA sequencing are mutually supportive, pointing to the same inflammatory pathways.

MBL is usually known as the main upstream initiator of the lectin pathway in the complement system,28 which provides an effective defense against invading pathogens and apoptotic cells in an organism.29,30 However, we did not find significant differences in the serum C3, C4, and C5b-9 levels or the degree of glomerular complement deposits in DN patients with different outcomes. Likewise, a previous study by Østergaard et al. also supported the increased activity of MBL within the kidney but no renal deposition of complement C3, C4, or C9 in streptozotocin-induced diabetic mice.23 In addition, Van et al. showed that inhibiting C5 does not reduce kidney damage, and the presence of kidney damage is earlier than the deposit of complement C3.31 Therefore, it is more likely that MBL contributes to DN progression via lectin pathway-independent effects.

Our study identifies monocytes/macrophages as a critical component downstream of MBL in DN. It has been known that MBL participates in a series of complement-independent opsonization and phagocytosis,18 as well as influences oxidative stress and coagulation systems.32,33 By directly treating macrophages as well as renal parenchymal cells in vitro, we identified macrophages as the major target of MBL in the kidney. This is also supported by our in vivo results, showing higher numbers of macrophages and higher levels of iNOS expression within these cells are observed in the kidney of db/db mice, and knockdown of MBL expression markedly reverses these changes. Interestingly, when overexpressing MBL in wild-type animals, renal macrophage infiltration increased significantly, but their M1 polarization was not observed. We speculate
FIGURE 6 Effects of modulating MBL1/2 on renal macrophage infiltration and fibrosis. (A) Representative F4/80 immunofluorescence images with mean data of macrophage quantification. ×400, scale bar = 50 µm. (B and C) Macrophage polarization was assessed by flow cytometry, with representative histogram and mean data of iNOS and CD206 expression shown, respectively. (D) The mRNA expression of IL-1β and CCL2 as determined by qPCR. (E) Representative images of FN and α-SMA immunohistochemistry. (F) Representative western blot of FN, α-SMA and GAPDH. FN and α-SMA levels were quantified and normalized to GAPDH. Mice in (A–F) were euthanized at 24 weeks. n = 6 per group. Data were analyzed using one-way ANOVA followed by multiple comparisons. *p < .05, **p < .01, ***p < .001, versus WT group; *p < .05, **p < .01, ***p < .001, versus db/db+AAV-U6-shRNA group. (G) Overexpression of MBL and its effects on metabolism in C57BL/6J mice. Validation of overexpression of serum MBL1 and MBL2 using ELISA. Body weights were recorded at 8 and 12 weeks of age. Blood glucose and UACR were measured at 12 weeks of age. (H) The mRNA expression of IL-1β and CCL2. (I) Renal infiltration of macrophages and (J) iNOS expression of these cells at 12 weeks of age were quantified by flow cytometry. n = 5 per group in (G–J), *p < .05, ***p < .001, as determined by Student’s t-test. AAV, adeno-associated virus; FN, fibronectin; MBL, mannose binding lectin; MFI, mean fluorescence intensity; UACR, urine albumin creatinine ratio; α-SMA, α smooth muscle actin.
that this requires a diabetic milieu, or it is still at the early stage of this pathological process.

Results from mRNA sequencing suggest that the NF-κB pathway activation and the TLR signaling are involved in MBL-induced macrophage polarization. RNA interference of p65 expression abrogates the direct effects of MBL on macrophages and subsequent effects on kidney cells. Previous literatures have found that NF-κB is activated in DN, and its activation is associated with the progression of DN, which is consistent with our results. Further studies are needed to identify the receptor or direct target of MBL that induces NF-κB activation. Results from KEGG pathway analysis indicate Toll-like receptor (TLR) signaling pathways, among other inflammatory pathways, are involved. It has been known that TLRs play crucial roles in various kidney diseases. TLR2/TLR4 are significantly increased in unilateral ureteral obstructive nephropathy, and the inflammation is reduced after knocking down TLR2, the fibrosis is improved after knocking down TLR4. Insulin resistance, urine protein, and renal fibrosis were improved by inhibiting TLR4 on macrophages. In a preliminary study, we found that rMBL-induced NF-κB activation is attenuated by a TLR4 antagonist TAK242 (data not shown), suggesting the involvement of TLR4 as a potential receptor on macrophages for MBL. However, considering the complexity of TLR signaling pathways, a comprehensive study is in need to warrant this putative MBL-TLR-NF-κB pathway.

Although MBL does not affect parenchymal cells damage directly, inflammation and fibrosis in these cells can be induced by macrophages in the presence of MBL. Macrophages accumulated in the kidney are essential in the development and progression of DN by releasing proinflammatory and profibrotic cytokines such as TNF-α, IL-β, and TGF-β. You et al. showed macrophages, mainly pro-inflammatory M1 subset, contribute to renal damage in DN directly. Further evidence indicates a critical role of macrophage-derived TNF-α in diabetic kidney injury and suggests blocking TNF-α as a potential therapy to treat DN. Consistently, our study indicates that MBL promotes inflammatory response in macrophages, and renal inflammation can be further amplified by crosstalk between the stimulated macrophages and renal parenchymal cells.

There are some limitations in the current study. First, this study is a single-center study based on limited samples. Secondly, we used AAV-MBL1/2-shRNA due to the lack of MBL1/2 knockout mice, which only achieved partial knockdown of MBL1/2. Moreover, the overexpression efficiency of MBL in DN mice is limited, and an extended observation would provide more knowledge on the long-term effects of MBL alone on the kidney.

Collectively, we elucidated MBL level and rs1800450 SNP are potential markers and therapeutic targets for diabetic kidney disease. In mechanism, MBL directly promotes the renal infiltration and M1 polarization of macrophages, which induces renal fibrosis via crosstalk with tubular epithelial cells and mesangial cells. More efforts in preventative treatment may benefit the patients with high serum MBL levels and/or rs1800450 GG genotype.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Fei Han, Liang Xiao, Yanhong Ma, Fanghao Cai, and Xiaohan Huang designed the study; Yanhong Ma, Fanghao Cai, Xiaohan Huang, Huijing Wang, Binfeng Yu, Junni Wang, and Wanyun Nie carried out experiments; Fei Han, Liang Xiao, Yanhong Ma, Fanghao Cai, Xiaohan Huang, Yi Yang, and Jianghua Chen analyzed the data; Fei Han, Liang Xiao, Yanhong Ma, Fanghao Cai, and Xiaohan Huang made the figures; Fei Han, Liang Xiao, and Yanhong Ma drafted and revised the paper; all authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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