SUPPLEMENTAL MATERIAL

Blockage of myeloid differentiation 2 attenuates diabetic nephropathy via reducing local RAS activation in mouse kidneys

Yi Wang[1,#,*], Qilu Fang[1,#], Yiyi Jin[1,#], Chunpeng Zou[2], Weihui Yu[3], Weixin Li[1], Xiaoou Shan[2], Ruijie Chen[2], Zia Khan[1], Guang Liang[1,*]

1 Chemical Biology Research Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, 325035, China;

2 The Second Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, 325000, China;

3 Department of Endocrinology, the First Affiliated Yueqing Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, 325035, China;

Supplementary Tables and Figures

Table S1: Primers used for real-time qPCR assay

Gene	Species	FW	RW
ACE	Rat	CACCGGCAAGGTCTGCTT	CTTGGCATAGTTTCGTGAGGAA
Renin	Rat	ACCAGGGCAACTTTCACTACGT	ACCCCCTTCATGGTGATCTG
AT1	Rat	ATGCCAGTGTGTTTCTGCTC	CCAATGGGGAGTGTTGAGTT
TGF-β	Rat	GCAACAACGCAATCTATGAC	CCTGTATTCCGTCTCCTT
β-actin	Rat	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC
AT1	Mouse	CCATTGTCCACCCGATGAAG	TGCAGGTGACTTTGGCCAC
IFN-γ	Mouse	GCCAAGTTTGAGGTCAACAAC	ATCAGCAGCGACTCCTTTTC
ACE	Mouse	ACCCAACCTCGATGTCACCA	GCGAGGTGAAGAATTCCTCTGA
CTGF	Mouse	ACTATGATGCGAGCCAACTGC	TGTCCGGATGCACTTTTTGC
Collagen1	Mouse	TGGCCTTGGAGGAAACTTTG	CTTGGAAACCTTGTGGACCAG
TNF-α	Mouse	TGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA
IL-6	Mouse	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
ICAM-1	Mouse	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA
VCAM-1	Mouse	TGCCGAGCTAAATTACACATTG	CCTTGTGGAGGGATGTACAGA
MCP-1	Mouse	TCACCTGCTGCTACTCATTCACCA	TACAGCTTCTTTGGGACACCTGCT
IL-1β	Mouse	AGAAGGAGCAGGACACCAGC	ATCACTCTTCATCATGCATTTTTTGCT
TGF-β	Mouse	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
β-actin	Mouse	CCGTGAAAAGATGACCCAGA	TACGACCAGAGGCATACAG
Renin	Mouse	GCTTTCTCAGCCAGGACATC	TGGGAGATGATGTTGTCGAA

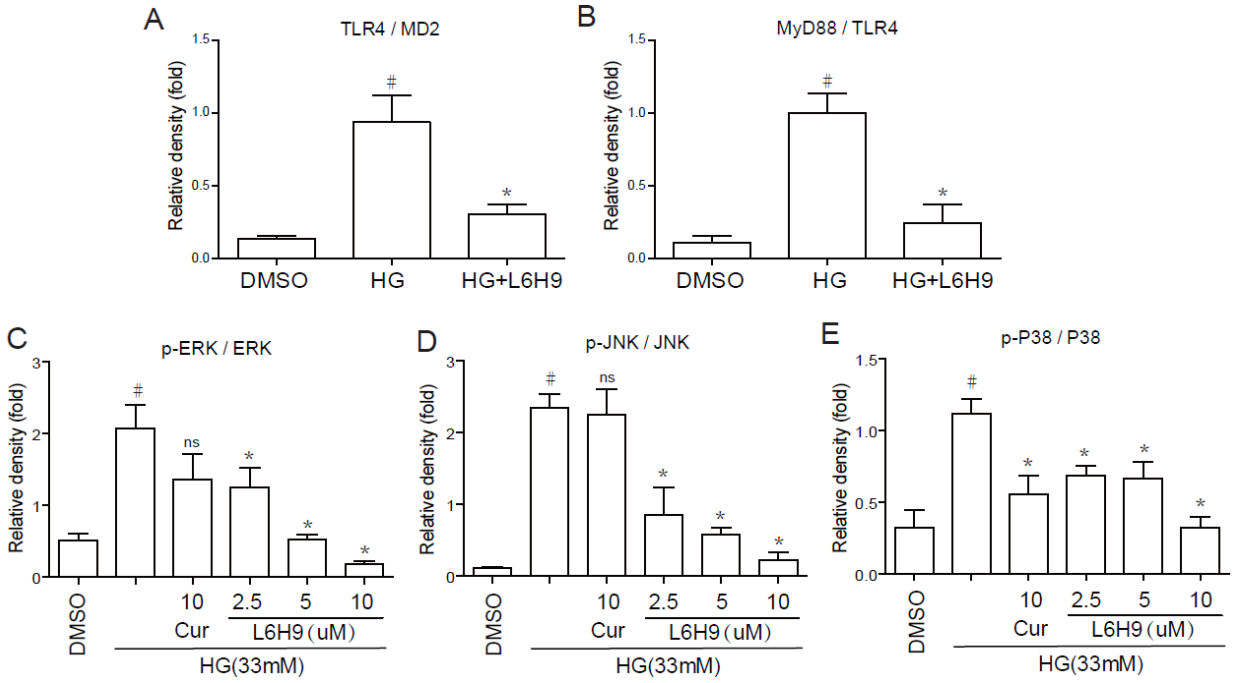

Figure S1: Densitometric quantifications for Figure 2B and 2C. Panel A and B, for Figure 2B; Panel C-E, for Figure 2C. (n=5, $^{\#}P<0.05$ vs the DMSO group; $^{*}P<0.05$ and ns =not significant, vs the HG group).

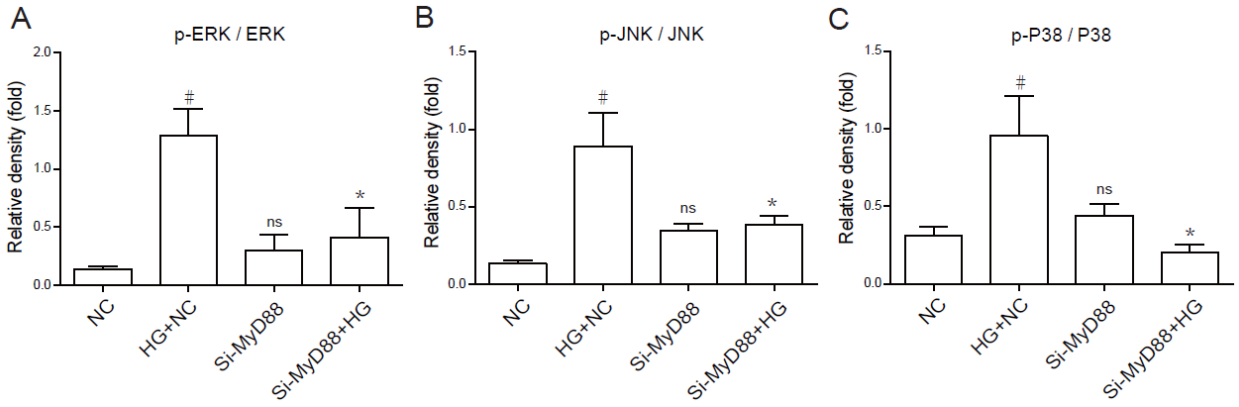

Figure S2: Densitometric quantifications for Figure 3G. (n=5, $^{\#}P<0.05$ and ns =not significant, vs the NC group; $^{*}P<0.05$, vs the HG+NC group).

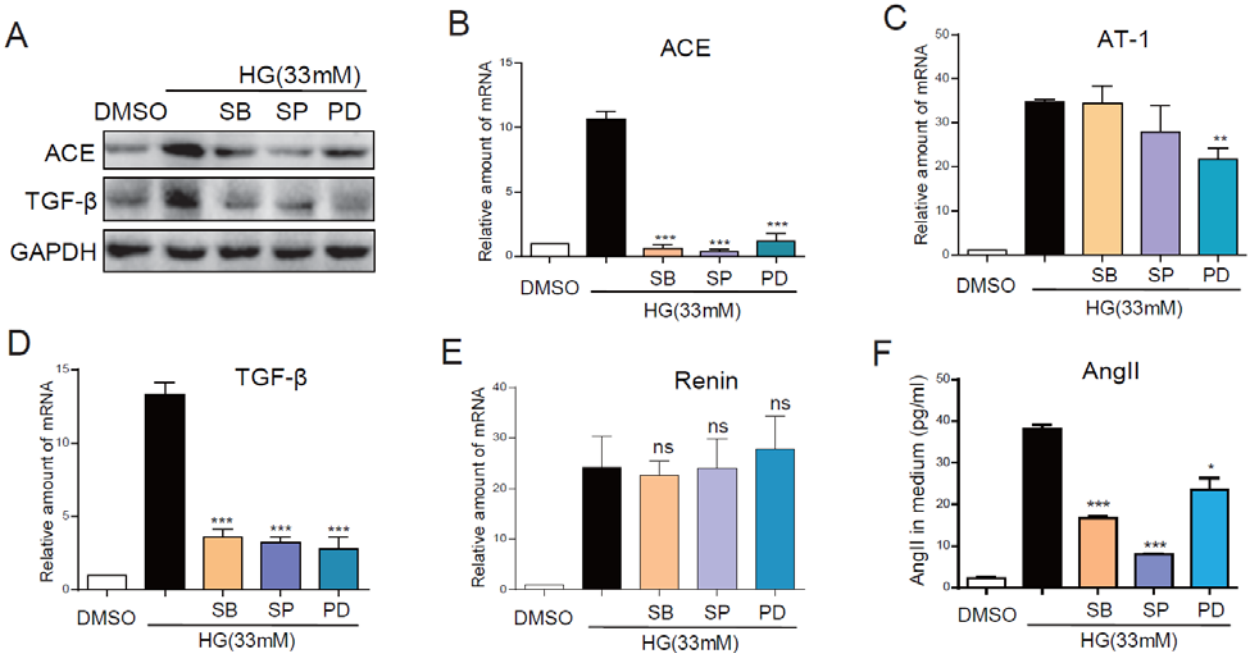

Figure S3. MAPKs inhibitors blocked HG-induced activation of RAS and expression of fibrotic factors. NRK-52E cells were firstly pretreated with SB203580 (SB), SP600125 (SP), or PD98059 (PD) for 1h and then stimulated by HG for 12h (mRNA detection) or 24h (protein detection), respectively. ACE and TGF-β protein levels were measured by western blot assay (A). The mRNA levels of ACE (B), AT-1 (C), TGF-β (D), and Renin (E) were measured by real-time qPCR assay. The cultural medium was collected for Ang II detection by ELISA (F). n=5 independent experiments; Bar graph shows mean values ± SE; *p<0.05, **p<0.01, ***p<0.001, and ns = not significant, versus HG group.

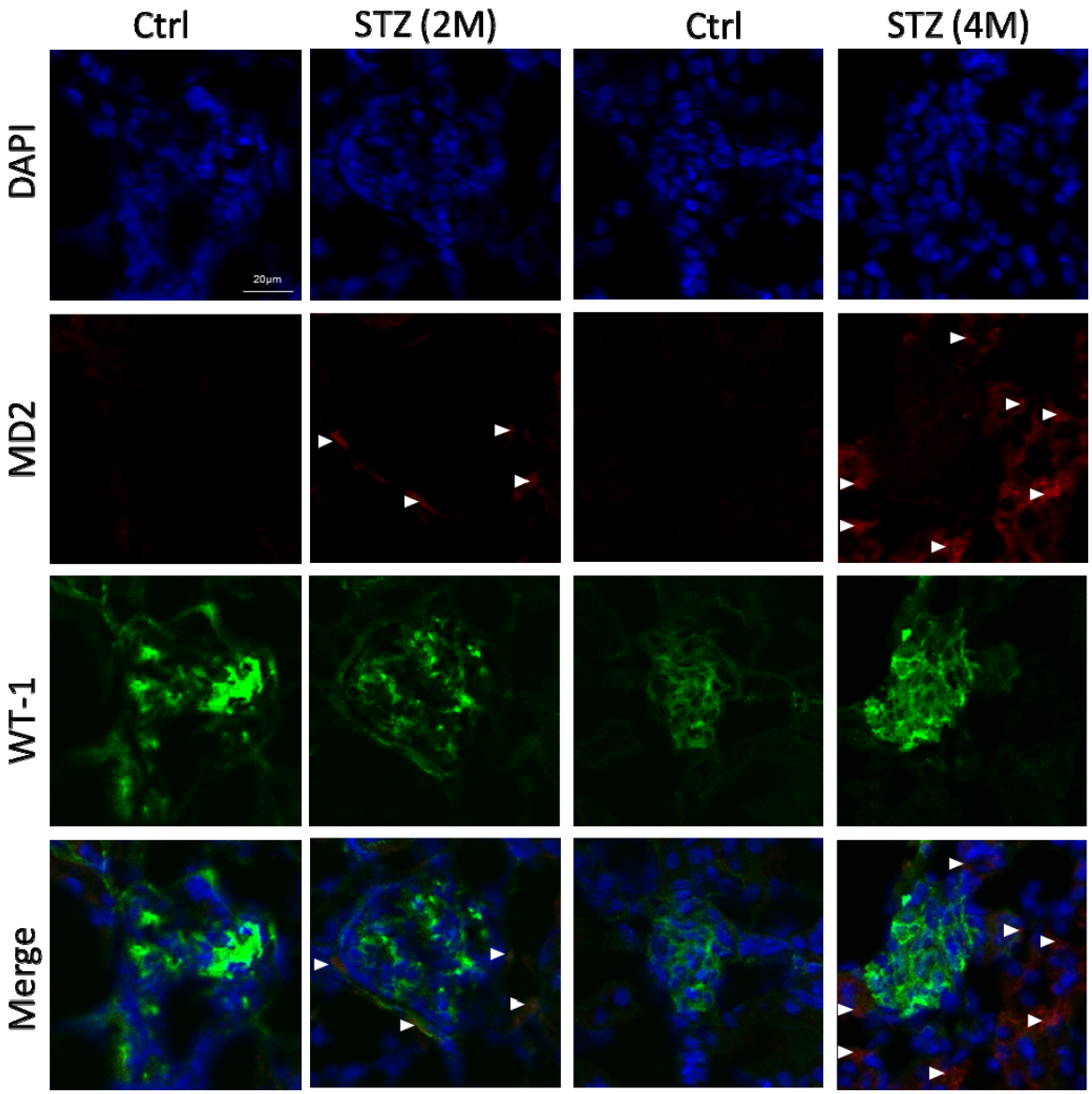

Figure S4: Co-immofluorescence staining analysis for MD2 and WT-1 in kidney tissues of diabetic mice. 14 mice were treated with a single intraperitoneal injection of STZ (100 mg/kg in citrate buffer, pH 4.5), while 14 control mice were received the same volume of citrate buffer. Seven days after STZ injection, mice with fasting-blood glucose >216 mg/dL were considered as diabetic. All mice were fed with standard animal low-fat diet. After 2 months, randomly 7 control mice and 7 diabetic mice were killed under ether anesthesia. After another 2 months, 7 control mice and 7 diabetic mice were killed under ether anesthesia. Renal tissues were collected at the time of sacrifice. Kidney tissue from each group was evaluated for distribution of MD2 and WT-1 (a marker for podocytes) by double immunofluorescence staining (the blue are DAPI staining for nuclei). Representative images were shown. The merged images showed no overlap of MD2 and WT-1 expression, indicating that podocytes do not express MD2 protein. Scale bar: 20 μm.

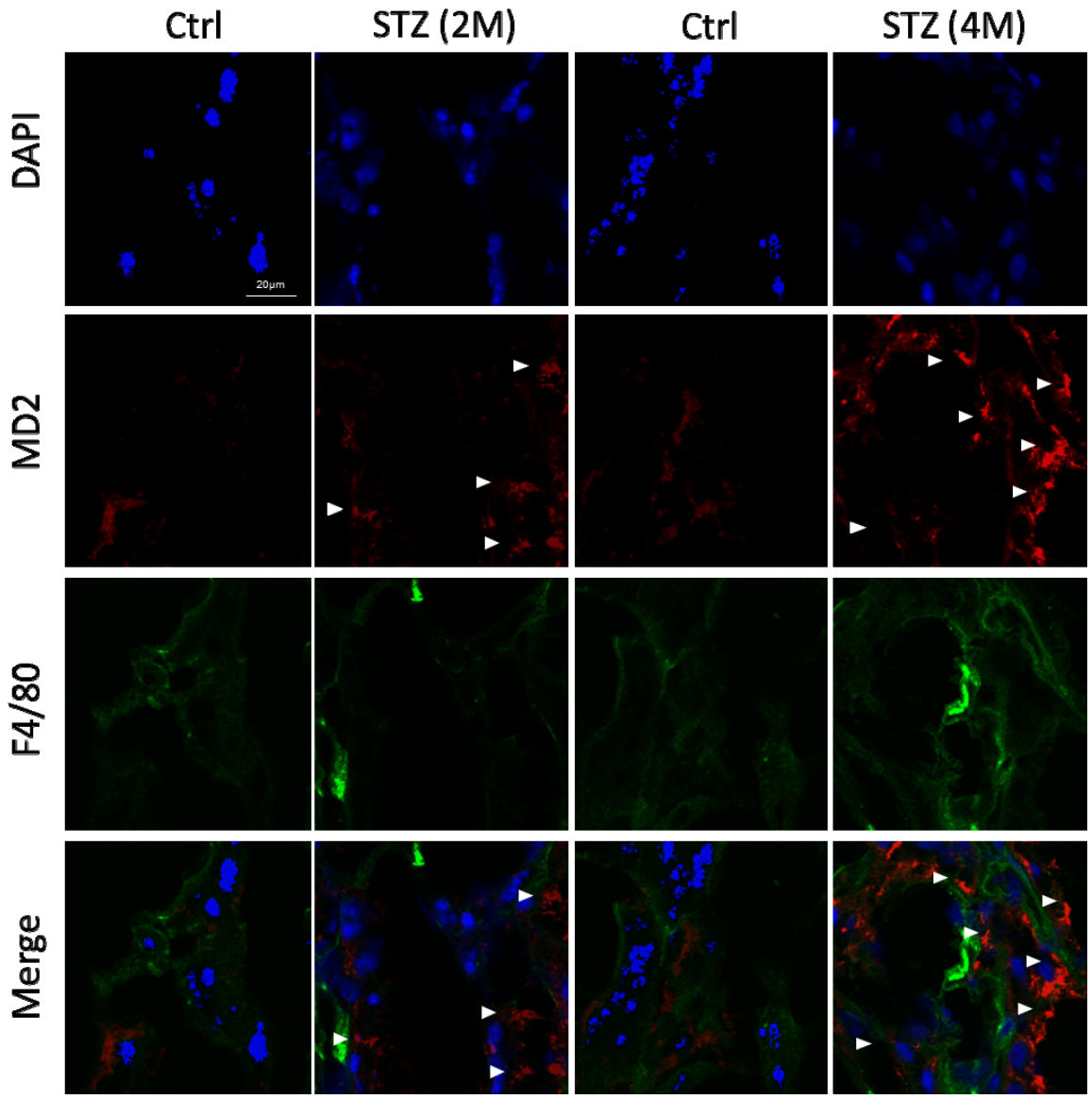

Figure S5: Co-immofluorescence staining analysis for MD2 and F4/80 in kidney tissues of diabetic mice. 14 mice were treated with a single intraperitoneal injection of STZ (100 mg/kg in citrate buffer, pH 4.5), while 14 control mice were received the same volume of citrate buffer. Seven days after STZ injection, mice with fasting-blood glucose >216 mg/dL were considered as diabetic. All mice were fed with standard animal low-fat diet. After 2 months, randomly 7 control mice and 7 diabetic mice were killed under ether anesthesia. After another 2 months, 7 control mice and 7 diabetic mice were killed under ether anesthesia. Renal tissues were collected at the time of sacrifice. Kidney tissue from each group was evaluated for distribution of MD2 and F4/80 (a marker for infiltrated macrophages) by double immunofluorescence staining (the blue are DAPI staining for nuclei). Representative images were shown. The merged images showed slight overlap of MD2 and F4/80 expression, indicating that infiltrated macrophages express less MD2 protein. Scale bar: 20 μm.

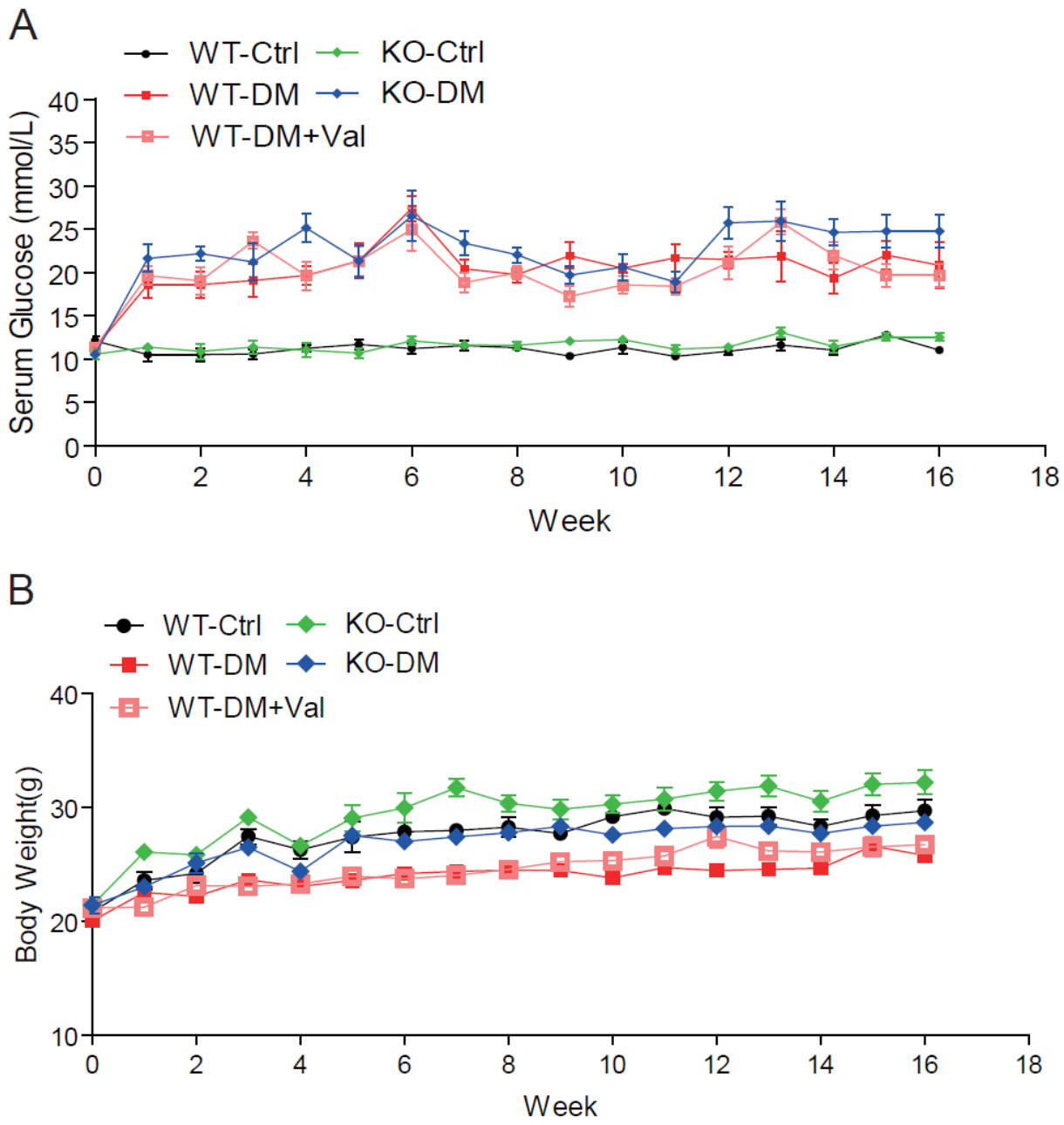

Figure S6: Glucose and body weight monitoring of animals. MD2 knockout mice (KO) and their wild type control (C57BL/6, B6) were induced diabetes by STZ injection (as described in Materials and Methods). MD2 knockout had no effect on A) serum glucose levels and B) body-weight (wild type non-diabetic =WT-Ctrol, wild type diabetic =WT-DM, MD2 knockout non-diabetic =KO-Ctrl and MD2 knockout diabetic = KO-DM, wild type diabetic mice with Valsartan treatment=WT-DM+Val) . Values are reported as means ± SEM; n=8 per group.

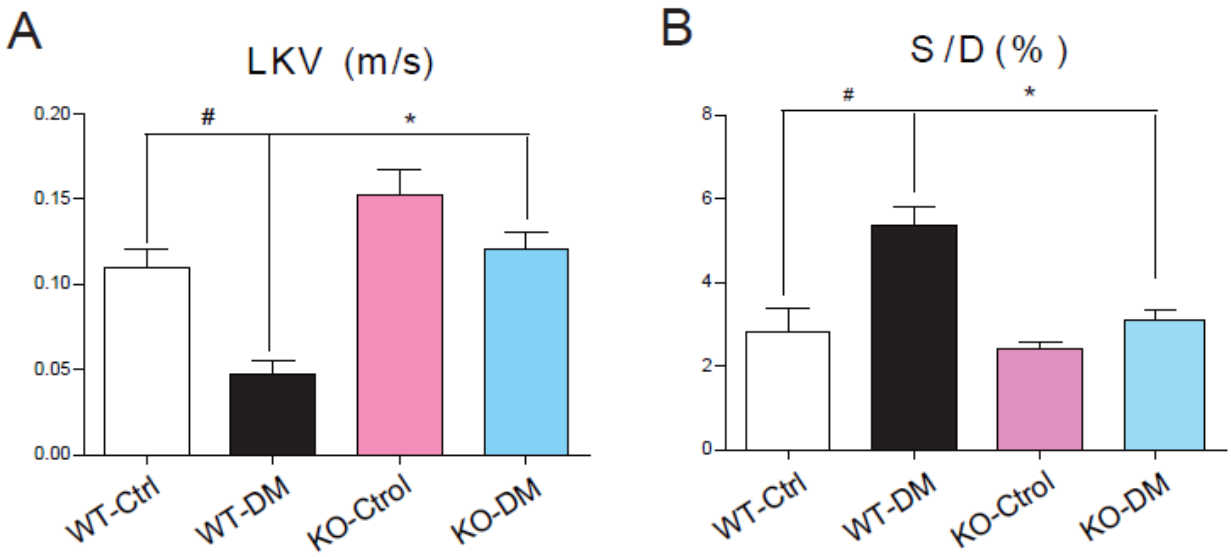

Figure S7: Ultrasound kidney function analysis showed that MD2 knockout attenuated kidney dysfunction in diabetic mice. MD2 knockout mice (KO) and their wild type control (C57BL/6, B6) were induced diabetes by STZ injection (as described in Materials and Methods). Before sacrifice, ultrasonography assay was performed on anesthetized mice at rest using a high-resolution imaging system for small animals (Vevo 770, Visual Sonics, Canada), equipped with a high-frequent ultrasound probe (RMV-707B). All hair was removed from the lower back using a chemical hair remover and the aquasonic clear ultrasound gel (Parker Laboratories, Fairfield, NJ) was applied to the lower back to optimize the visibility of the renal artery. Mice were placed in the supine position on a heating pad to maintain body temperature at 36–37°C. Left kidney blood flow velocity (LKV), and systolic and diastolic pressure ratio (S/D) from four experimental groups were detected by ultrasonographic measurement. Wild type non-diabetic =WT-Ctrl, wild type diabetic =WT-DM, MD2 knockout non-diabetic =KO-Ctrol and MD2 knockout diabetic = KO-DM; Values are reported as means ± SEM; n=8 per group. $^{\#}P<0.05$ vs the WT-Ctrl group; $^{*}P<0.05$ vs the WT-DM group.

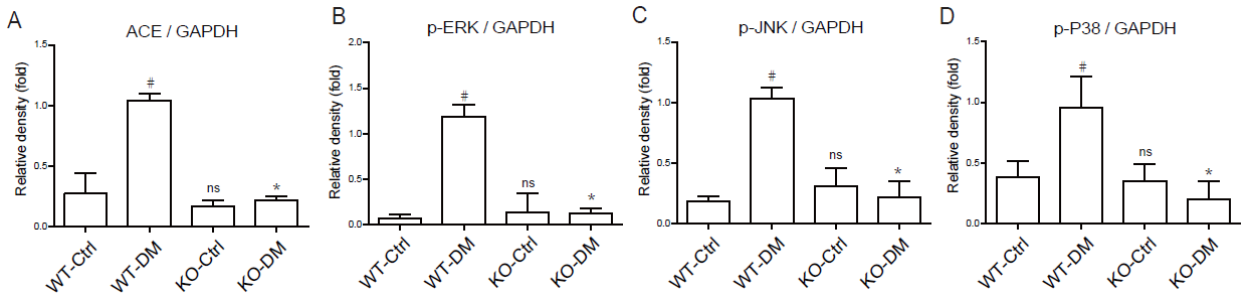

Figure S8: Densitometric quantifications for Figure 5H. (n=7 per group, $^{\#}P<0.05$ and ns =not significant, vs the WT-Ctrl group; $^{*}P<0.05$, vs the WT-DM group).

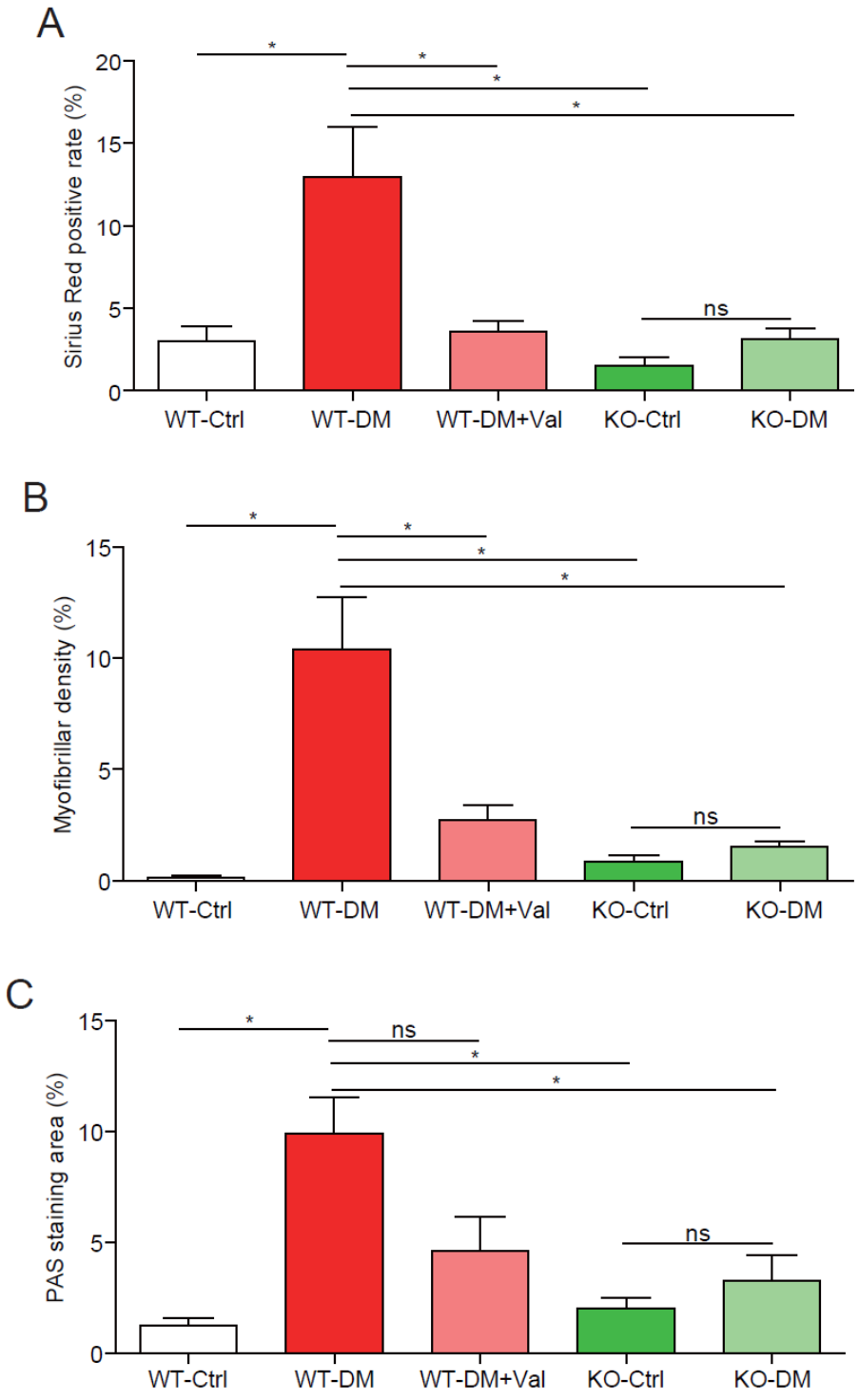

Figure S9: Quantification for staining results in Figure 6A. Data from 8 mice per group; values reported as mean ± SEM; *P<0.01; ns=no significance.

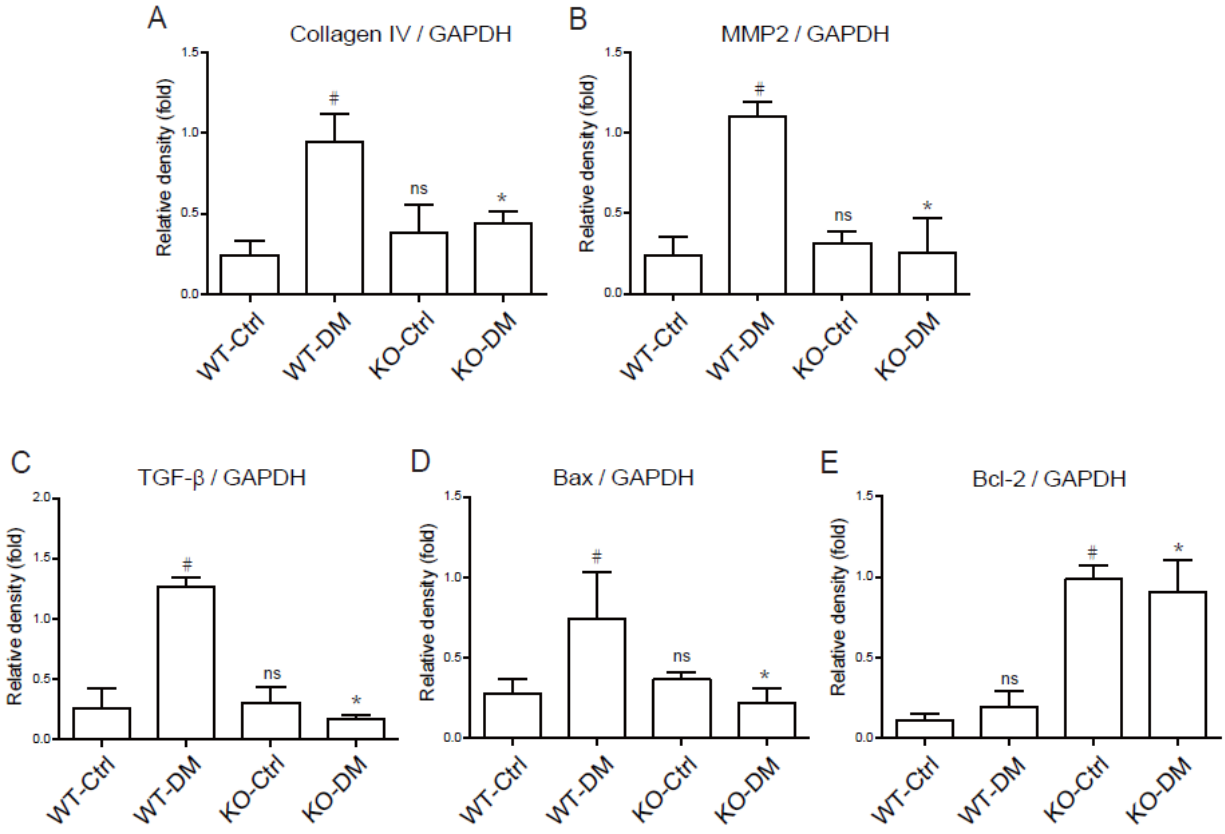

Figure S10: Densitometric quantifications for Figure 6C. (n=7 per group, $^{\#}P<0.05$ and ns =not significant, vs the WT-Ctrl group; $^{*}P<0.05$, vs the WT-DM group).

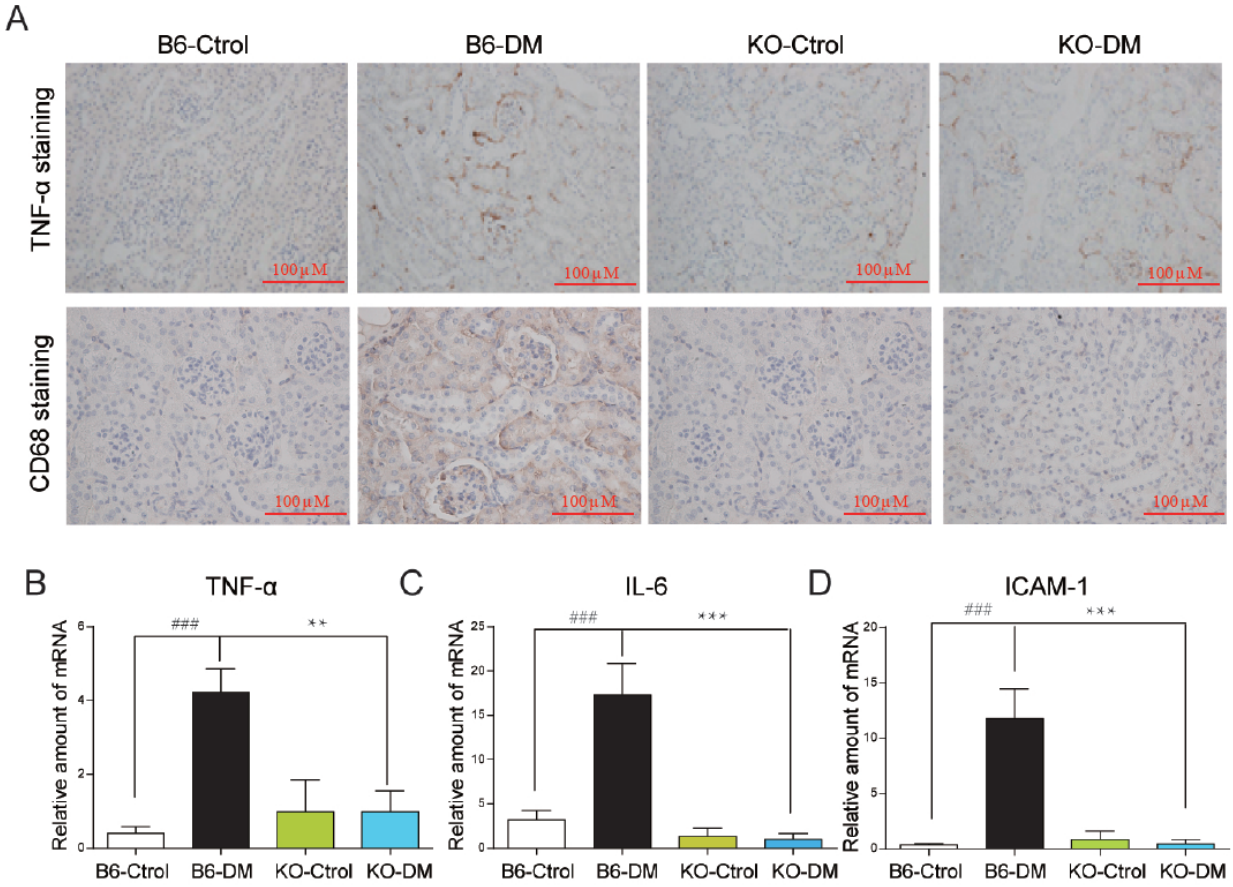

Figure S11: MD2 knockout mice reduced inflammatory changes in diabetes. MD2 knockout mice (KO) and their wild type control (C57BL/6, B6) were induced diabetes by STZ injection (as described in Materials and Methods). A) Immunohistochemical analysis showing diabetes induced increased TNFα and CD68 expression are prevented in the MD2 knockout mice. After deparaffinization and rehydration, slides were treated with 3% H2O2 for 10 min and with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min. Slides were then incubated overnight at 4°C with anti-CD68 and anti-TNF-α antibodies (1:200) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500) for 1 h at room temperature. Hematoxylin was used as nuclear stain. The sections were dehydrated with graded alcohol and xylene and mounted with neutral resins. The slides were examined microscopically (×200, Nikon, Tokyo, Japan). B-D) In parallel diabetes induced increased renal B) TNF-α, C) IL-6 and D) ICAM-1 mRNA expressions were prevented in the MD2 knockout mice. (mRNA data are normalized to β-actin, wild type non-diabetic =B6-Ctrol, wild type diabetic =B6-DM, MD2 knockout non-diabetic =KO-Ctrol and MD2 knockout diabetic = KO-DM). Values are reported as means ± SEM; n=8 per group; $^{###}P<0.001$, ** $P<0.01$, and ***$P<0.001$.

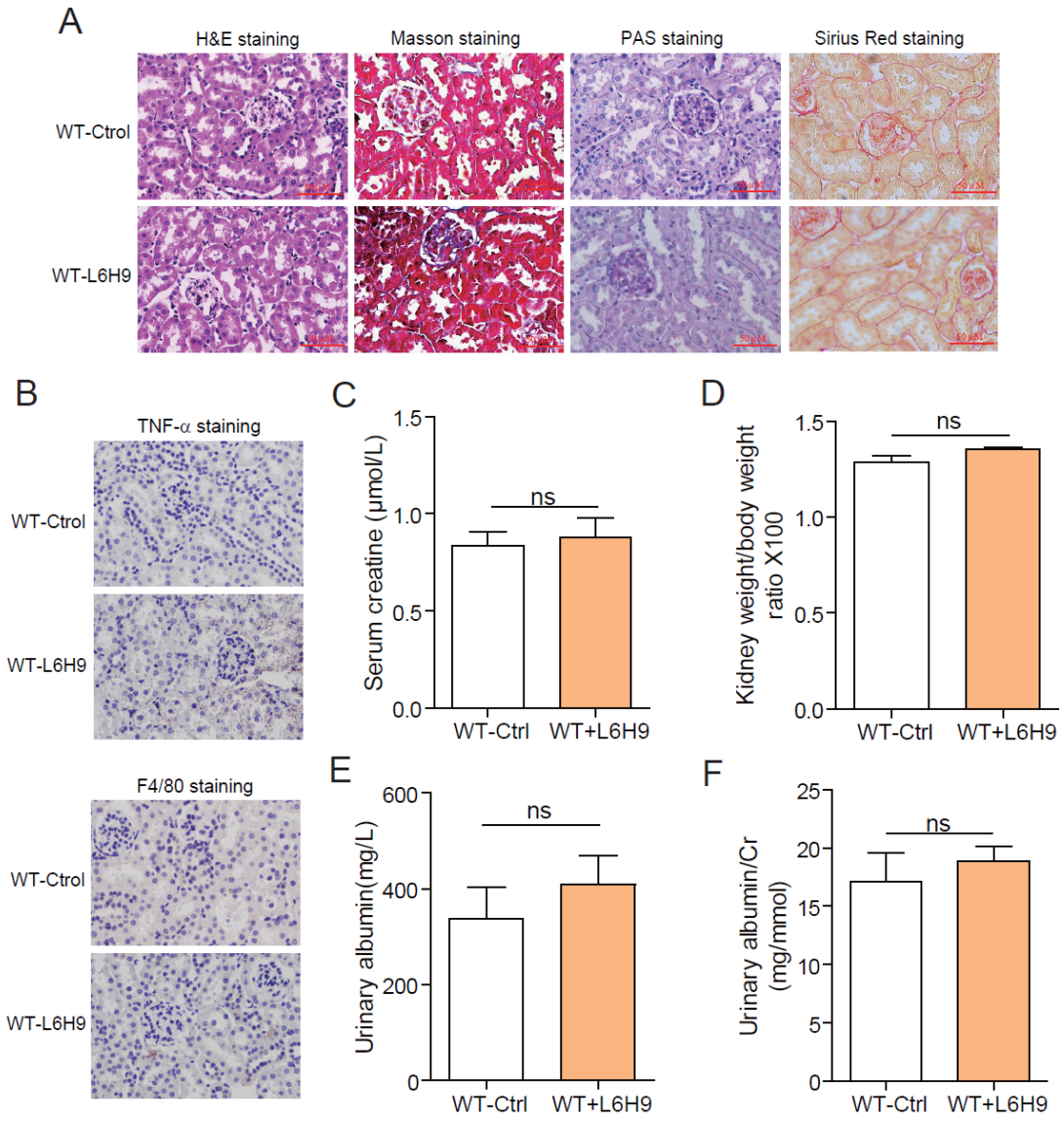

Figure S12： L6H9 administration showed no renal side-effect in mice. 7 male C57BL/6 mice were administrated with L6H9 (20mg/kg) by oral gavage once every 2 days for 4 months. The age-matched control group (n=7) received 1% CMC-Na solution alone according to the same schedule. No death and no difference in body weight changes between these two groups were observed during the experiment. After 4 months, animals were killed under ether anesthesia. Blood, urine, and renal tissues were collected at the time of sacrifice. A) Representative light micrograph of histochemical assessment of kidney tissues: hematoxylin and eosin (H&E) staining, PAS for glycogen (purple), Masson's trichrome stain (Blue) for detection of connective tissue, and Sirius Red staining were used for the detection of fibrosis (Red); 400 x magnification (The methodology for histopathological analysis was referred the description in Materials and Methods). B) Immunohistochemical analysis showing L6H9 did not increase TNF-α and F4/80 expression in mouse kidney (The methodology for histopathological analysis was referred the description in Figure S6A). C) Serum creatinine level was detected by corresponding kits. D) The ratio of kidney weight to body weight was recorded. E) Urinary albumin level was detected by corresponding kit. F) Urinary creatinine level was detected by commercial kit and the ratio of urinary albumin to urinary creatinine was calculated. Mean values ± SE; n=7 per group; ns = not significant.

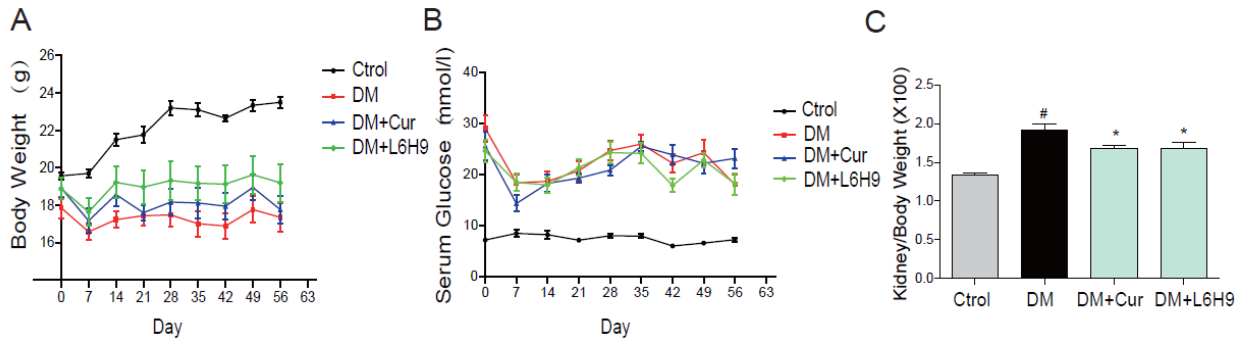

Figure S13. Phenotypic changes in L6H9-treated type 1 diabetic mice. Curcumin, which has been reported to show protective effect on diabetic nephropathy, was used as a positive comparison. *Method:* Male C57BL/6 mice weighing 18–22 g were obtained from the Animal Centre of Wenzhou Medical University. Animals were housed at a constant room temperature with a 12:12 h light–dark cycle and were fed with a standard rodent diet and water. All animal care and experimental procedures were approved by the Wenzhou Medical University Animal Policy and Welfare Committee. To induce type 1 diabetes, mice were treated with a single intraperitoneal injection of STZ (100 mg/kg in citrate buffer, pH 4.5), while the control animals were received the same volume of citrate buffer. The blood glucose level was monitored using glucometer. One week after STZ injection, mice with fasting-blood glucose >216 mg/dL were considered as diabetic. Randomly selected diabetic animals were divided into three groups: DM (n=8), curcumin-treated DM (DM+Cur, n=8) and L6H9-treated DM (DM+L6H9, n=8). In the DM+Cur group and DM+L6H9 group, curcumin (50mg/kg) and L6H9 (20mg/kg) were administered as oral gavage once every 2 days, respectively. The DM group and age-matched control group (n=8) received 1% CMC-Na solution alone according to the same schedule. Bodyweight and blood glucose levels were recorded weekly. After 8 weeks of treatment, animals were killed under ether anesthesia. Blood and renal tissues were collected at the time of sacrifice. A portion of renal tissues from all animals were fixed in 4% paraformaldehyde for paraffin embedding and histopathological analysis (The methodology for histopathological analysis was referred the description in Materials and Methods). Remaining renal tissues were snap-frozen in liquid nitrogen for gene and protein expression analysis. A and B. L6H9 or curcumin treatment had no effect on body weight change (A) and serum glucose level (B). C. Both treatments however reduced kidney/body ratio, which indicated less kidney injury. Values are reported as means ± SEM; n=8 in four groups; $^{\#}$ $P<0.05$, versus Ctrl group; * $P<0.05$, versus DM group. Neither L6H9 nor curcumin (used as a positive control) treatment altered body weight or blood glucose levels in the diabetic mice. However, kidney to body weight ratio was significantly reduced following L6H9 treatment.

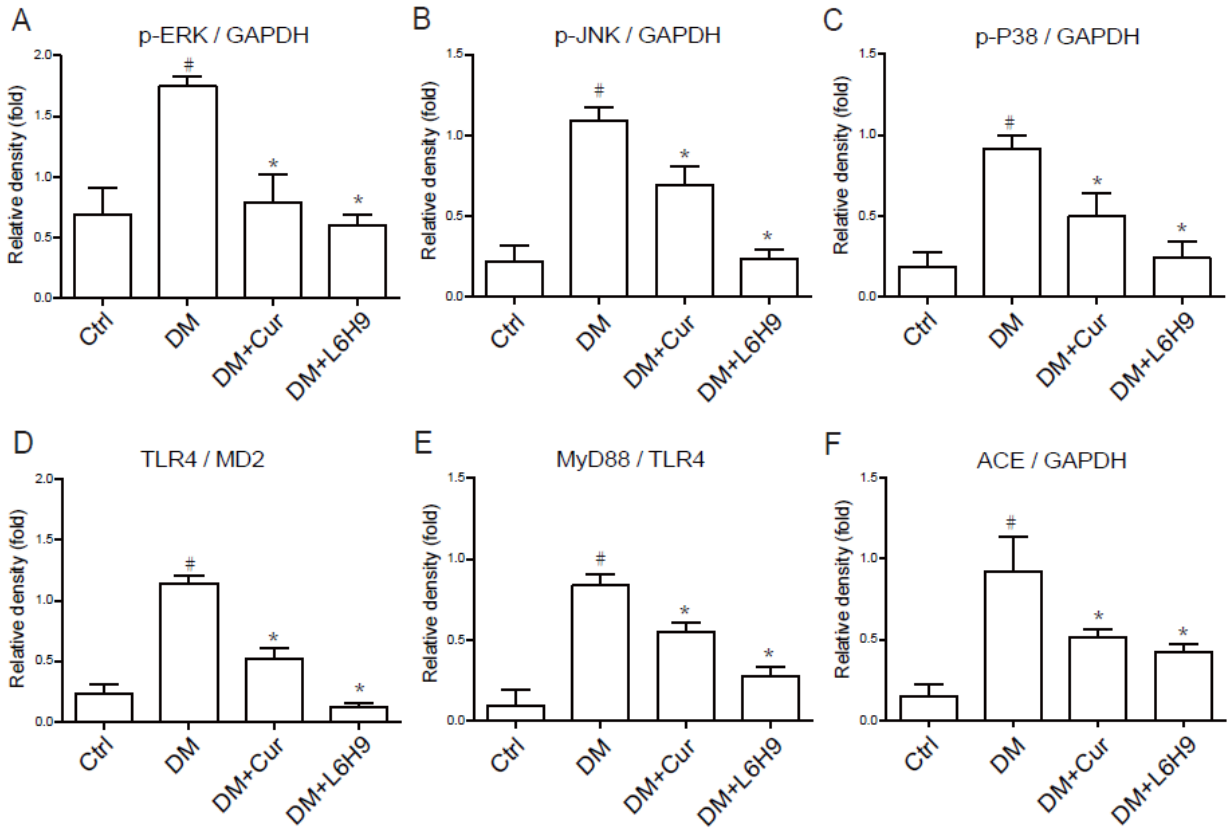

Figure S14: Densitometric quantifications for Figure 7B, 7C, and 7F. Panel A-C, for Figure 7B; Panel D and E, for Figure 7C; Panel F, for Figure 7F. (n=7 per group, $^{\#}P<0.05$, vs the Ctrl group; $^{*}P<0.05$, vs the DM group).

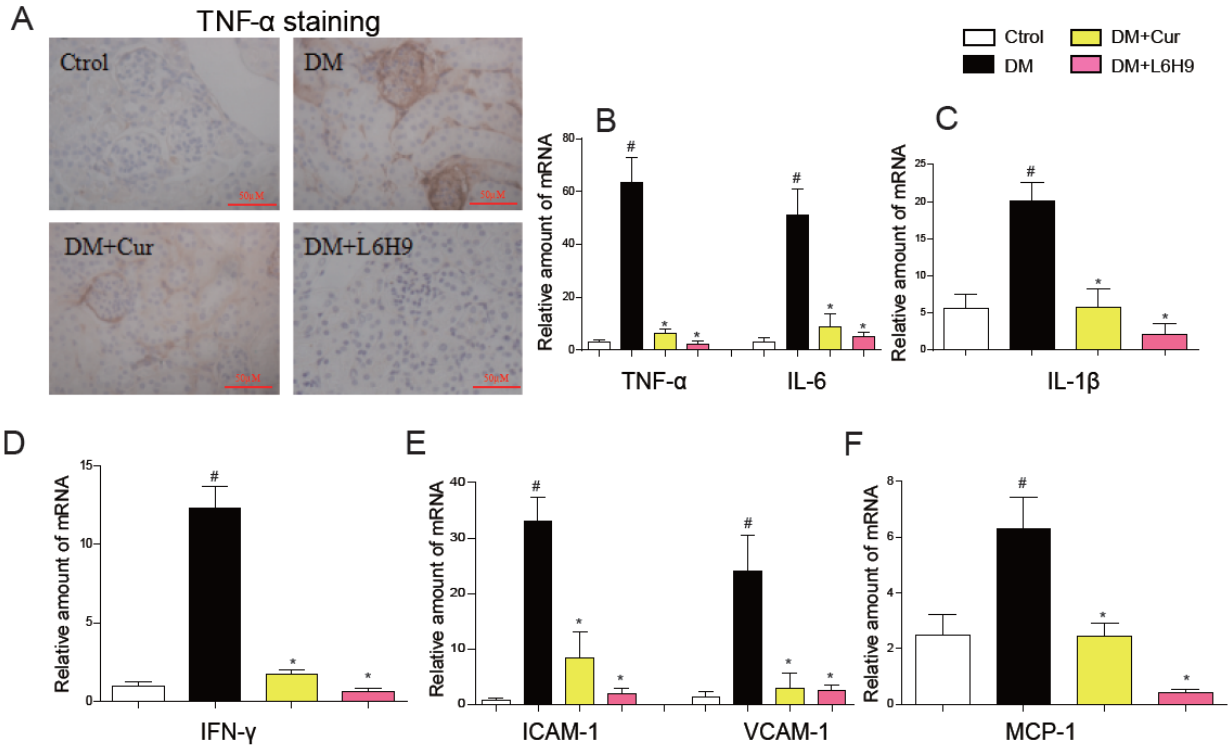

Figure S15：Oral administration of L6H9 inhibited diabetic-induced inflammation in C57BL/6 mouse kidney. The methodology of animal experiment was described in Figure S13. L6H9 lowered inflammatory response in kidneys in diabetes as evidenced by decreased hyperglycemia-induced TNF-α production, mRNA expression of inflammatory cytokines (TNF-α, IL-6, IL-1β and IFN-γ), adhesion molecules (ICAM-1 and VCAM-1) and chemokine (MCP-1) at a level comparable to those of curcumin treatment. A) Immunohistochemical analysis showing diabetes induced increased TNFα protein expression is prevented following L6H9 and curcumin treatments. In parallel diabetes induced increased renal B) TNFα, C) IL-1β D) IFN-α, E) ICAM-1 and VCAM-1 and F) MCP-1 mRNA expressions were prevented following curcumin and L6H9 treatment (mRNA data are normalized to β-actin, DM=diabetic)**.** Values are reported as means ± SEM; n=8 in four groups; $^{\#}$ P<0.05 versus Ctrl group; * P<0.05 versus DM group.

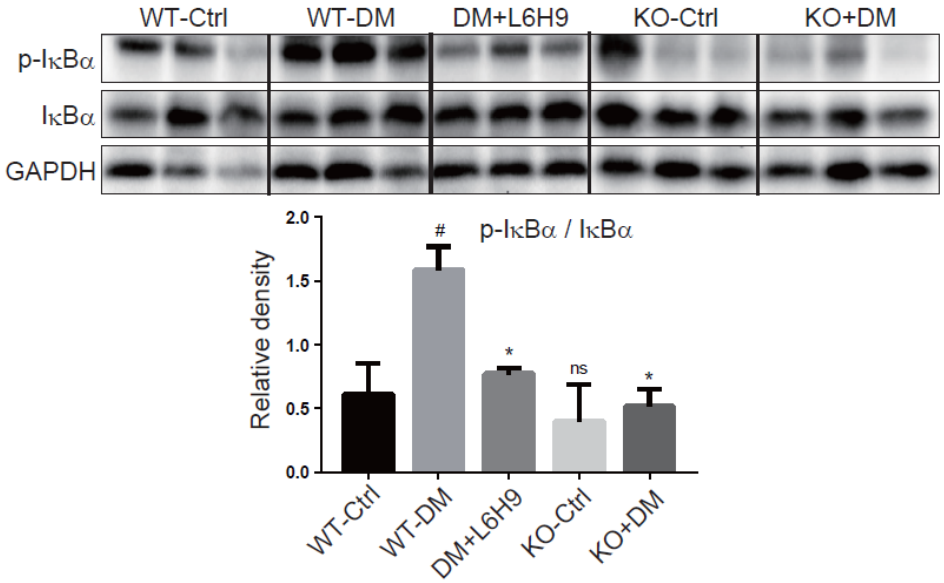

Figure S16 : Either L6H9 administration or MD2 knockout inhibited diabetic-induced IκBα phosphorylation in C57BL/6 mouse kidney. The methodology of animal experiments was described in Methods section. The mouse kidney tissues were performed to determine IκBα phosphorylation using Western blot assay. Below is the densitometric quantification. (n=8 per group, $^{\#}P<0.05$ and ns =no significance, vs the WT-Ctrl group; $*P<0.05$, vs the WT-DM group).