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Effect of SOCS1 on diabetic renal injury through regulating TLR signaling pathway

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Abstract. – OBJECTIVE: To clarify the effect of suppressor of cytokine signaling 1 (SOCS1) on diabetic nephropathy (DN)-induced renal injury by regulating the Toll-like receptor (TLR) signaling pathway.

MATERIALS AND METHODS: The Sprague-Dawley rats were divided into control group (n=10) and DN group (established with streptozotocin injection, n=20). The DN rats were administrated with SOCS1 lentivirus to upregulate the *in vivo* expression. The rat blood glucose was detected to confirm the successful preparation of the DN model. The hepatic and renal function indexes, including blood urea nitrogen (BUN), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and creatinine (CR) were detected. The pathological lesions in the kidney were observed via hematoxylin-eosin (HE) staining. Besides, the serum levels of the inflammatory factors in rats were detected via enzyme-linked immunosorbent assay (ELISA). The relative levels of genes in the TLR signaling pathway were detected via RT-PCR and Western blotting.

RESULTS: The blood glucose level in rats of the DN group was significantly enhanced, indicating the successful modeling. The expression of SOCS1 was significantly upregulated in rats administrated with SOCS1 lentivirus. The contents of BUN, ALP, ALT, and CR in rats of SOCS1 overexpression group were significantly lower than those in the DN group. The inflammatory infiltration in the kidney and the glomerular injury were pronounced in the DN group. The serum levels of interleukin-1 (IL-1), interferon-γ (INF-γ), and tumor necrosis factor-α (TNF-α) were significantly declined in SOCS1 overexpression group. Besides, the mRNA expressions of myeloid differential protein-88 (MyD88), TLR2, and INF-γ, and the protein expression of TLR2 were all remarkably downregulated in SOCS1 overexpression group.

CONCLUSIONS: SOCS1 can promote renal injury repair in DN rats by inhibiting the TLR pathway. Therefore, SOCS1 is expected to be a new target for the repair of DN renal injury.

Key Words
SOCS1, TLR signaling pathway, Diabetes mellitus, Renal injury, Rats.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases due to defects in insulin secretion or function[1]. DM has become one of the most common chronic metabolic diseases in the world[2]. The traditional treatments for DM mainly include insulin preparations and oral hypoglycemic drugs. However, the latter will lead to lactic acidosis, pulmonary edema, etc[3]. In recent years, cell therapy using human embryonic stem cells and adult stem cells has been well concerned[4]. Although cell therapy can effectively reduce renal injury, promote tubular proliferation, and improve renal function, its high medical expense significantly restricts the clinical application. Therefore, searching for new treatment methods for DM has become a top priority. The complications of DM include diabetic nephropathy (DN), proteinuria and end-stage renal failure characterized by the decline in glomerular filtration rate and kidney structural changes (i.e., basement membrane thickening and mesangial sclerosis). Hyperglycemia is the main cause of advanced glycation end (AGE) products and diabetic renal insufficiency[5,6]. However, Bos et al[7] have demonstrated that many patients are poorly responded to these drugs, which aggravates the end-stage renal disease. According to Makni et al[8], the dietary antioxidants seem to be a potential adjuvant therapy that can prevent or delay DM complications, such as renal diseases. Moreover, the statins are reported to improve blood glucose control by stimulating the gastrin axis, which can prevent renal injury and provide support for kidney shielding[9]. However, no definite conclusion has been made, and there is a great controversy

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in the above studies. Therefore, the clarification of these molecular regulatory networks is critical for the treatment of DN.

The specific innate immune recognition receptors are able to recognize multiple risk signals of DM by triggering the pro-inflammatory cascade during DN[10]. The Toll-like receptors (TLRs) can recognize the pathogenicity-related and damage-related molecular patterns. TLRs can promote the activation of the leukocytes and innate renal cells in non-immune renal disease, thus triggering the pro-inflammatory signaling pathway of microbial pathogens[11,12]. It is recently recognized that TLRs are activated by non-microbe-derived endogenous ligands[13]. Therefore, the increased contents of TLRs in renal cells and leukocytes are thought as the main cause of acute and chronic kidney diseases[14]. TLR2 and TLR4 in monocytes are positively correlated with the level of glycosylated hemoglobin A1c, and myeloid differential protein-88 (MyD88) is the downstream molecule of TLRs. TLRs may be important regulators in renal immune cascade and ultimately lead to DN[15]. Recent reports suggested that TLR-mediated signals can be regulated by the suppressor of cytokine signaling (SOCS). SOCS1 mediates the inflammatory response induced by interferon (IFN)[16]. Nevertheless, some studies have shown that the regulatory capacity of SOCS1 is not limited to IFN. The ability of SOCS1 to regulate the TLR-mediated signal transduction and to produce pro-inflammatory cytokines has aroused much interest[17-19]. SOCS1 is abnormally down-regulated in the kidney of DN mice. More interestingly, SOCS1 can suppress the progression of DN by inhibiting the TLR pathway. However, the mechanism of SOCS1 in inhibiting the TLR pathway remains unclear, nor the correlation between renal injury and SOCS1/TLR pathway-mediated inflammation. Besides, the regulatory effect of SOCS1 on DN has not been clarified.

The present work aims to investigate the effect of SOCS1 on renal injury in DN rats through the TLR pathway and its specific mechanism.

Materials and Methods

Animal Grouping and Modeling

The male Sprague-Dawley rats were divided into the blank control group (n=10) and DN group (intraperitoneal injection of streptozotocin, n=20). 10 rats in the DN group were administrated with SOCS1 overexpression lentivirus (SOCS1 overexpression group, n=10). SOCS1 complementary deoxyribonucleic acid (cDNA) was designed and used for the gene-specific primer amplification to produce N-terminal hemagglutinin. After purification, the amplified SOCS1 cDNA was subcloned into green fluorescence protein (GFP) to express the adenovirus transfer vector, and the adenovirus containing SOCS1 was transfected into the rats according to the adenovirus transfection instructions. The rat blood and kidney samples were collected for later use. This study was approved by the Animal Ethics Committee of Sichuan University Animal Center.

Determination of Animal Model Preparation

To observe the successful preparation of the DN model, the rat blood was taken from the caudal vein after modeling and centrifuged to separate the serum. The serum level of blood glucose was determined to verify the successful establishment.

Detection of Transfection Efficiency of SOCS1 Adenovirus

The adenovirus containing SOCS1 was transfected into the rats. The transfection efficiency of SOCS1 in DN was detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using the primer sequences of SOCS1 shown in Table I.

Table I. Primer sequences in RT-PCR.

Target gene	Primer sequence (5'–3')
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCTGTTGCTGTAGCCAAA-3'
INF-γ	F: 5'-GAGGGATCCATGAAATATACAAGCTAT-3' R: 5'-GACGAATTCTTACGTTGATGCTCTCC-3'
SOCS1	F: 5'-GACCCGTAAATCTGAAGCTAATGC-3' R: 5'-AATTAAGGCATCACAGTCCGAGTC-3'
TLR2	F: 5'-CTGAACCAGGGCATACCTGT-3' R: 5'-GAGAAGTCCATGTCCGCAAT-3'
MyD88	F: 5'-AGCTGGAGCAGACGGAGTG-3' R: 5'-GAGGCTGAGAGCAAACTTGGTC-3'

Detection of Hepatic and Renal Functions

To predict the occurrence of DN in advance in clinical practice and provide important references for early diagnosis, the hepatic function indexes alkaline phosphatase (ALP), the alanine aminotransferase (ALT), the renal function indexes creatinine (CR), and the blood urea nitrogen (BUN) were detected. The serum samples stored in the low-temperature refrigerator were thawed and centrifuged. Finally, the serum levels of the abovementioned indexes were detected using the full-automatic biochemical analyzer according to the instructions.

Observation of Pathological Renal Changes Via Hematoxylin-Eosin (HE) Staining

The kidney tissues were fixed in formalin, washed with running water for 24 h, transparentized, immersed, and embedded in paraffin. The embedding block was sliced into pathological sections (about 5 μm in thickness), stained with hematoxylin for 10 min, washed with water, and counterstained with eosin for 3 min. Subsequently, the block was dehydrated with alcohol, transparentized, and sealed with neutral resin, followed by the tissue observation under a light microscope.

Detection of Inflammatory Factors Via Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of the inflammatory factors were detected via ELISA (R&D Systems, Minneapolis, MN, USA). The serum samples previously collected and frozen at -80°C were took out, slowly thawed at 4°C and centrifuged again at a low speed. Then the supernatant was collected, incubated (100 μL) at 37°C for 90 min, and washed. The serum levels of the inflammatory factors in each group were detected using a microplate reader.

RT-PCR

About 200 mg of sterile kidney tissues were weighed accurately under low temperature. The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was qualified. The RNA was reversely transcribed into cDNA, followed by a primer amplification using the 20 μL system (2 μL of cDNA, 10 μL of mix, 2 μL of primer, 6 μL of ddH_2O). The sequences of the target genes and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to those published in the GenBank (Table I). The expression levels of the target genes were detected via qRT-PCR using the ABI 7500 real time-PCR system and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Firstly, the ratio of lysis buffer was calculated according to the instructions of the protein extraction kit. About 150 mg of sterile kidney tissues were accurately weighed, placed into a 10 mL Eppendorf (EP) tube, taken under low temperature, and rapidly smashed using a homogenizer under low temperature. The homogenate was lysed in the protein lysis buffer and centrifuged. Secondly, the supernatant was collected and placed into the EP tube, followed by the detection of protein concentration according to the instructions of the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Thirdly, the protein sample was loaded, subjected to electrophoresis, and transferred onto a membrane. After blocking in 5% skim milk for 2 h, the membrane was incubated with the primary antibody and the horseradish peroxidase-labeled rabbit secondary antibody. Then, the protein band was scanned and quantified using the scanner (Bio-Rad, Hercules, CA, USA). The protein level was normalized to that of GAPDH, and the gray value of the target protein band was analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for the processing of the raw experimental data. The experimental results obtained were expressed as mean ± standard deviation ($\bar{\chi}\pm SD$). $p<0.05$ suggested a statistically significant difference. The bar graph was plotted using the GraphPad Prism 7.04 (La Jolla, CA, USA).

Results

Successful Establishment of Animal Model

The blood glucose of the rat was measured to verify whether the DN model was successfully established. As shown in Figure 1, the level of blood glucose in the DN group was significantly higher than that in the healthy rats ($p<0.05$).

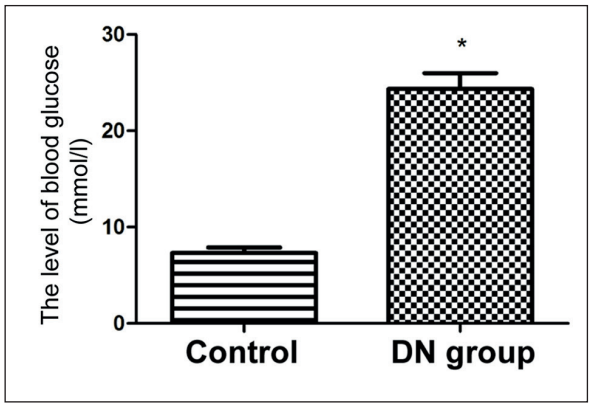

Figure 1. The level of blood glucose in the DN group is significantly higher than that in healthy rats. *p<0.05: There is a statistically significant difference.

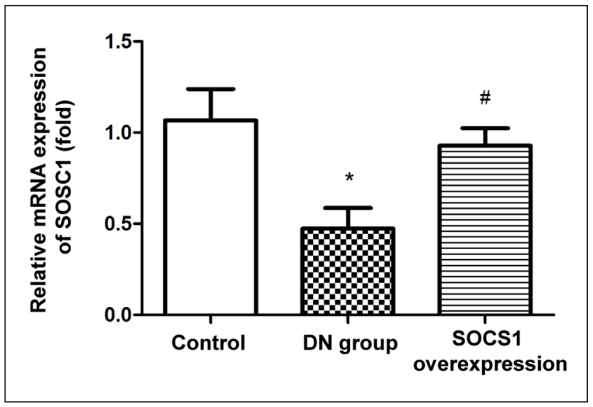

Figure 2. Transfection efficiency of SOCS1 lentivirus. The expression level of SOSC1 is significantly increased in the SOCS1 overexpression group (p<0.05). #p<0.05 *vs.* DN group, *p<0.05 *vs.* control group.

Transfection Efficiency of SOCS1 Adenovirus

To further explore the role of SOCS1 in kidney tissues of DN, SOSC1 was overexpressed in rats by adenovirus transfection. The transfection efficiency of SOSC1 was detected via RT-PCR. As shown in Figure 2, the expression level of SOSC1 was significantly upregulated in SOCS1 overexpression group (p<0.05).

Hepatic and Renal Functions

The serum levels of biochemical indexes CR, BUN, ALT, and ALP were detected here. As shown in Table II, the contents of CR, BUN, ALT, and ALP significantly declined in the SOSC1 overexpression group (p<0.05). It is suggested that the hepatic and renal functions were severely damaged in DN rats.

HE Staining Results

After HE staining, the morphological changes in rat kidney of each group were observed under the light microscope (Figure 3). In the DN group, the glomerular injury, glomerular hypertrophy, and proximal tubular injury were pronounced. The glomerular basement membrane was remarkably thickened. The tubular cystic dilatation, the vacuolization of the tubular epithelial lining, and the tubular hyaline change were shown in DN rats (Figure 3A). In SOSC1 overexpression group, the abovementioned pathological lesions were much more alleviated (Figure 3B).

Serum Levels of Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6)

The serum levels of TNF-α, IL-1, and IL-6 increased in the DN group (p<0.05), while they declined in the SOSC1 overexpression group, and closed to those in the control group (p<0.05) (Table III).

TLR Pathway Changes in DN

The expressions of interferon-γ (INF-γ) and key genes in TLR pathway TLR2 and MyD88 were remarkably declined in the SOSC1 overexpression group (p<0.05) (Figures 4, 5), indicating the activated TLR pathway following DN.

Table II. Changes in content of CR, BUN, GPT and ALP.

Group	CR (μmol/L)	BUN (mmol/L)	ALP (U/L)	ALT (U/L)
Control group	22.36±4.16	19.34±3.42	88.36±5.23	50.67±4.28
DN group	100.54±2.34[a]	39.57±5.27[a]	225.18±2.52[a]	142.38±5.37[a]
SOSC1 overexpression group	43.28±5.24[b]	26.58±2.15[b]	105.14±4.56[b]	67.59±5.34[b]

Note: The content of CR, BUN, GPT and ALP decline significantly in SOSC1 overexpression group (p<0.05). [a]p<0.05 *vs.* control group, [b]p<0.05 *vs.* DN group.

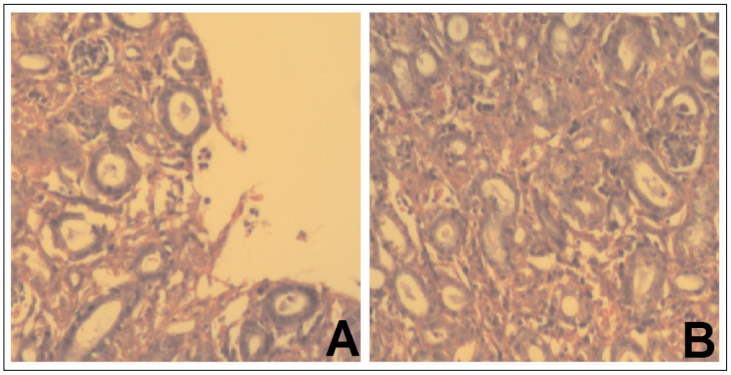

Figure 3. Morphological observation of the kidney. **A**, In the DN group, the glomerular injury, glomerular hypertrophy, and proximal tubular injury occur (magnification×100). **B**, In the SOSC1 overexpression group, the glomerular and tubular morphology is normal, and no pathological changes are observed (magnification × 100).

Table III. Serum TNF-α, IL-1, and IL-6 content.

Group	TNF-α (fmol/mL)	IL-6 (mg/L)	IL-1(mg/L)
DN group	52.84±1.72[a]	98.53±3.56[a]	100.69±5.76[a]
SOSC1 overexpression group	20.48±2.15[b]	35.25±6.47[b]	38.34±3.58[b]
Control group	15.34±3.14	29.15±2.58	30.14±5.64

Note: The content of TNF-α, IL-1 and IL-6 is increased in DN group ($p<0.05$), while it declines in SOSC1 overexpression group ($p<0.05$). [a]$p<0.05$ *vs.* control group, [b]$p<0.05$ *vs.* DN group

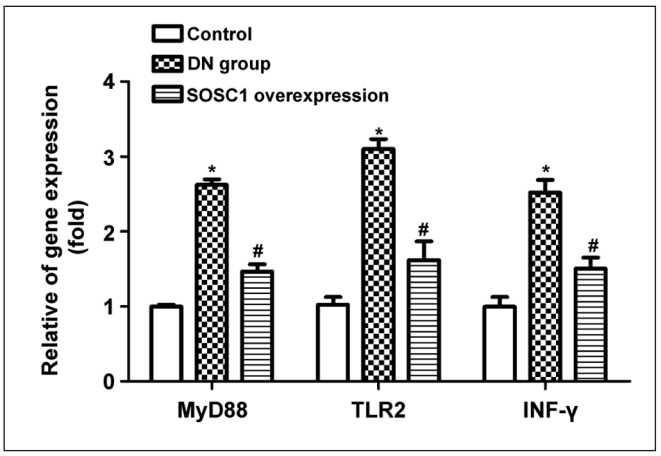

Figure 4. Results of gene expression. The expressions of INF-γ and pathway genes TLR2 and MyD88 remarkably decline in the SOSC1 overexpression group ($p<0.05$). There is a statistically significant difference vs. control group (*$p<0.05$) and *vs.* DN group (#$p<0.05$).

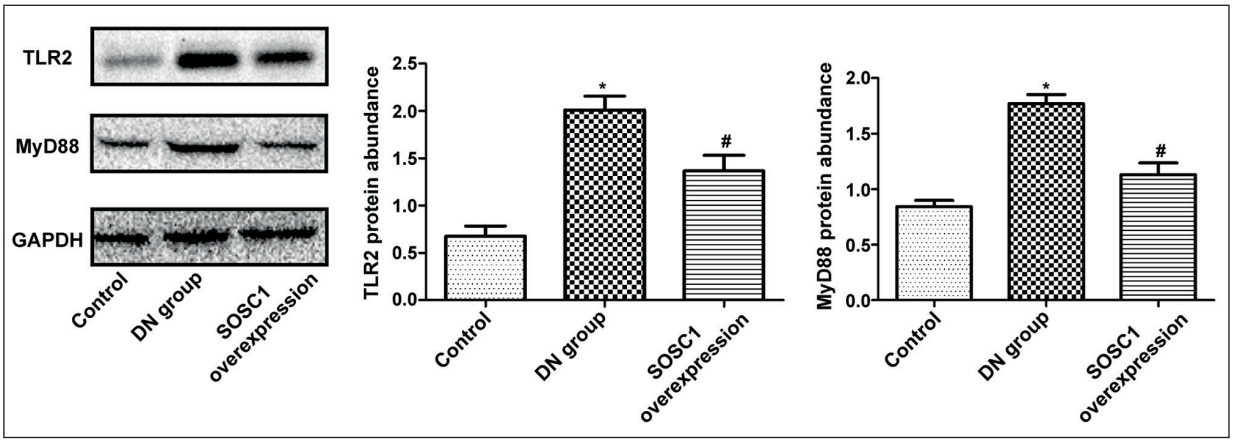

Figure 5. Protein expressions. Protein expressions of TLR2 and MyD88 remarkably decline in the SOSC1 overexpression group ($p<0.05$). There is a statistically significant difference *vs.* control group (*$p<0.05$) and vs. DN group (#$p<0.05$).

Discussion

DM is a lifelong chronic disease characterized by a high level of blood glucose. DM has become one of the diseases with the highest mortality rate in the world, causing various complications. It is also the main cause of non-invasive amputation, blindness, visual impairment, and end-stage renal disease in adults[20]. The treatment of DM complications is a major challenge. STZ-induced DN leads to the necrosis of pancreatic β-cells in rats, resulting in the degeneration and loss of function to secrete insulin[21]. STZ significantly increases the level of fasting blood glucose and it is applied for the establishment of the DN animal model[22]. Preventing the occurrence and development of DN has been well concerned. The increased levels of blood glucose, CR, BUN, and uric acid are markers of DN. Previously Zhang et al[23] confirmed that the BUN level is elevated in renal injury mice. The kidneys are more susceptible to aging than other tissues. The renal disease is a complex and dynamic process regulated by a variety of cellular components and cytokines, involving the influence of multiple genes and regulatory factors on renal injury[24,25]. Therefore, a deep understanding of the specific molecular regulatory network of SOSC1 is essential for the treatment of the renal injury. In the present study, the level of blood glucose in the DN group was significantly elevated, indicating that the DN model was successfully established. To further explore the role of SOSC1 in renal injury, *in vivo* SOSC1 was overexpressed using the adenovirus transfection, and the transfection efficiency in the DN rat model was detected. The results revealed that the expression level of SOSC1 in SOSC1 overexpression group was significantly higher than that in the DN group. In addition, renal injury repair is regulated by a variety of genes or proteins. Searching for the key genes and proteins that are related to renal injury, and designing drugs targeting these genes or proteins will provide new ideas for renal injury repair. In this experiment, the contents of CR, BUN, ALT, and ALP in SOSC1 overexpression group significantly declined ($p<0.05$), indicating the occurrence and development of the disease. Some studies have demonstrated that inflammation plays an indispensable role in the occurrence and development of DN. With the aggravation of the inflammatory response, a persistent infiltration initiated from neutrophils and macrophages gradually invaded the interstitial space[26]. In this study, the levels of IL-6, IL-1, and TNF-α were elevated in DN group, demonstrating that the increased levels of IL-6 and TNF-α will further promote the development of DN and the inflammatory response. The overexpression of SOSC1 alleviated the above pathological condition, indicating that SOSC1 had a good protective effect on DN. Moreover, IL-6 can also stimulate the excessive production of other inflammatory mediators. Our conclusion identically demonstrated that SOSC1 was able to inhibit the excessive inflammatory cytokines and prevent excessive production from causing irreversible damage to cells. In addition, the morphological observation showed that the glomerular injury, glomerular hypertrophy, and proximal tubular injury occurred in the DN group, which was consistent with the previous studies[27].

The TLR inflammatory pathway plays an important role in renal disease, and it triggers different downstream signaling cascades, including MyD88-dependent and independent pathways. It produces downstream pro-inflammatory cytokines and chemokines through NF-κB[28,29]. TLR2 significantly reduces the serum levels of cytokines in mice, causing oliguria and renal histological damage[30]. Based on this, many genes or proteins that can regulate this pathway are expected to be potential targets for the treatment of renal diseases. In this research the high-level SOCS1 protected the kidney by inactivating the TLR signaling pathway. During the development of the renal disease, the expressions of the important index INF-γ and pathway genes TLR2 and MyD88 were significantly downregulated in the SOSC1 overexpression group. Moreover, the expression levels of TLR2 and MyD88 were remarkably declined in SOSC1 overexpression group, indicating that the overexpression of SOSC1 can promote the recovery of renal injury in renal diseases, which was consistent with the study of Mudaliar et al[31]. Therefore, SOCS1, as a key regulator of the TLR signaling pathway, seems to be an effective target for the treatment of DN injury. To sum up, the above results indicated that SOCS1 affected renal injury in renal diseases by down-regulating the TLR signaling pathway.

Conclusions

We demonstrated that SOSC1 protects the progression of DN by mediating the TLR2-MyD88 pathway to alleviate the inflammatory response. This study provides a theoretical basis for the prevention and treatment of DN and its complications

Conflict of Interests

The Authors declared that they have no conflict of interests.

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