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Original Article

Role of toll-like receptor 4-dependent signal pathways in bone marrow-derived macrophage activation induced by high glucose

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Abstract: Chronic microinflammatory state plays an important role in occurrence and development of diabetic nephropathy. Macrophages are the main regulatory cells in the inflammatory response. Toll-like receptors (TLRs) play an important role in innate immune response and inflammation. In the present study, bone marrow-derived macrophages (BMDMs) were separated from C57BL/6J and B10ScNNju (TLR4 knockout mice). These were divided into a normal control group (LG), high glucose group (HG), TLR4 knockout group (TLR4-/-), and high glucose stimulated TLR4 knockout BMDM group (TLR4-/-+HG). The M1 phenotype of macrophages was detected by flow cytometry and co-expression of TLR4 while macrophage activation marker inducible nitric oxide synthase (iNOS) was observed by immunofluorescence. Tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-1 (IL-1β) were assessed by RT-PCR and ELISA, together with mRNA levels of iNOS. Western blot was performed to analyze protein levels of TLR4, myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-b (Trif), p-IRAK-1, p-IRF3, IRF3, NF-κB p65, NF-κB p-p65, and iNOS. Compared to the LG group, high glucose increased the percentage of M1 macrophages and mRNA levels of TNF-α, MCP-1, IL-1β, and iNOS. In addition, expression of TLR4, MyD88, Trif, p-IRAK-1, p-IRF3, IRF3, NF-κB p65, NF-κB p-p65, and iNOS proteins were enhanced. Knockout of TLR4 genes eliminated the effects of macrophage activation induced by high glucose. The present study suggests that high glucose can promote BMDM to M1 phenotype polarization and knockout of TLR4 genes can inhibit the M1 phenotype of macrophage activation and production of inflammatory cytokines induced by high glucose.

Keywords: BMDM, diabetic nephropathy, high glucose, inflammation, TLR4

Introduction

Diabetic nephropathy (DN) is a severe microvascular complication, common in diabetic patients, but the exact pathogenesis remains unclear. In addition to changes in renal hemodynamics, glucose and lipid metabolism disorders, oxidative stress, and genetic predisposing factors, recent studies have found that chronic microinflammatory state plays an important role in occurrence and development of DN [1]. Macrophages are the main regulatory cells in the inflammatory response and macrophage infiltration occurs in renal tissue in the early stages of DN, promoting the secretion of cytokines, inflammatory mediators, and oxygen free radicals [2-4], resulting in renal tissue structural and functional damage. The method of infiltration and activation of macrophages during DN has been the focus of attention in many studies.

Toll-like receptors (TLRs) are a classic family of membrane receptors in the innate immune system. Some studies have shown that TLRs, especially TLR2 and TLR4, are increased in type 1 diabetes and type 2 diabetes mellitus [5]. In addition, it has been demonstrated that TLRs are a pathway for macrophage activation in diabetic atherosclerosis [6]. Their activation triggers a signaling cascade resulting in cytokine production and initiation of an adaptive immune response [7]. Mohammad et al. [8] showed that expression of TLR2 and TLR4 increased in type 1 diabetic nonobese mice. This triggered increased nuclear factor kB activation in response to the TLR4 ligand, LPS, resulting in increased production of proinflammatory cytokines. Devaraj et al. [9] demonstrated that levels of MyD88, IRAK-1 protein phosphorylation, Trif, IRF3, and NF-κB activity were significantly reduced in TLR4(-/-)+STZ mice compared to WT+STZ mice. Levels of serum and macrophage IL-1β, IL-6, MCP-1, and TNF-α in WT+STZ mice significantly increased compared to WT mice. This was significantly attenuated in TLR4(-/-)+STZ mice. Dasu et al. [10] reported that high glucose can cause increased expression of TLR2 and TLR4 in THP-1 mononuclear cells and induce activation of MyD88/IRAK-1/NF-κB signaling pathways. Many studies have shown that TLR2 and TLR4 of TLRs are likely to be correlated with key factors of the innate immune system and microvascular inflammatory response, in diabetic patients, and a favorable target for anti-inflammatory therapy.

High glucose is a prerequisite for development and progression of DN [11]. Therefore, it was hypothesized that high glucose can act as an endogenous ligand of TLR4 in combination with activated downstream signaling pathways, leading macrophages polarized to M1 type. Additionally, this study may provide an experimental basis for further research on blocking activation of TLR4 signaling pathways of macrophages in the prevention of DN.

Materials and methods

Reagents

FITC stained anti-mouse F4/80 antibody, APC stained anti-mouse CD11b antibody, PE stained anti-mouse CD11c antibody, and isotype controls were purchased from BioLegend (BioLegend, San Diego, California, USA). DMEM medium and fetal bovine serum (FBS) were obtained from WISENT (WISENT, Canada). D-glucose and mannitol were purchased from Sigma (Sigma, USA). TRIzol Reagent was purchased from Invitrogen (Invitrogen, California, USA). cDNA synthesis kit was obtained from Promega (Promega, Madison, USA). SYBR Green PCR Master Mix Kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Real-time fluorescence quantitative PCR primers were bought from Sangon Biotech and United States GeneCopoeia. Anti-TLR4, anti-MyD88, anti-Trif, and anti-iNOS antibodies were purchased from Abcam Biotechnology (Abcam, Cambridge, UK). Anti-p-IRAK1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-pIRF3, anti-IRF3, and anti-NF kappa B p65 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-action antibody, horseradish peroxidase-labeled goat anti-rabbit IgG, and anti-mouse IgG conjugated to horseradish-peroxidase were purchased from Wuhan Sanying Biotechnology Inc (Wuhan, China). Bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). ECL enhanced chemiluminescence kit was obtained from Thermo (Thermo Scientific, USA). TNF-α and IL-1β Enzyme Linked Immunosorbent Assay Kit (ELISA Kit) were purchased from R&D Systems (R&D Systems, USA). MCP-1 ELISA Kit was purchased from RIBIO TECH (RIBIO TECH, Beijing, China).

Isolation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated from male wild-type littermates SPF C57BL6/J mice, 6 to 8 weeks old, and male SPF B10ScNNju (TLR4-/-) mice, 6 to 8 weeks old. They were provided by Nanjing University Model Animal Research Institute. Mice weights were controlled at 18~20 g. The mice were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 minutes. The femur and tibia were separated and soaked in 70% ethanol for 3 minutes. Cells were washed out into 2% fetal bovine serum and cold PBS 3-5 mL/mouse. Supernatant was discarded by centrifugation and resuspended in low glucose-Dulbecco's modified Eagle media, containing 15% L929 cell culture medium, 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cell concentration was adjusted to 1*10^6 cells/mL. They were then inoculated into six-well plates and cultured in a 37°C incubator containing 5% CO_2. Cells were acquired on day 7. Double-labeled F4/80 and CD11b were positive for mature bone marrow-derived macrophages.

Optimization of experimental conditions

Using different concentrations of glucose to stimulate mature bone marrow-derived macrophages, mannitol supplementation was used as osmolality control. Cells were collected and total protein was extracted. The glucose concentration promoting the highest expression of TLR4 and iNOS protein was selected as the

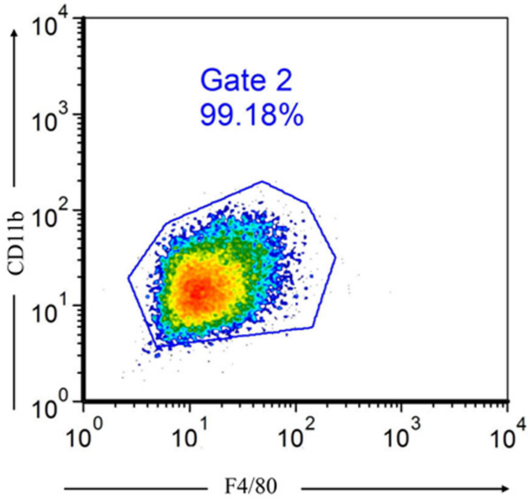

Figure 1. Identification of BMDMs. Mature BMDMs were defined as F4/80+CD11b double positive cells.

high glucose stimulating concentration in subsequent experiments. Bone marrow macrophages were stimulated with the same high glucose concentration and cells were harvested at different time points. Total protein was extracted and expression of TLR4 and iNOS proteins were observed to determine optimal stimulating time.

Confocal microscopy analysis

Cells were plated at 1 × 10^5 cells/well on a Petri dish. Cell samples were fixed with ice-cold methanol for 15 minutes. After blocking with 5% donkey derum albimin for 2 hours, the macrophages were incubated with anti-iNOS and anti-TLR4 primary antibody at 4°C overnight. After washing with PBS, FITC-conjugated IgG and PE-conjugated IgG were added for 2 hours in the dark. Nuclear were stained by 4-, 6-diamidino-2-phenylindole (DAPI) and the cells were then observed under Leica TCS SP5 laser confocal microscope (Leica, Germany).

Flow cytometry (FCM) analyses

Anti-mouse CD16/CD32 receptor blocking antibody was incubated with BMDMs for 30 minutes. This was followed by the addition of FITC-labeled anti-mouse F4/80 antibody, APC-labeled CD11b, and PE-labeled CD11c antibody. Cells were incubated at room temperature for 30 minutes in the dark. Supernatant was discarded by centrifugation and resuspended in 500 μl of PBS. CD11c-positive, F4/80-positive, and CD11b-positive cells were detected as positive macrophages with the help of the Beckman FACS Calibur. Percentage of positive macrophages was also calculated.

RNA extraction and RT PCR

Total RNA was extracted using TRIzol Reagent, according to manufacturer protocol. cDNA was synthesized from total RNA by reverse transcriptase. To determine the quantity of mRNA, SYBR Green method was used. Primer sequences to detect mRNA were: GAPDH: Forward primer 5'-ACCCCAGCAAGGACACTGAGCAAG-3'; Reverse primer 5'-GGCCCCTCCTGTTATTATGGGGGT-3'; TNF-α: Forward primer 5'-CCCTCCTGGCCAACGGCATG-3' Reverse primer 5'-TCGGGGCAGCCTTGTCCCTT-3'. Primers MCP-1 (MQP027672), IL-1β (MQP027422), and iNOS (MQP029793) were purchased commercially from GeneCopoeia, Inc (Rockville, MD, USA). Expression levels of all genes were normalized with the reference gene GAPDH using the 2-ΔΔCt method.

Western blots

Cells were collected and total protein was extracted after lysis. Proteins were separated by 10-12% SDS-PAGE and electro blotted onto a nitrocellulose membrane, incubated with primary antibody anti-TLR4 (1:1000), anti-MyD88 (1:1000), anti-NF-κB p-p65 (1:1000), anti-NF-κB (1:1000), anti-pIRF1 (1:1000), and β-actin (1:35000) antibodies overnight at 4°C. Next, they were washed with TBST three times and incubated at 37°C with horseradish peroxidase labeled secondary antibody. The final step was observation of the image, requiring the help of enhanced chemiluminescence. Protein content was quantitated using the documentation system.

Enzyme linked immunosorbent assay (ELISA)

At the end of the experiment, the macrophage culture medium was gathered and levels of TNF-α, IL-1β, and MCP-1 in the medium were determined by ELISA kits, according to manufacturer instructions.

Statistical analyses

Data were analyzed using SPSS 16.0 software. Results are expressed as mean ± standard deviation (SD). All compared data were con-

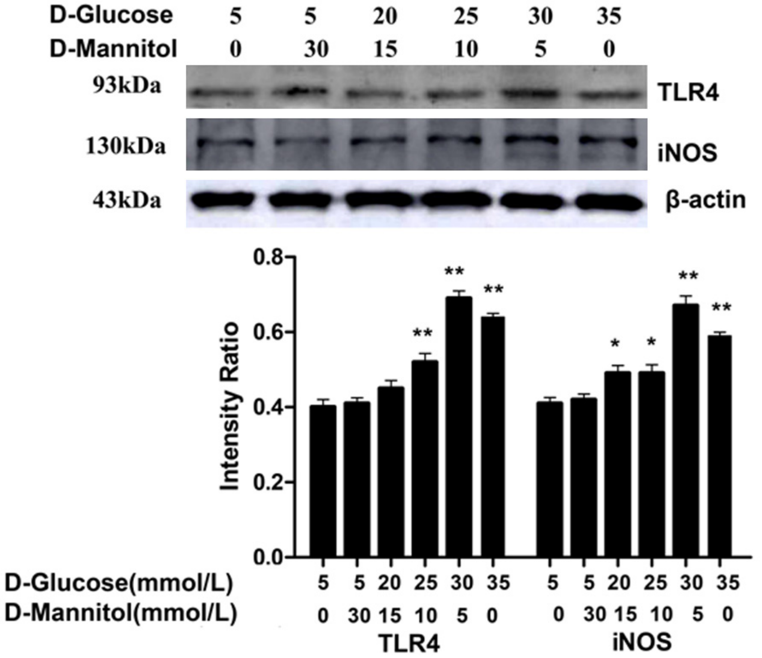

Figure 2. Effects of different concentrations of high glucose stimulation on TLR4 expression and activation of BMDMs. Expression of TLR4 and iNOS was significantly increased in BMDMs at concentrations higher than 20 mmol/l. Results are expressed as mean ± SD in at least three repeated experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. negative control.

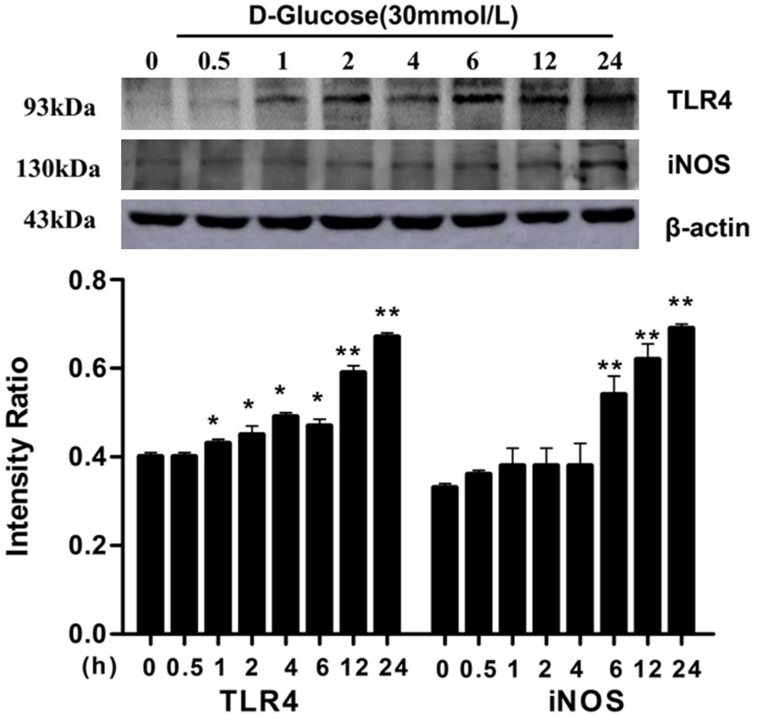

Figure 3. Effects of high glucose on TLR4 expression and BMDM activation at different time points. The concentration of high glucose was 30 mmol/L, which was observed to activate TLR4 and iNOS at different time points. The stimulation time of 24 hours was chosen as the experimental endpoint. Results are expressed as mean ± SD in at least three repeated experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. control group.

ducted by ANOVA analysis. Differences between groups were tested by LSD and Levene method for homogeneity test of variance, with *P* values under 0.05 indicating a significant difference.

Results

Flow cytometry analysis of BMDMs differentiation

BMDMs were identified by flow cytometry. FITC-labeled anti-mouse F4/80 and APC-labeled anti-mouse CD11b were used. Results showed that 99.18% of BMDMs were presented as F4/80 and CD11b double positive cells (**Figure 1**).

Optimization of experiment conditions

BMDMs were treated with D-glucose, at different concentrations, with complementary D-Mannitol as the osmotic control to select suitable conditions in which HG significantly increases TLR4 expression and alters macrophage behaviors obviously. As shown in **Figure 2**, Western blot results showed that expression of TLR4 began to increase at 25 mmol/L glucose concentration and peaked at 30 mmol/L glucose concentration ($P<0.05$). Expression of iNOS began to increase at 20 mmol/L glucose concentration ($P<0.05$) and peaked at 30 mmol/L glucose concentration ($P<0.05$). Next, BMDMs were collected at 8 stimulation time points of 0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h after stimulation. Western blot analysis showed that TLR4 began to increase at 1 hours and peaked at 24 hours ($P<0.05$), while iNOS began to increase at 6 hours

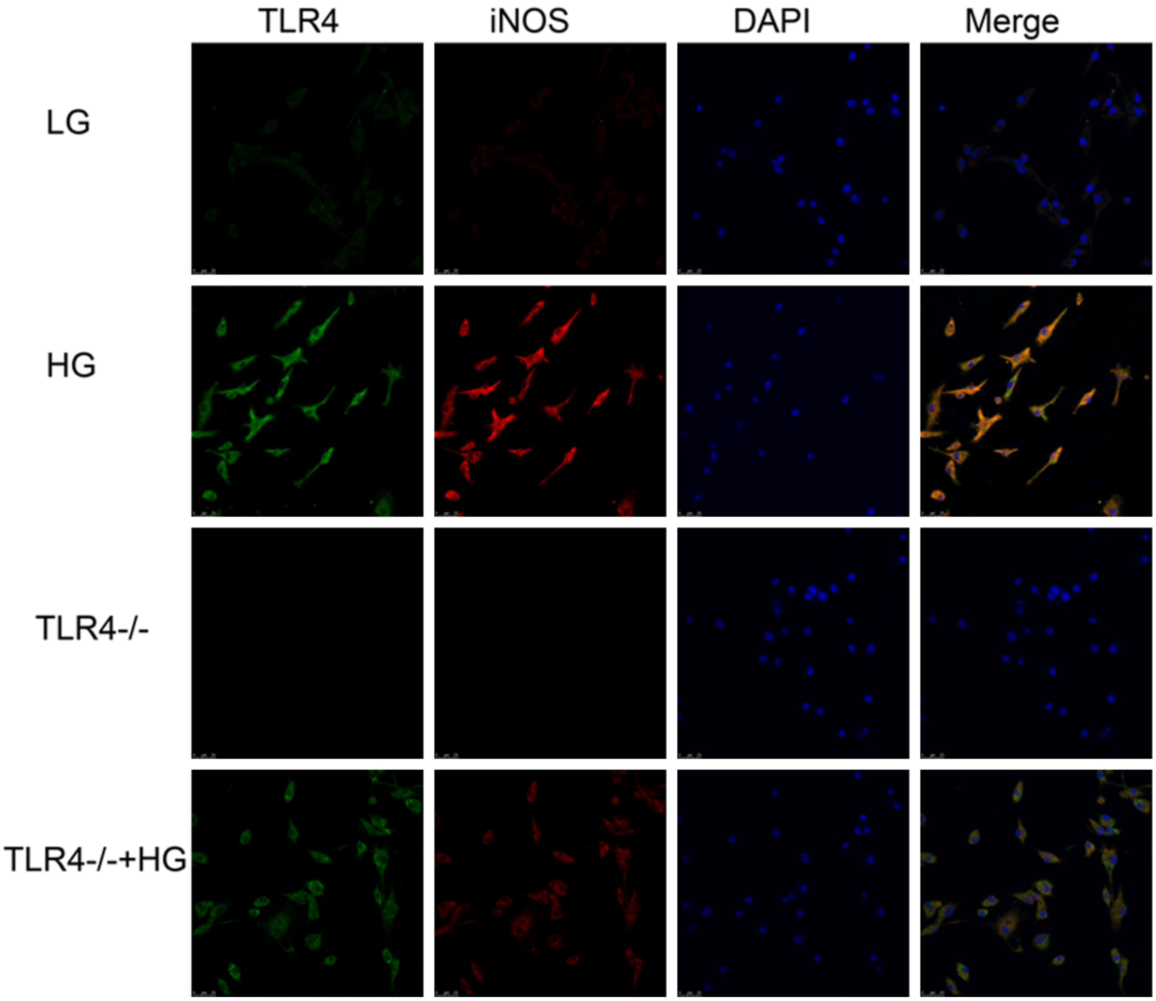

Figure 4. Co-expression of TLR4 and iNOS in high glucose-stimulated BMDMs by confocal microscopy.

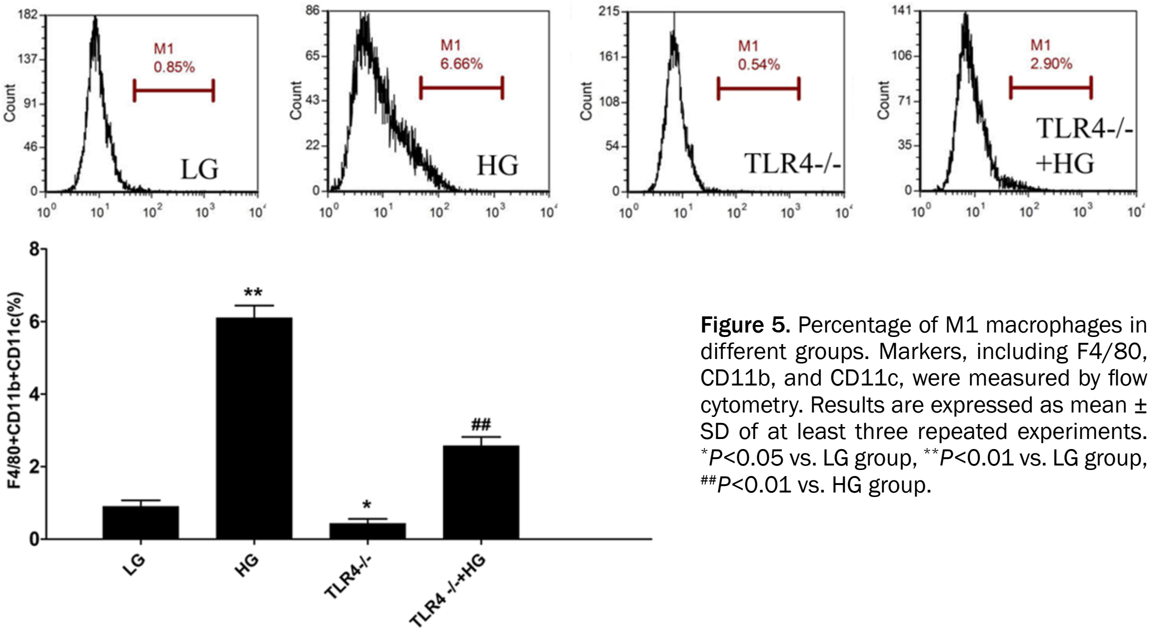

Figure 5. Percentage of M1 macrophages in different groups. Markers, including F4/80, CD11b, and CD11c, were measured by flow cytometry. Results are expressed as mean ± SD of at least three repeated experiments. $^{*}P<0.05$ vs. LG group, $^{**}P<0.01$ vs. LG group, $^{\#\#}P<0.01$ vs. HG group.

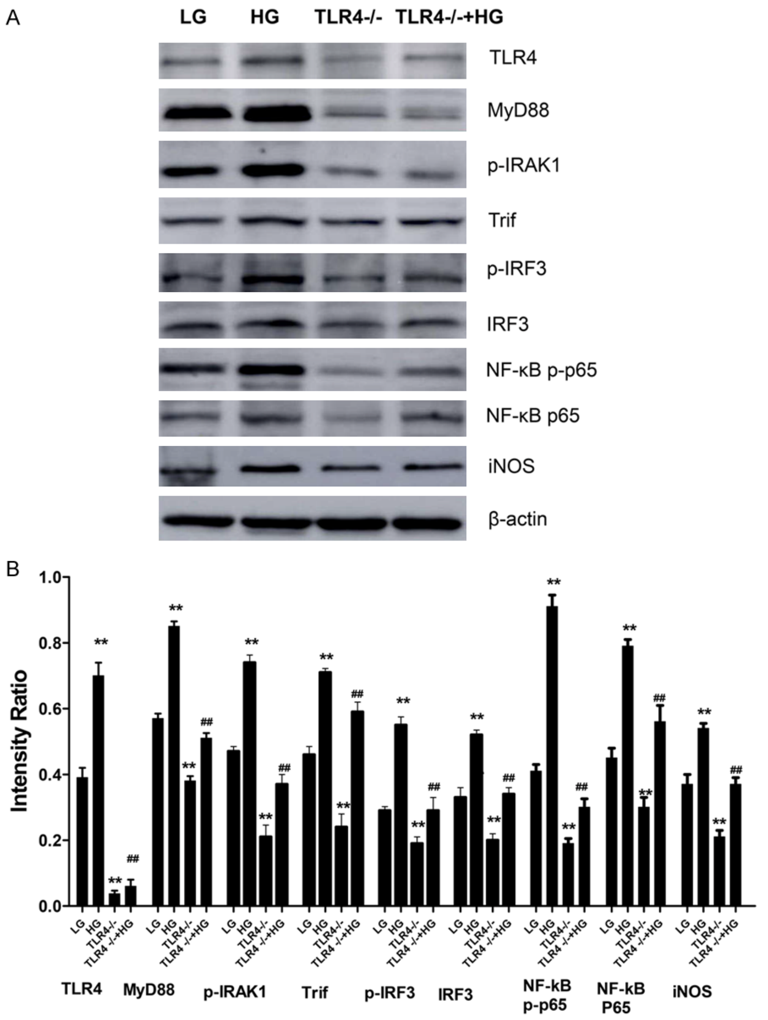

Figure 6. Knockout of TLR4 genes inhibited HG-induced activation of TLR4 signaling pathways in BMDMs. Protein levels of TLR4, MyD88, p-IRAK1, Trif, p-IRF3, IRF3, NF-κB p-p65, NF-κB p65, and iNOS in different groups. Results were normalized to β-actin levels and are expressed as mean ± SD from at least three repeated experiments. $^{**}P<0.01$ vs. LG group, $^{\#\#}P<0.01$ vs. HG group.

and peaked at 24 hours ($P<0.05$). This study chose 30 mmol/L glucose concentration as the medium glucose concentration and stimulation time of 24 hours as the experimental endpoint (**Figures 2**, **3**).

Knockout of TLR4 genes decreased HG-induced BMDMs differentiation towards pro-inflammatory phenotype

Fluorescence intensities of TLR4 and M1 macrophages maker iNOS in the HG group were significantly higher than those in the LG group under the confocal laser microscope, while knockout of TLR4 genes significantly reduced iNOS fluorescence intensity, as shown in **Figure 4**. The percentage of M1 macrophages in the HG group was higher than that in the LG group, significantly ($P<0.05$). The percentage of M1 macrophages after knockout of TLR4 genes was significantly decreased ($P<0.05$), compared to the HG group (**Figure 5**).

Knockout of TLR4 genes affected expression of TLR4, MyD88, p-IRAK-1, Trif, p-IRF3, IRF3, NF-κB p-p65, NF-κB p65, and iNOS proteins in BMMs

Western blot results showed that expression of TLR4, MyD88, p-IRAK-1, Trif, p-IRF3, IRF3, NF-κB p-p65, NF-κB p65, and iNOS proteins in the HG group were significantly higher than those in the LG group ($P<0.05$). Compared to the HG group, knockout of TLR4 genes significantly inhibited expression of the above proteins ($P<0.05$) (**Figure 6A**, **6B**).

Effects of TLR4 on mRNA levels of pro-inflammatory cytokines in BMDMs

MicroRNA levels of TNF-α, IL-1β, MCP-1, and iNOS in the HG group were significantly higher than those in LG group ($P<0.05$). Levels were significantly decreased after knockout of TLR4 genes compared to those in the HG group ($P<0.05$) (**Figure 7**).

BMDMs secreted TNF-α, IL-1β, and MCP-1 in response to stimulation of high glucose and knockout of TLR4 genes affected it

ELISA results showed that levels of TNF-α, IL-1β, and MCP-1 were significantly increased in the HG group ($P<0.05$), compared to the LG group. Levels of TNF-α, IL-1β, and MCP-1 in the culture medium were decreased by TLR4 gene knockout, compared to the HG group ($P<0.05$) (**Figure 8**).

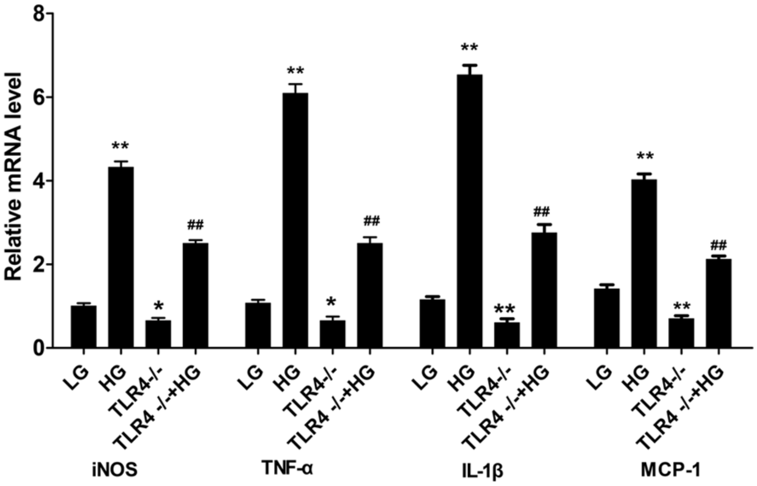

Figure 7. Effects of high glucose on expression of iNOS mRNA, TNF-α mRNA, IL-1β mRNA, and MCP-1 mRNA. Values are expressed as mean ± SD from at least three repeated experiments. $^{*}P<0.05$ vs. LG group, $^{**}P<0.01$ vs. LG group, $^{\#\#}P<0.01$ vs. HG group.

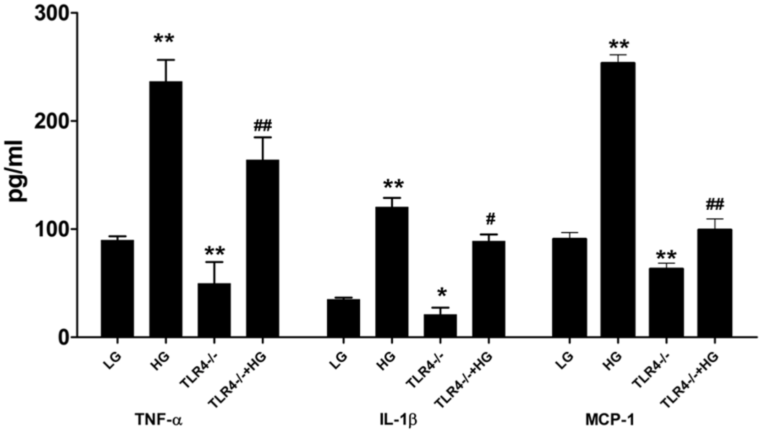

Figure 8. Detection of secreted TNF-α, IL-1β, and MCP-1. Expression of secreted TNF-α, IL-1β, and MCP-1 was determined by ELISA. Values are represented as mean ± SD of at least three repeated experiments. $^{*}P<0.05$ vs. LG group, $^{**}P<0.01$ vs. LG group, $^{\#\#}P<0.01$ vs. HG group.

Discussion

The pathogenesis of DN, the most common diabetic microvascular complication of diabetes, is not entirely clear. Studies have shown that occurrence and development of DN may combine with genetic background, hemodynamic changes, oxidative stress, and immune and inflammatory response. Inflammation has been considered the main influencing factor and has drawn more and more attention in recent years [12, 13]. Macrophages are the main inflammation cells. Macrophage infiltration in the kidneys of patients with DN has been recognized as the main characteristic of inflammation. Nguyen et al. found glomerular and interstitial macrophage infiltration in renal biopsies of diabetic patients, with degree of infiltration and post-renal function decline positively correlated [14]. In a diabetic animal model, increased macrophage infiltration of renal tissue was found, compared to the control group [15], while inhibition of macrophage migration inhibitory factor inhibited macrophage activation, reduced the release of inflammatory factors, and reduced urinary albumin excretion rate, thus delaying development of DN [16]. It has been demonstrated that a high glucose environment could induce macrophage activation and promote polarization of macrophages to the M1 phenotype, leading to inflammatory response and tissue damage [17]. In this study, high glucose was used to stimulate BMDMs. This study observed the proportion of M1 macrophages increasing significantly and upregulated expression of inflammatory cytokines of TNF-α, IL-1β, and MCP-1 in the HG group, compared to the control group. It was concluded that activation and infiltration of macrophages induced by high glucose were involved in the inflammatory process of DN. However, molecular level mechanisms of macrophages involved in the development of DN remain unclear.

Toll-like receptors are a family of receptors involved in innate immune responses that can be expressed on immune-presenting cells surfaces, such as macrophages, dendritic cells, and innate cells. Previous studies have shown that TLR signaling pathways could initiate inflammatory cascades, leading to kidney damage and DN progression [18-20]. TLR4 binds to endogenous or exogenous ligands activated

NF-κB signaling pathways, finally releasing proinflammatory cytokines and chemokines through MyD88-dependent and non-MyD88-dependent pathways, leading to inflammatory reaction in DN [21]. Lin et al. found that TLR4 signaling could promote tubulointerstitial inflammation [22]. Mudaliar et al. also confirmed that TLR4 could regulate inflammation in the high-glucose culture of endothelial cells [23]. The roles of TLR4 on macrophages inducing inflammatory response and participating in the process of DN have not been reported. Jeb et al. found that the lack of TLR4 can promote selective activation of macrophages in adipose tissue [24] and TLR4-dependent macrophage activation leads to renal damage [25]. Thus, it was hypothesized that TLR4 deficiency inhibited macrophage polarization towards M1 phenotype by inhibiting macrophage infiltration and activation of downstream signaling pathways, reducing the inflammatory state. This study observed that high glucose could promote TLR4 and increase significantly downstream signaling pathways MyD88, p-IRAK-1, Trif, p-IRF3, IRF3, NF-κB P-p65, and NF-κB p65, along with inflammatory factors IL-1β, MCP-1, and TNF-α. Knocking out TLR4 inhibited the activation of NF-κB in macrophages. TLR4 and iNOS double-labeled laser confocal results showed that knockout of TLR4 weakened the fluorescence intensity of iNOS, further confirming that TLR4 could lead to macrophage activation by regulating its downstream MyD88-dependent pathways and non-MyD88-dependent pathways, triggering inflammation response.

In conclusion, the present study demonstrated that high glucose induces the polarization of BMDM to M1 phenotype. It also demonstrated that TLR4 is involved in macrophage polarization, promoting the synthesis and release of inflammatory factors in a high-glucose environment. Therefore, knockout of TLR4 genes through inhibiting the activation of macrophages can reduce the production of inflammatory factors, delay the progress of DN, and provide a new research direction for the prevention and treatment of DN.

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Disclosure of conflict of interest

None.

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