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Original Article

Toll-like receptors promote apoptosis of retinal ganglion cells in diabetic retinopathy via regulating Caspase-3 and Bcl-xL

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Abstract: As a complication of diabetes, diabetic retinopathy (DR) is a leading cause of poor vision and blindness. In order to figure out the pathogenesis of DR, this study started with the expression of Toll-like receptor 2 (TLR2) and TLR4 in retinas of diabetic mice induced by streptozotocin *in vitro*. RGCs were isolated from wild-type (WT) mice and TLR-knockout ($TLR^{-/-}$) mice, and cultured in high glucose conditions or normal conditions. Cell viability assay and apoptosis assay were performed to analyze the influences of TLRs on RGC viability and apoptosis. The regulatory mechanisms of TLRs and apoptosis-related factors, cysteine-aspartic acid protease-3 (Caspase-3) and the long isoform of B-cell CLL/lymphoma 2-like 1 (Bcl-xL), were further analyzed. Results showed up-regulated expression levels of TLR2 and TLR4 in retinas of diabetic mice ($P < 0.05$). RGCs from WT mice under high glucose conditions were suppressed in viability and promoted in apoptosis compared to RGCs cultured in normal conditions ($P < 0.05$). While the viability or apoptosis of RGCs from $TLR^{-/-}$ mice was not affected by high glucose culturing conditions. Related regulatory analyses revealed TLRs could up-regulate Caspase-3 and down-regulate Bcl-xL ($P < 0.05$). Furthermore, the promoted expression of Caspase-3 and the suppressed expression of Bcl-xL were detected in RGCs from WT mice under high glucose conditions, but not in RGCs of normal conditions, or RGCs from $TLR^{-/-}$ mice. These data implied that TLRs promoted apoptosis of RGCs via regulating Caspase-3 and Bcl-xL, which might be a pathogenic mechanism of DR.

Keywords: Diabetic retinopathy, retinal ganglion cell, Toll-like receptor, apoptosis

Introduction

Diabetic retinopathy (DR) is a retina disease caused by diabetes. It affects a considerable part of diabetic patients and can eventually lead to blindness [1, 2], thus becoming the leading cause of blindness for middle-aged people in the United States [3]. The progression of DR can be divided into two stages, namely non-proliferative DR and proliferative DR. No typical sign or symptom is visible to the patients of non-proliferative DR, though macular edema may begin to occur during this stage [4]. But when DR progresses to the next stage, bleeding occurs in the eye and makes the eyesight of patients even worse [5]. Numerous studies have discussed the pathogenesis of DR. Potential regulation targets of DR have been studied, such as intercellular adhesion molecule 1 and its leukocyte counter-receptor CD18 [6], placental growth factor-1 [7], and vascular endothelial growth factor [8]. The number reduction and pathological changes of retinal ganglion cells (RGCs) have been found in DR [9], and researchers detect nuclear translocation of GAPDH in these RGCs [10]. However, the pathogenesis of DR, especially the regulatory mechanism of RGCs, is still unclear.

Family of Toll-like receptor (TLR) consists of members vital for activating innate immune responses [11]. The pivotal roles of TLRs in regulating viral pathogenesis make them promising therapeutic targets for treating immune diseases [12]. Among the TLRs, TLR2 and TLR4 have been well studied in eye diseases. TLR2, together with heat shock 27 kDa protein 4, is related to sterile inflammation of the cornea

[13]. TLR2 and TLR4 may associate with fungal keratitis [14], and they are also up-regulated in experimental dry eye with corneal epithelial loss and thinning [15]. Besides, hyperglycemia-induced high expression of TLR-2 and TLR-4 in human microvascular retinal endothelial cells has been detected, which may contribute to DR [16]. But the roles and mechanisms of TLRs in regulating RGCs remain to be clarified.

This study aims to uncover the regulatory mechanisms of TLRs in RGCs, and to reveal the possible pathogenesis of DR. The diabetic mice were experimentally induced by streptozotocin for detecting TLR2 and TLR4 expression. RGCs were isolated from wild-type (WT) mice and *TLR*-knockout (*TLR*$^{-/-}$) mice and cultured in high glucose conditions to analyze changes in cell viability and apoptosis *in vitro*. Further, the regulatory relationships between TLRs and apoptosis-related factors, cysteine-aspartic acid protease-3 (Caspase-3) and the long isoform of B-cell CLL/lymphoma 2-like 1 (Bcl-x), Bcl-xL, were discussed. These findings will provide valuable information on the regulatory mechanisms of RGCs and the pathogenesis of DR.

Material and methods

Animals

Clean grade WT C57BL/6J mice (The Jackson Library, Bay Harbor, ME, USA) weighing from 20 to 25 g were randomly grouped into the nondiabetic control group and the experimentally induced diabetic group (six individuals for each group). To generate diabetic mice, freshly prepared streptozotocin (Sigma-Aldrich, Shanghai, China) dissolved in citrate buffer (pH 4.5) was intraperitoneally injected (60 mg/kg) to mice that had been deprived of food for 12 h. The injection was performed once every day for 5 d. To keep the mice insulin deficient but not catabolic, neutral protamine hagedorn (NPH) insulin (Wanbang, Xuzhou, China) of 0.1 units was subcutaneously injected once per week. Then the mice were raised with free access to food and water. Food consumption, body weight and glycated hemoglobin (Bio-swamp, Wuhan, China) were measured every week to estimate the level of hyperglycemia. Retinas of these mice were sampled for expression analyses. *TLR*$^{-/-}$ mice were purchased from Biomodel Organism (Shanghai, China). The experiments were approved by a local animal committee for ethics and performed according to the guidelines of our institute.

Culture of RGCs

Twelve WT mice and 12 *TLR*$^{-/-}$ mice were cryoanesthetized for sampling. Retinas were isolated and digested by 0.08% trypsin for 20 min. Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS) was used to terminate the trypsinization. Cells were collected by centrifugation and pipetted gently to generate single-cell suspension after adding DMEM supplemented with 10% FBS, 1 mg/mL glucose, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 μg/mL amphotericin (Sigma-Aldrich). Cells were counted and adjusted to the concentration of 1×10^5/mL, and cultured on plates coated with mouse tail collagen (Sybio, Hangzhou, China). After 24 h, 5-Bromo-2'-deoxyuridine (20 μg/mL, Sigma-Aldrich) was added to inhibit the growth of non-neuronal cells, and the medium was changed after 48 h. Cells were cultured at 37°C with 5% CO_2. RGCs were identified by morphology and the monoclonal anti-THY1 antibody (Sigma-Aldrich). For RGCs in normal conditions, RGCs from six WT mice or six *TLR*$^{-/-}$ mice were designated as RGC (WT) and RGC (*TLR*$^{-/-}$), respectively. For RGCs in high glucose conditions, RGCs form six WT mice or six *TLR*$^{-/-}$ mice were cultured in DMEM (high glucose) with 4.5 mg/mL glucose, designated to be RGC (WT) +HG and RGC (*TLR*$^{-/-}$) +HG, respectively.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from retinas using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed by PrimerScript RT Reagent Kit (TaKaRa, Dalian, China) according to the manuals. Each 20 μL PCR reaction contained 20 ng complementary DNA and the specific primers for mouse *TLR2* (forward: 5'-GAT AAT GAA CAC CAA GAC CTA CC-3' and reverse: 5'-GCA GTT CTC AGA TTT ACC CA-3'), *TLR4* (forward: 5'-ACT CTG ATC ATG GCA CTG TTC TT-3' and reverse 5'-GCT CAG ATC TAT GTT GGT TGA-3') or *GAPDH* (forward: 5'-TCA ACA GCA ACT CCC ACT CTT CCA-3' and reverse: 5'-ACC CTG TTG CTG TAG CCG TAT TCA-3'). Reactions were conducted on LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland) following the procedures: pre-denaturation at 94°C for 2 min, followed by 40 cycles com-

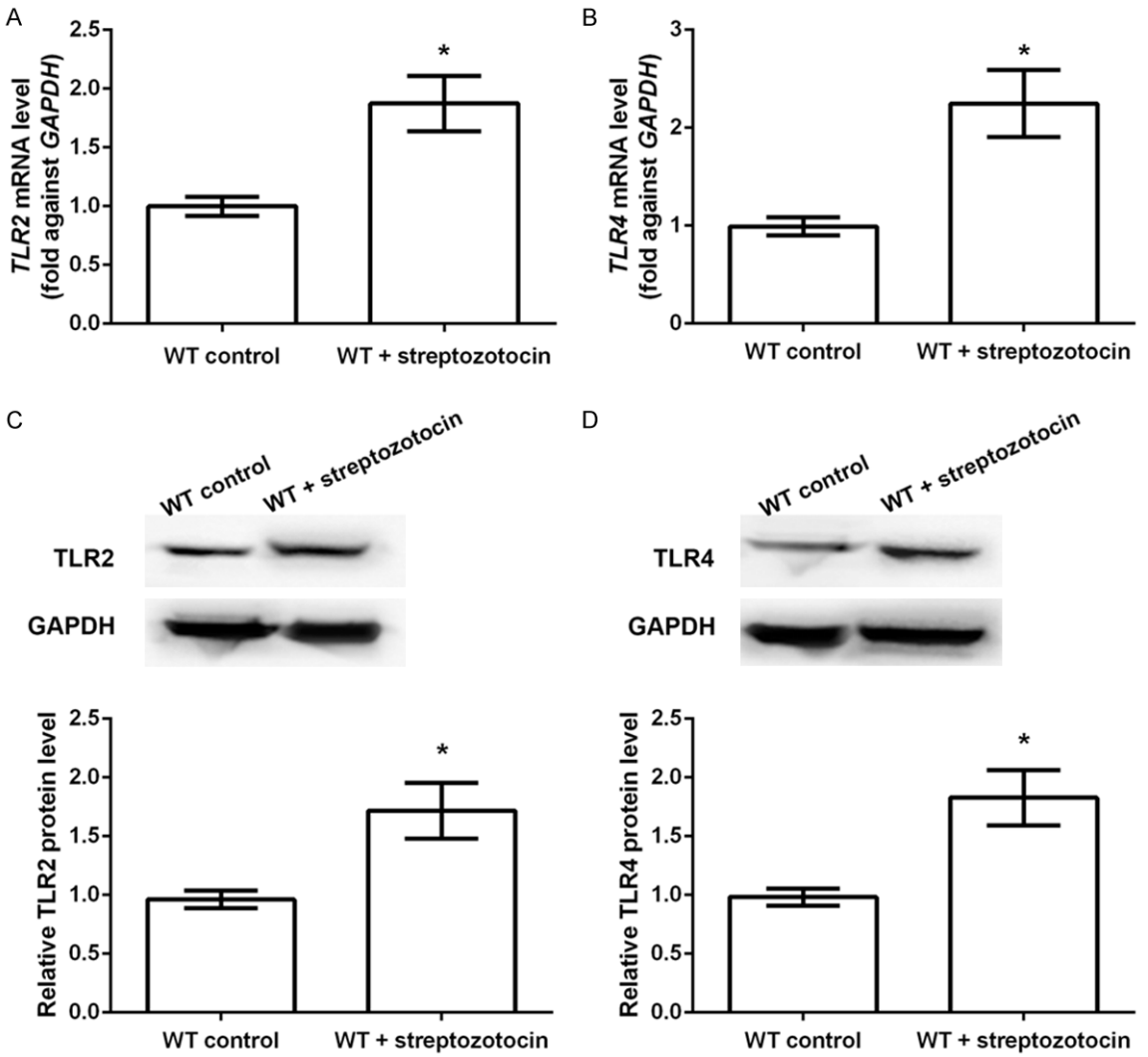

Figure 1. Up-regulation of TLR2 and TLR4 in retinas of diabetic mice (Diabetic) compared to the control mice (Control). A. qRT-PCR showing *TLR2* mRNA was up-regulated in retinas of diabetic mice. B. qRT-PCR showing *TLR4* mRNA was up-regulated in retinas of diabetic mice. C. Western blot showing TLR2 protein expression was higher in retinas of diabetic mice. D. Western blot showing TLR4 protein expression was higher in retinas of diabetic mice. *, significant differences compared to the control group ($P < 0.05$). GAPDH was used as the internal reference of qRT-PCR and western blot. TLR2, Toll-like receptor 2. TLR4, Toll-like receptor 4.

prised of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, and a step of final extension at 72°C for 7 min. All samples were tested in triplicate. *GAPDH* was used as the internal control and data were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

Protein samples of 20 μg from retinas and RGCs were denatured at 100°C for 5 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted to polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk for 2 h at room temperature and incubated in the primary antibody overnight at 4°C. The primary antibodies were mouse monoclonal anti-TLR2, TLR4, Caspase-3 or Bcl-xL (Invitrogen), and GAPDH was the internal reference. Then the blot was incubated in the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were detected by enhanced chemiluminescence reagent and analyzed by ChemiScope 3600 Mini (CLINX, Shanghai, China).

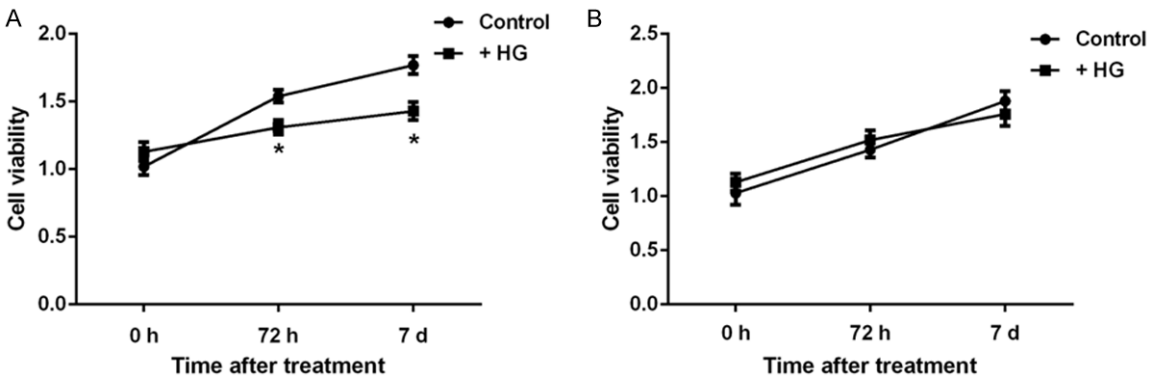

Figure 2. Viability changes of RGCs in high glucose conditions compared to normal conditions. A. Viability changes of RGCs from WT mice. B. Viability changes of RGCs from *TLR*$^{-/-}$ mice. Cell viability was detected at 0 h, 72 h and 7 d post high glucose treatment. *, Significant differences compared to RGC (WT) ($P < 0.05$). RGC, retina ganglion cell. HG, high glucose. WT, wild type. TLR, Toll-like receptor. *TLR*$^{-/-}$, *TLR*-knockout.

Cell viability assay

RGCs were seeded on the 96-well plate and adjusted to the concentration of 1×10^4/well. The cells were cultured at 37°C for 24 h. Then cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manuals. MTT (5 mg/mL) was added to the medium. After 4 h of incubation, formanzan dissolving solution (100 μL) was add to each well and the plate was shaken slowly for 4 h. Then the absorbance was detected at 570 nm with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Cell apoptosis assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) was conducted using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) to analyze cell apoptosis. Cell smears were prepared and fixed using Immunol Staining Fix Solution (Beyotime). TUNEL reaction mixture (50 μL) was added, and the slides were incubated in a humidified atmosphere for 60 min at 37°C in the dark. Then the slides were observed with a microscope and five view fields were randomly selected for each sample to count the apoptotic (TUNEL-positive) cell number.

Statistical analysis

Statistical analysis was performed by Statistical Product and Service Solutions (SPSS) 19 (IBM, New York, USA). The significance of difference between groups of qRT-PCR, western blot, MTT and TUNEL experiments was tested by *t* test, before which *F* test was performed to test the homogeneity of variance. Differences were considered significant if $P < 0.05$.

Results

TLR2 and TLR4 are up-regulated in RGCs of DR

We first compared the expression of TLR mRNA and proteins in RGCs between the WT control and the diabetic mouse induced by streptozotocin. Both *TLR2* and *TLR4* mRNA levels detected by qRT-PCR were significantly up-regulated in retinas of diabetic group compared to the control group ($P < 0.05$, **Figure 1A** and **1B**). Besides, the protein expression levels of TLR2 and TLR4 detected by western blot were also increased significantly in the diabetic group ($P < 0.05$, **Figure 1C** and **1D**). These results indicated that TLR2 and TLR4 were up-regulated in the diabetic retinas, implying their potential roles in regulating DR.

TLRs inhibit viability and promote apoptosis of RGCs in DR

After the RGCs were cultured in high glucose conditions or normal conditions, we conducted cell viability assay with MTT method to analyze the relationship between TLRs and RGCs. Results showed the cell viability of RGC (WT) +HG was obviously inhibited compared to RGC (WT) ($P < 0.05$, **Figure 2A**), indicating high glucose conditions could lead to inhibition of RGC viabil-

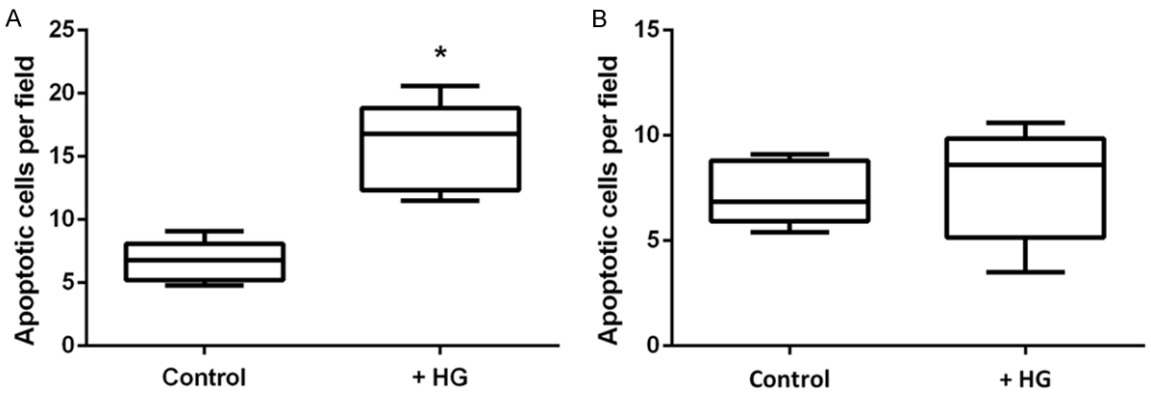

Figure 3. Apoptosis changes of RGCs in high glucose conditions compared to normal conditions. A. Apoptosis changes of RGCs from WT mice. B. Apoptosis changes of RGCs from *TLR*$^{-/-}$ mice. Detection was conducted at 3 d post the high glucose treatment. *, Significant differences compared to the control group ($P < 0.05$). RGC, retina ganglion cell. HG, high glucose. WT, wild-type. TLR, Toll-like receptor. *TLR*$^{-/-}$, *TLR*-knockout.

ity. But the viability of RGCs from *TLR*$^{-/-}$ mice was not affected by high glucose, with no significant difference between RGC (*TLR*$^{-/-}$) and RGC (*TLR*$^{-/-}$) +HG ($P > 0.05$, **Figure 2B**). These data revealed that TLRs were closely related to the reduced RGC viability in DR.

Similarly, apoptosis of RGCs was analyzed by TUNEL after cells were treated by high glucose for 3 d. The apoptosis of RGC (WT) +HG was significantly promoted by the high glucose conditions compared to RGC (WT) ($P < 0.05$, **Figure 3A**). While the apoptosis showed no significant difference between RGC (*TLR*$^{-/-}$) +HG and RGC (*TLR*$^{-/-}$) ($P > 0.05$, **Figure 3B**). So TLRs might play roles in inducing the RGC apoptosis in DR.

TLRs mediate RGCs via regulating Caspase-3 and Bcl-xL

Since TLRs might mediate cell apoptosis in DR, we further analyzed its regulation on two apoptosis-related factors, Caspase-3 and Bcl-xL. Caspase-3 was significantly up-regulated in RGC (WT) +HG compared to RGC (WT) ($P < 0.05$, **Figure 4A** and **4B**). But it was significantly inhibited when TLRs were knocked out ($P < 0.05$). While RGCs were cultured in normal conditions, it seemed that knockout of TLRs did not affect the expression of Caspase-3. Similar experiments were conducted on Bcl-xL (**Figure 4A** and **4C**). RGC (WT) +HG expressed Bcl-xL in a significant lower level compared to RGC (WT) ($P < 0.05$). In normal culture conditions, the expression levels of Bcl-xL were not affected when TLRs were knocked out ($P > 0.05$). But in high glucose conditions, Bcl-xL was up-regulated by the knockout of TLRs ($P < 0.05$). Taken together, the expression levels of Caspase-3 and Bcl-xL were up-regulated and down-regulated, respectively, when RGCs were cultured in high glucose conditions. In normal culturing conditions, the expression of Caspase-3 or Bcl-xL was not affected by TLRs. However, in high glucose conditions, knockout of TLRs could inhibit Caspase-3 and promote Bcl-xL. It could be deduced from these results that Caspase-3 and Bcl-xL participated in the regulation of RGCs in DR, and that TLRs could mediate RGCs via up-regulating Caspase-3 and down-regulating Bcl-xL. The regulatory functions of TLRs, Caspase-3 and Bcl-xL on RGC apoptosis might be pivotal to the pathogenesis of DR.

Discussion

It has been postulated that TLR2 and TLR4 are associated with eye diseases induced by diabetes [14, 17]. In the present study, we use streptozotocin-induced diabetic mice and detect the high expression levels of TLR2 and TLR4 in retinas. *In vitro* experiments on RGCs from WT and *TLR*$^{-/-}$ mice indicate TLRs inhibit the viability and promote the apoptosis of RGCs in DR. Then experiments on cell apoptosis-related factors show *TLR*-knockout down-regulates Caspase-3 and up-regulates Bcl-xL in RGCs under high glucose condition, implying TLRs mediate the apoptosis of RGCs in DR via regulating Caspase-3 and Bcl-xL. But in normal RGCs, Caspase-3 and Bcl-xL are not affected by TLRs, suggesting the regulation of RGCs by TLRs,

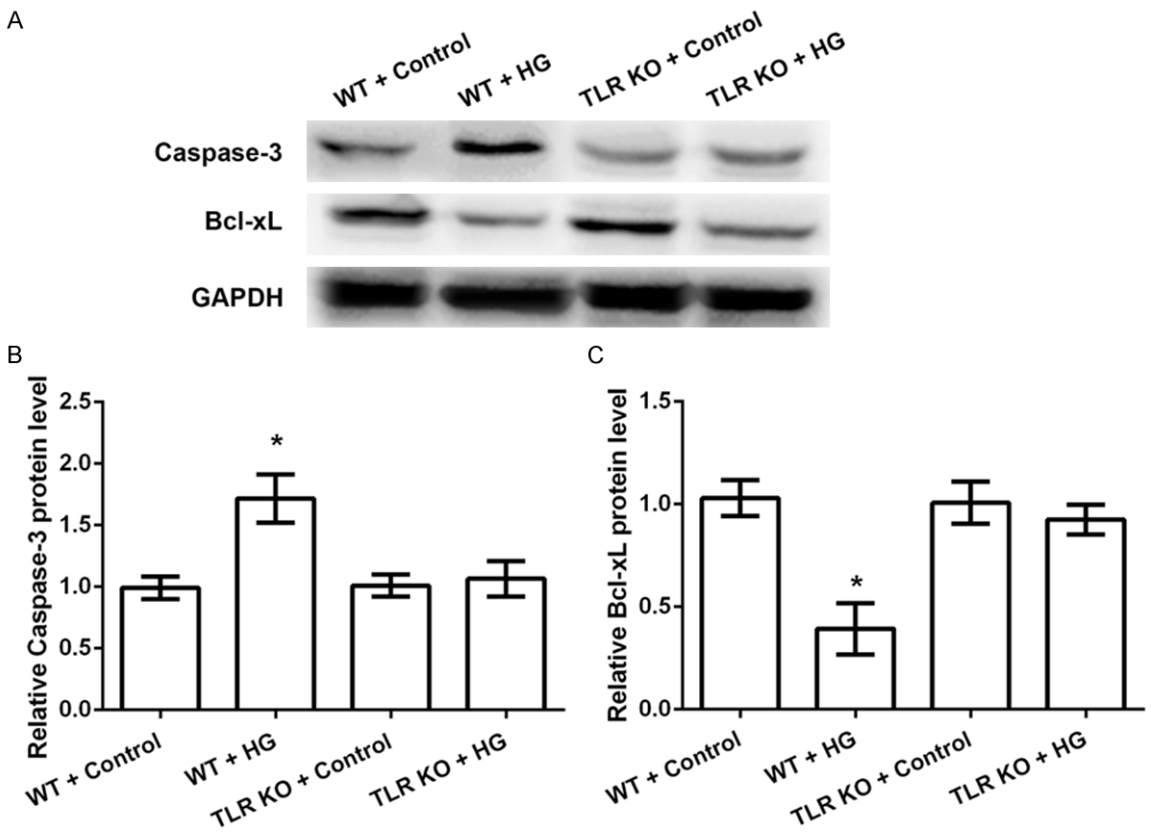

Figure 4. TLRs regulating Caspase-3 and Bcl-xL in RGCs under high glucose conditions. A. Western blot showing Caspase-3 and Bcl-xL were regulated by TLRs in RGCs under high glucose conditions. B. Histogram showing the relative signal intensity of Caspase-3 in the Western blot results. C. Histogram showing the relative signal intensity of Bcl-xL in the western blot results. *, Significant differences compared to the other groups ($P < 0.05$). RGC, retina ganglion cell. WT, wild-type. HG, high glucose. TLR, Toll-like receptor. *TLR*$^{-/-}$, TLR-knockout. Caspase-3, cysteine-aspartic acid protease-3. Bcl-xL, the long isoform of B-cell CLL/lymphoma 2-like 1.

Caspase-3 and Bcl-xL may be significant to DR pathogenesis. These data reflect the association between TLRs and RGC apoptosis in DR, providing possible therapeutic targets for DR treatment.

Caspase-3 and Bcl-xL, the two cell apoptosis-related factors used in this study, are capable of regulating cell apoptosis. Caspase-3 could be activated by Caspase-9, participating in efficient execution of cell apoptosis [18]. Active Caspase-3 has been used for detecting apoptosis induced by a wide variety of apoptotic signals [19]. Its roles in regulating RGCs have been reported [20-22]. Inhibition of Bcl-2 and Bcl-xL increases the apoptosis of human myeloid leukemia cells [23]. The possible functions of Bcl-xL in RGCs have also been studied. Loss of Bcl-xL significantly increase the death rate of RGCs after axonal injury, implying its anti-apoptosis roles in RGCs [24]. Results of this study suggested that TLRs inhibited viability and promoted apoptosis of RGCs, which was achieved by up-regulating Caspase-3 and down-regulating Bcl-xL. These results were consistent with previous studies, indicating the pro-apoptosis roles of Caspase-3 and the anti-apoptosis roles of Bcl-xL. Additionally, the altered expression levels of Caspase-3 and Bcl-xL were only detected in high glucose conditions, so apoptosis of RGCs regulated by TLRs, Caspase-3 and Bcl-xL seemed pivotal to DR pathogenesis.

TLRs can regulate cell apoptosis via a variety of apoptosis-related factors. TLR2 is capable of accelerating the apoptosis of human monocytic cell line THP-1 via inhibiting p38 mitogen-activated protein kinase and tumor necrosis factor-α [25]. In myelomonocytic leukaemia cells, TLR2 inhibits the transcriptional factor NF-κB and suppresses apoptosis of tumor cells [26]. With regard to TLR4, it mediates apoptosis of

intestinal epithelial cells via cyclooxygenase 2 [27], and suppresses Wnt signaling to regulate apoptosis of photoreceptors [28]. In DR, the TLR-mediated NF-κB activation is further regulated by microRNA-146 [29]. Therefore, TLRs and multiple regulatory factors composing the complex regulatory parts of cell apoptosis. Though there are studies indicating down-regulation of TLR4 and Caspase-3 suppresses neuronal apoptosis [30], no explicit evidence refers to the associations between TLRs and Caspase-3 or Bcl-xL. In this study, we discovered the regulatory functions of TLRs on Caspase-3 and Bcl-xL, providing new references for RGC apoptosis and DR pathogenesis.

To sum up, this study detects higher expression levels of TLR2 and TLR4 in retinas of diabetic mice and uncovers the anti-viability and pro-apoptosis roles of TLRs. The regulatory functions of TLRs in RGC apoptosis of DR patients are executed via mediating Caspase-3 and Bcl-xL. These findings contribute to the elucidation of DR pathogenesis and provide possible therapeutic targets for DR treatment.

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Disclosure of conflict of interest

None.

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