



Toll Like Receptors Promote High Glucose-Induced Vascular Endothelial Cell Dysfunction by Regulating Neutrophil Extracellular Traps Formation

Shirou Wu^{1,2} · Yahui Chen³ · Xiuming Jin^{1,2} · Jiayun Yu^{1,2} · Xueping Chen^{1,2} · Ting Wan^{1,2}

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Abstract

Diabetic retinopathy (DR) is a major cause of blindness globally. Neutrophils and neutrophil extracellular traps (NETs) are believed to play a role in the development of DR. However, the specific contribution of NETs to hyperglycemia-induced vascular endothelial cell dysfunction remains unclear. In this study, we cocultured high glucose-activated neutrophils (HGNs) with human umbilical vein endothelial cells (HUVECs) to investigate the role of NETs in high glucose-induced HUVEC dysfunction. Our findings indicate that high glucose levels promote NETs formation, which can be inhibited by a toll-like receptor (TLR) 2 antagonist and a TLR4 antagonist. It was observed that reactive oxygen species production plays a role in TLR2- but not TLR4-mediated NETs formation. Additionally, HGNs were found to promote HUVEC proliferation through phagocytosis rather than NETs. We also discovered that NETs contribute to high glucose-induced HUVEC dysfunction by enhancing neutrophil-HUVEC adhesion, inhibiting HUVEC migration, and compromising the barrier function of the cells by reducing zonula occludens-1 expression. This dysfunction could be partially mitigated by TLR2 and TLR4 antagonists. In conclusion, high glucose stimulates NETs formation, leading to vascular endothelial cell damage, and TLRs may facilitate high glucose-induced endothelial dysfunction by modulating NETs formation.

Keywords Neutrophils · Neutrophils extracellular traps · Diabetic retinopathy · HUVEC · Permeability · Toll-like receptor

Shirou Wu and Yahui Chen contributed equally to this work.

✉ Ting Wan
wanting1982@zju.edu.cn

Shirou Wu
wusr0105@zju.edu.cn

Yahui Chen
yahui.chen@iqvia.com

Xiuming Jin
lzyjxm1@zju.edu.cn

Jiayun Yu
22218969@zju.edu.cn

Xueping Chen
22218662@zju.edu.cn

¹ Eye Center of Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

² Zhejiang Provincial Key Laboratory of Ophthalmology, Zhejiang Provincial Clinical Research Center for Eye Diseases, Zhejiang Provincial Engineering Institute on Eye Diseases, Hangzhou 310009, China

³ Quintiles Medical Research & Development Beijing Ltd, Liangshuihe No.2 Street, Beijing 100176, China

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease, and the prevalence of DM has been steadily increasing in China and globally, with a trend toward a younger age of onset [1]. It is estimated that the number of patients with DM worldwide will reach 700 million by 2045 [2]. Diabetic retinopathy (DR) is one of the most severe microvascular complications of DM; it can lead to vision loss due to hemorrhage, exudates, edema, and retinal detachment [3, 4]. As the incidence of DM is rising and patients with DM are surviving longer, an increasing number of people are experiencing DR and related vision loss [5]. Nowadays, laser photocoagulation, vitrectomy, intravitreal injection of anti-vascular endothelial growth factor (VEGF) drugs or steroids are used to treat DR [6, 7]. However, laser photocoagulation and vitrectomy result in unavoidable damage, the curative effect of anti-VEGF drugs and steroids is temporary and intravitreal steroid injections are associated with complications, such as an increased risk of developing cataracts and glaucoma [8]. Additionally, stem cell therapy and photobiomodulation are

emerging treatments for DR [9, 10]. However, the former is costly and time-consuming, while the latter still requires extensive clinical validation. Thus, there are ongoing efforts to identify the molecular mechanisms involved in the pathogenesis of DR and develop better treatment modalities for this condition.

Chronic inflammation induced by chronic hyperglycemia is considered a significant contributing factor of DR [11, 12]. Several inflammatory cell types and molecules have been implicated in the pathogenesis of DR; they have been shown to cause retinal vascular cell dysfunction, retinal hemorrhage, macular edema, retinal neovascularization, and retinal detachment in DR patients [13, 14]. Moreover, it has been demonstrated that the development of DR in animal models can be prevented by applying anti-inflammatory therapies [15].

Neutrophils are important immune and inflammatory cells in the human body, and they have been implicated in the pathogenesis of DR. More specifically, peripheral blood neutrophil levels were found to be positively associated with disease severity in both DM and DR [16]. Neutrophil extracellular traps (NETs) is another effector mechanism of neutrophil, which were first proposed by Brinkmann et al. in 2004 [17]. NETs are extracellular structures released by neutrophils upon stimulation, with DNA as the scaffold and containing histones, antimicrobial proteins, and other cytokines (such as myeloperoxidase, MPO). PAD4 (peptidyl arginine deiminase 4) plays a crucial role in the formation of NETs and promotes their release [18]. It is demonstrated that NETs not only participate in the body's defense mechanisms but also serve as a key pathogenic factor in a range of non-infectious inflammatory diseases, including autoimmune disorders, self-inflammatory conditions, and metabolic diseases, such as DM [12]. High glucose has been reported to increase the formation of NETs both in vivo and in vitro [18]. Furthermore, DR patients had higher levels of NET components in the peripheral blood than diabetic patients without DR [19]. Moreover, NETs have been shown to trigger proinflammatory immune responses and induce endothelial dysfunction, which subsequently leads to increased endothelial permeability, endothelial barrier collapse, impaired wound repair, and angiogenic dysfunction in several non-infectious inflammatory diseases [20–22]. However, the role that NETs play in high glucose-induced endothelial cell dysfunction and the underlying mechanism are not clear.

Toll-like receptors (TLRs) are fundamental pattern recognition receptors that help cells recognize microbial structures and mediate subsequent inflammatory responses [23, 24]. As important components of neutrophil-related inflammatory processes, TLRs have been reported to participate in the formation of NETs after stimulation with *Trypanosoma brucei* lipophosphoglycan and gut-derived lipopolysaccharide [25, 26]. Our previous research also confirmed that

TLRs participate in regulating NETs formation induced by *Staphylococcus aureus* [27]. Furthermore, TLRs have been found to be involved in the pathogenesis of DM and DR [28]. Zhu et al. reported that TLR4 expression was elevated in the peripheral blood of patients with type 2 DM [29], and Kim et al. reported that TLR2 is associated with the death of pancreatic β cells [30]. Moreover, it has also been demonstrated that TLR4 gene polymorphism is associated with the elevated incidence of DR and that TLR4 deletion alleviates the development of DR in mice [31, 32]. These findings suggest that TLRs may play a crucial regulatory role in the development of DR by regulating NETs formation under conditions of persistent high blood glucose. However, the role that TLRs play in high glucose-induced NETs formation and vascular endothelial cell dysfunction remains unknown.

Therefore, we conducted this study to elucidate the role that NETs play in high glucose-stimulated vascular endothelial cell dysfunction. We also examined the effect of TLR2 and TLR4 on high glucose-stimulated NETs formation and subsequent vascular endothelial cell dysfunction.

Materials and Methods

Reagents

Peripheral blood neutrophil isolation kit was purchased from TBD sciences. 4% paraformaldehyde (PFA), Triton X-100, horseradish peroxidase (HRP) were purchased from Solarbio (Beijing, China). DNase I, cytochalasin D, dichlorofluorescein diacetate (DCF-DA), bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). TMB Chromogen Solution, Stop Solution for TMB Substrate, CCK-8 assay were purchased from Beyotime (Shanghai, China). TLRs antagonists were purchased from InvivoGen (San Diego, CA, USA). Anti-histone H3 antibody, horseradish peroxidase (HRP), and secondary antibodies coupled to AF488 or AF555 were purchased from Santa Cruz Biotechnology (CA, USA). Anti-zonula occludens-1 (ZO-1) tight junction protein antibody and anti-myeloperoxidase (MPO) antibody were purchased from Abcam (Cambridge, UK). FITC anti-CD45 and APC anti-CD16 were purchased from Absin (Shanghai, China). Optimal cutting temperature compound (OCT) was purchased from Sakura (LA, USA). Diphenyliodonium chloride (DPI), were purchased from MCE(NJ, USA), SYTOX Green, Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12), Roswell Park Memorial Institute (RPMI) 1640, microplates were purchased from ThermoFisher Scientific (Basingstoke, UK). Cell proliferation reagent water-soluble tetrazolium salt-1 (WST-1) was purchased from Roche (Basel, Switzerland).

Data Acquisition

GSE221521 as an independent DR dataset was downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). We selected a subset of 20 DR membrane specimens and 24 control samples randomly [33, 34]. The raw data from the GSE221521 dataset was downloaded for further analysis. Table S1 shows the relevant information of the dataset. Using matching platform files to obtain gene symbols of each probe matrix. The differential expression genes (DEGs) were identified using the “DESeq2” in R-4.4.2.

Identification of DR-Related DEGs

Differential expression analysis was performed using the DESeq2 package. DEGs were identified using the criteria of an adjusted *P*-value < 0.05 and an absolute $\log_2$ Fold Change ≥ 1 . To visualize the results, we employed the R software packages “ggplot2” to create volcano plots. Specific genes of interest, such as TLR2/4, MPO and PADI4 were labeled on the volcano plot for further interpretation. The Spearman correlation analysis was performed to explore the correlation between TLR2/4 and PADI4.

Pathway Enrichment Analysis

We employed the Metascape database (<https://metascape.org/>) to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the TLR2/4 and PADI4. A *p*-value threshold of < 0.05 was applied to identify significantly enriched pathways. The enrichment results were visualized using bar charts.

Human Primary Neutrophils Isolation

This study was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (protocol code 20,210,011; 2 January 2021). All procedures for sample collection complied with the principles of the Helsinki Declaration on Human Subjects. All human subjects taking part in the present study signed the informed consent. Neutrophils were isolated from peripheral blood of healthy volunteers using peripheral blood neutrophil isolation kits. The neutrophil content was more than 95% as determined by Wright-Giemsa staining. Isolated neutrophils were cultured in RPMI-1640 medium with 2% bovine serum albumin.

Flow Cytometry and Neutrophil Purity Assessment

Neutrophils were isolated and resuspended in PBS. After centrifugation, the cells were incubated with a mixture of fluorophore-conjugated antibodies (anti-CD45 and

anti-CD16) for 30 min at 4 °C temperature to identify and assess neutrophil purity. Following staining, the cells were washed with PBS to remove excess antibody, then resuspended for analysis. Flow cytometry analysis was performed on a Beckman Coulter CytoFlex LX, and the data were analyzed using CytExpert Software (v2.4, Beckman).

Neutrophils Stimulation

Neutrophils (1×10^6 cells/well in 1 mL) placed in a humidified incubator at 37 °C with 5% CO₂ were treated with different concentrations of glucose for 2 h. In some experiments, neutrophils were pretreated with TLR2 antagonist (1 µg/mL), TLR4 antagonist (1 µg/mL), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor (diphenyleneiodonium, DPI, 10 µM), or vehicle (controls) for 1 h.

Immunofluorescence for NETs Formation

After stimulation, neutrophils were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with 10% goat serum, incubated with primary antibodies against Histone H3 and MPO overnight at 4 °C, and followed by secondary antibodies incubation for 1 h at room temperature. Meanwhile, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Subsequently, specimens were fixed and analyzed under an inverted fluorescence microscope (Leica DMi8).

NETs formation was also detected by using SYTOX Green, which is a commonly used membrane-impermeable DNA-binding dye. After stimulation, SYTOX Green (5 µM) was added to the medium, and the NETs structure were observed and imaged by using an inverted fluorescence microscope 5 min later.

ROS Production

Neutrophils were incubated with 10 µM DCF-DA in serum-free RPMI-1640 at 37 °C for 20 min, followed by washing with phosphate-buffered saline (PBS) for three times and transferring to a 96-well plate (1×10^6 cells per well in 100 µl). Subsequently, the cells were pretreated with TLR2 antagonist or TLR4 antagonists for 1 h and then stimulated with vehicle or 35 mM glucose. After 2 h stimulation, the fluorescence signal of DCF-DA was measured by using SpectraMax M3 Multi-Mode Microplate Reader with 480 nm excitation wavelength and 520 nm emission wavelength.

HUVEC Stimulation

HUVEC (purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd) were incubated in DMEM/F12

supplemented with 10% bovine serum albumin. To investigate the effect of high glucose-activated neutrophils (HGNs) on HUVEC and the underlying mechanism, HUVEC were co-cultured with HGNs pretreated with TLR2 antagonist, TLR4 antagonist or vehicle. In order to distinguish the effect of NETs from neutrophil phagocytosis, DNase I (100 U/mL) was used to degrade the structure of NETs and Cytochalasin D (100 µg/mL) was used to inhibiting neutrophil phagocytosis. In order to determine whether TLR2/4 antagonist has a direct effect on HUVEC, we established a co-culture system of HUVECs with TLR2/4 antagonist.

HUVEC Viability Assessment

HUVEC were seeded into a 96-well plate and incubated for 24 h to allow cell adhesion. Then the HUVEC were co-cultured with vehicle, TLR2/4 antagonist for 24 h. Following the treatment period, 10 µL of CCK-8 solution was added to each well. The plate was incubated for an additional 2 h. After the reaction was complete, the optical density (OD) at 450 nm was measured using a microplate reader. The cell viability was calculated as follows: Cell viability (%) = [(OD450 of the experimental group—OD450 of the blank control) / (OD450 of the control group—OD450 of the blank control)] * 100.

HUVEC Proliferation Assessment

HUVEC were cultured overnight in a 96-well plate according to a concentration gradient. Then the HUVEC were co-cultured with vehicle, TLR2/4 antagonist, HGNs, HGNs with DNase I or HGNs with Cytochalasin D for 24 h. Subsequently, the cells were incubated with 10 µL WST-1 at 37 °C for another 4 h and the absorbance at 450 nm and 690 nm were measured by using SpectraMax M3 Multi-Mode Microplate Reader to quantify the HUVEC proliferation.

Neutrophil-HUVEC Adhesion Assay

HUVEC with green fluorescent protein (HUVEC-GFP) (5.0×10^4 cells/well) were incubated overnight in 12-well plates, and the neutrophils were labeled with Hoechst 33,342 (4×10^6 cells/mL). Subsequently, the monolayer HUVEC were co-cultured in DMEM/F12 with neutrophil, TLR2/4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist for 2 h. After that, non-adherent neutrophils were gently removed by washing with DMEM/F12 and adherent neutrophils were imaged by an inverted fluorescence microscope (Leica DMi8) and analyzed by Image J (National Institutes of Health, the USA). The ratio of the number of neutrophils to HUVEC was calculated as the adhesion rate.

HUVEC Migration Assay

HUVEC-GFP (5×10^4 cells/well) were cultured in a 12-well plate overnight to reach confluence. The wounding was created by drawing a line with a 200 µL pipette tip, and the cells debris was removed by gently wash twice with PBS. Then the HUVECs were incubated with vehicle, TLR2/4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist. Twenty-four hours after incubation, the wound size was observed by taking images with an invert fluorescence microscope (Leica DMi8) and analyzed by Image J (National Institutes of Health, the USA). HUVEC migration was evaluated by calculating the wound closure percentage on the scratch.

HUVEC Permeability Assay

HUVEC cultured on polycarbonate cell culture inserts with pores of 4.0 µm were allowed to reach confluence overnight. Then HUVEC were treated with vehicle, TLR2/4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist overnight, followed by the addition of 100 ng/mL HRP. After incubating at 37 °C for 30 min, 50 µL medium from the lower chamber was collected and incubated with the TMB chromogen solution for 3 min. The absorbance values of HRP were read by a microplate reader at a wavelength of 450 nm, which indirectly reflected the permeability of HUVEC.

Furthermore, the filter membrane material with attached HUVECs was cut from the chamber. After washing with PBS, cells were fixed with 4% PFA for 15 min, followed by incubation in Triton X-100 for 5 min. Then, serum blocking was carried out for 30 min and ZO-1 antibody was added for overnight incubation at 4 °C. After washing with PBS, cells were incubated with secondary antibodies for 1 h at room temperature. Following PBS washing and DAPI counterstaining, ZO-1 protein was observed under a fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, the USA). Data are presented as mean ± standard error (SE). We used Student's t-test to compare the values between two group and analysis of variance (ANOVA) followed by Bonferroni's post hoc test to compare among multiple groups. Levels of significance was set at 0.05.

Results

Identification of DR-Related DEGs and Pathway Enrichment Analysis

A total of 37,598 DEGs were identified and 1400 of them were significantly upregulated in the DR samples, which including TLR2/4, MPO (the main components of NETs) and PADI4 (the key enzymes involved in NETs formation). 534 of DEGs showed downregulation and 35,664 showed no significant difference (Fig S1a). Moreover, we conducted a correlation analysis, and the results showed that TLR2/4 and PADI4 exhibited a significant positive correlation (Fig S1b).

The bar chart (Fig S1c) depicted the results of Metascape enrichment analysis. The enrichment analysis of TLR2/4 and PADI4 revealed significant associations with biological processes related to the NETs formation pathways.

High Glucose Level Promoted NETs Formation

To ensure the accuracy of our results, the purity of neutrophils was confirmed to be 95% through flow cytometry analysis (Fig. S2). The NETs formation that occurred during incubation with different concentrations of glucose (11, 15, 25, or 35 mM) for 120 min is shown in Fig. 1a, 1b. NETs formation was validated by immunofluorescence staining of

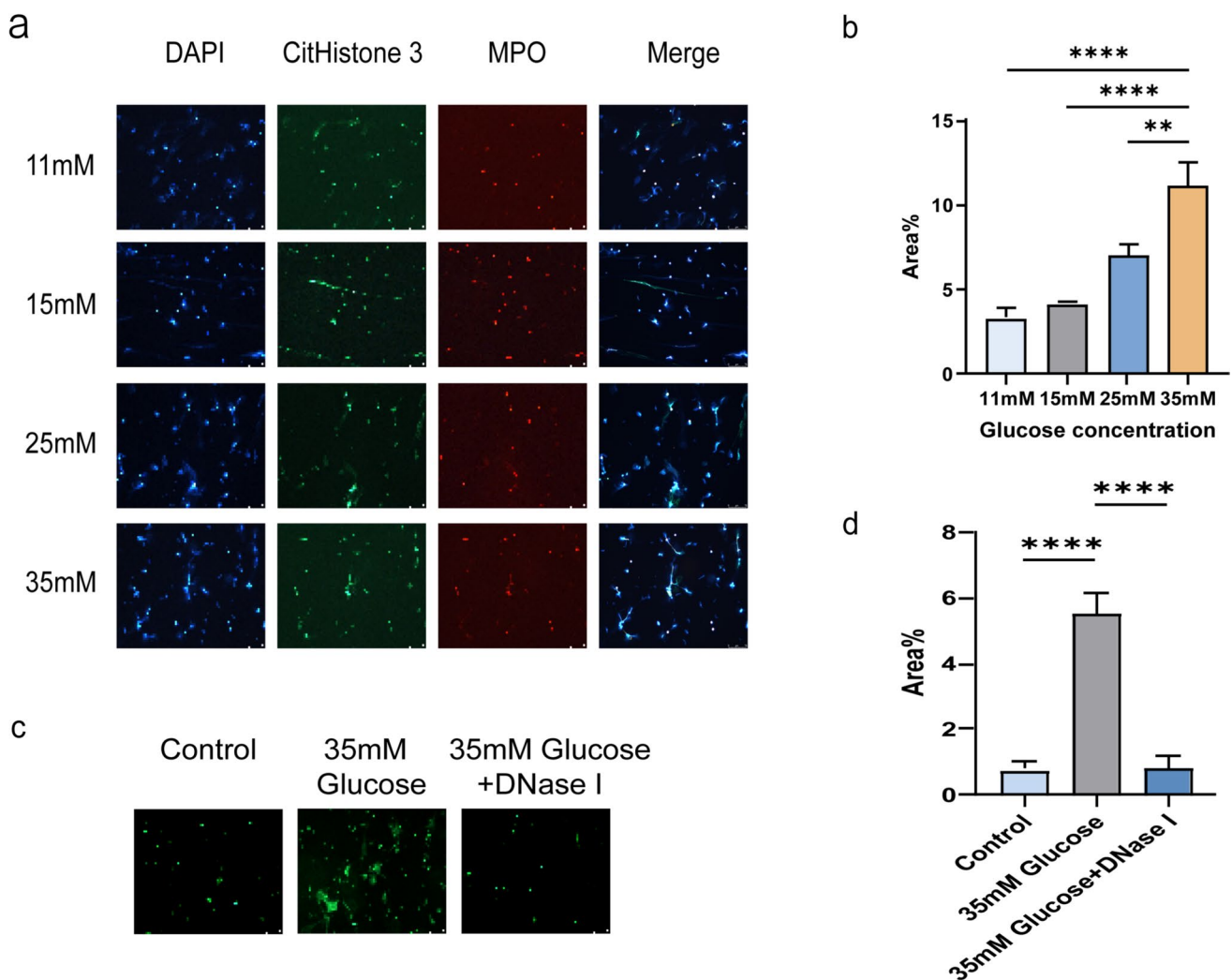


Fig. 1 High glucose stimulated neutrophil extracellular traps (NETs) formation. **(a)** Human neutrophil suspended in media were treated with high glucose (11,15,25,35 mM) for 2 h. Neutrophils were labeled with 4',6'-diamidino-2-phenylindole (DAPI) to identify DNA (blue) and with antibodies to identify neutrophil histone (green) and MPO (red). **(b)** Quantification of NETosis area in the observed

area. Data represent mean $\pm$ SD of triplicate experiments. ** $p < 0.01$, **** $p < 0.0001$ by one-way ANOVA. **(c)** 35 mM glucose stimulated NETs formation could be degraded by DNase I as observed by SYTOX green staining. Bar: 75 μ m. **(d)** Quantification of NETosis percentage of neutrophils. Data represent mean $\pm$ SD of triplicate experiments. **** $p < 0.0001$ by one-way ANOVA

MPO (red) and histones (green) with respective antibodies, and of DNA (blue) with DAPI. The NETs formation that occurred during incubation with 35 mM glucose was further confirmed by SYTOX Green staining (Fig. 1c, 1d), which could be degraded by DNase I. As the NETs present after incubation with 11, 15, and 25 mM glucose were little, 35 mM glucose and a stimulation time of 120 min were used for subsequent experiments.

High Glucose-Induced NETs Formation was Inhibited by a TLR2 Antagonist and a TLR4 Antagonist

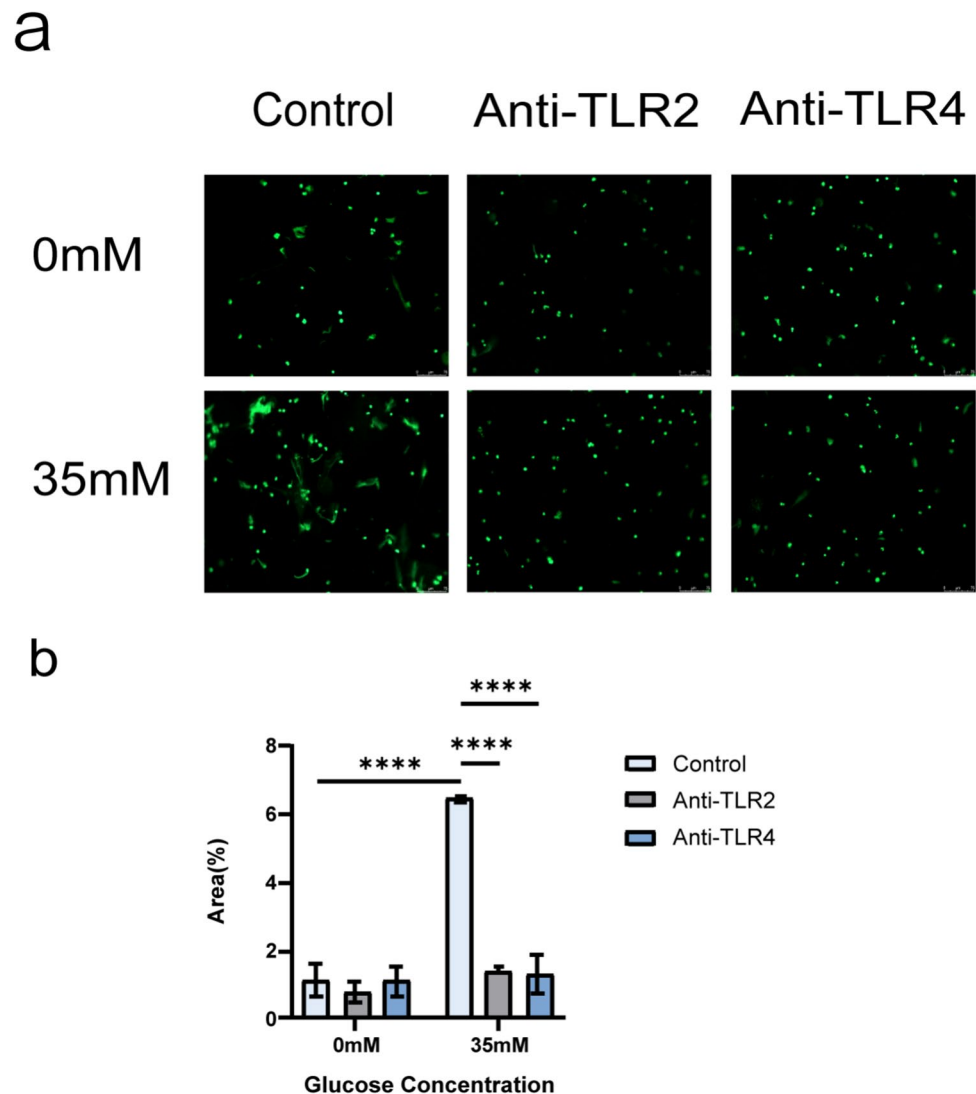
To validate the impact of TLRs on NETs production under high glucose conditions, neutrophils were pretreated with a vehicle, TLR2 antagonist, or TLR4 antagonist for 1 h. NETs formation was observed using SYTOX Green staining under a fluorescence microscope after stimulating neutrophils with or without 35 mM glucose (Fig. 2a). As expected, both the

TLR2 and TLR4 antagonists reduced the high glucose-induced NETs formation (Fig. 2b).

ROS Production was Involved in TLR2- but not TLR4-Regulated NETs Formation

The formation of NETs can be either an ROS-dependent or ROS-independent process; thus, we used a DCF-DA fluorescence assay to determine whether high glucose-stimulated NETs formation is ROS dependent. We also assessed the impact of an NADPH oxidase inhibitor (diphenyleneiodonium [DPI]) on high glucose-induced NETs formation. As expected, an oxidative burst was observed in neutrophils upon exposure to a high glucose concentration (Fig. 3a). The NETs formation that was induced by high glucose was reduced by pretreatment with DPI (Fig. 3b, 3c), which indicated that the process was ROS dependent. We also explored whether TLRs regulate high glucose-stimulated

Fig. 2 High glucose-induced NETs formation was inhibited by a TLR2 antagonist and a TLR4 antagonist. Human neutrophils suspended in media were pretreated with TLR2 antagonist or TLR4 antagonist for 1 h, and then NETs formation after stimulation with 35 mM glucose for 2 h. Neutrophils without any stimulation were used as control. **(a)**. NETs were examined using SYTOX green staining. After high glucose stimulation, the production of NETs increased. TLR2 and TLR4 antagonists inhibited NETs formation. Bar: 75 μ m. **(b)**. Quantification of NETosis percentage of neutrophils. The result showed that TLR2 and TLR4 antagonists markedly inhibited the NETs formation. Data represent mean $\pm$ SD of triplicate experiments. **** p < 0.0001 by two-way ANOVA



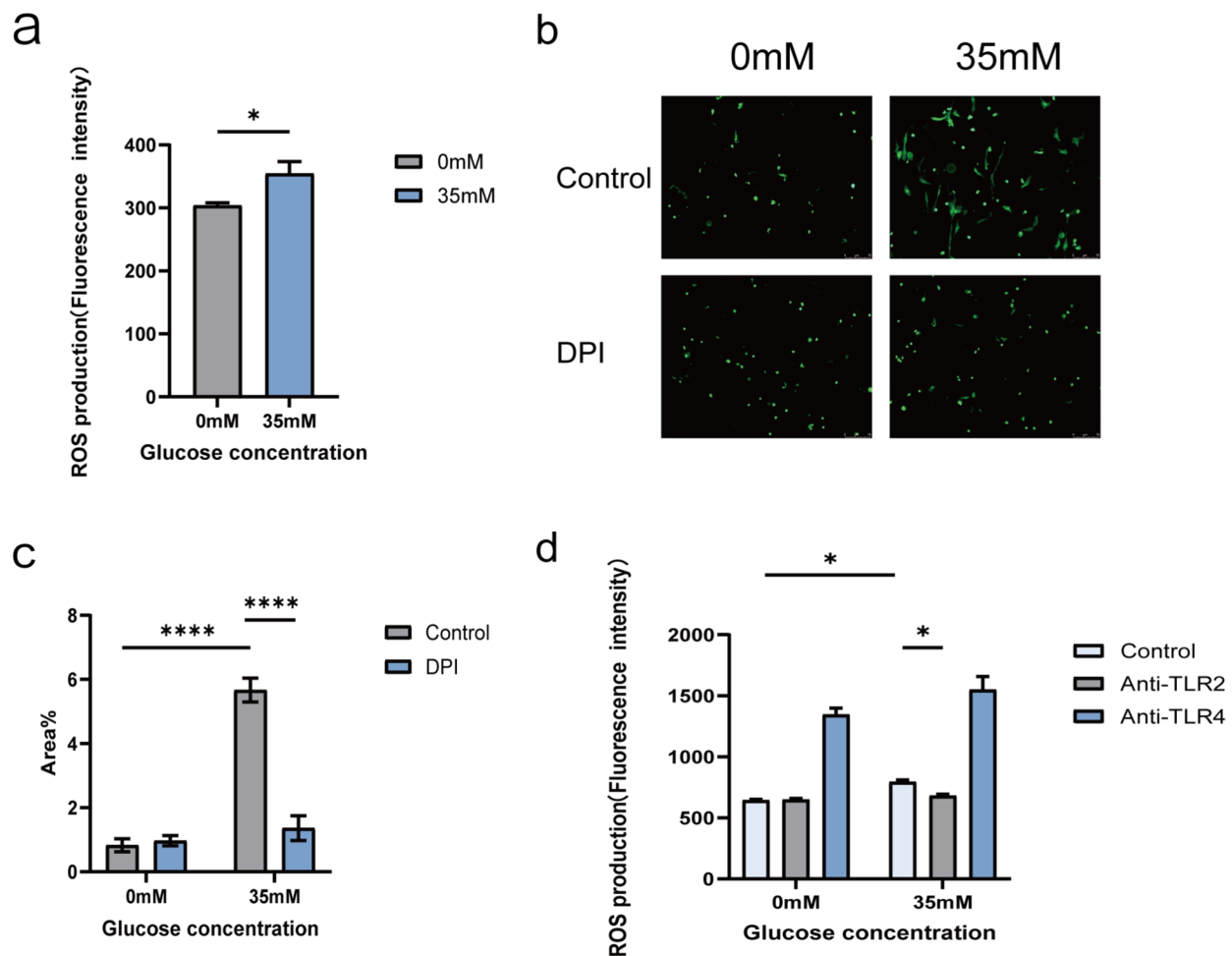


Fig. 3 High glucose-induced NETs formation through a ROS-dependent pathway. ROS production was involved in TLR2- but not TLR4-regulated NETs formation. **(a)** Neutrophils were unstimulated or stimulated with 35 mM high glucose for 2 h, and ROS production was measured using the DCF-DA fluorescence assay. 35 mM glucose triggered neutrophil oxidative burst. Data represent mean $\pm$ SD of triplicate experiments. * $p < 0.05$ by t-test. **(b)** Neutrophils were pretreated with DPI for 1 h to inhibit NADPH oxidase, then stimulated with or without 35 mM high glucose. NETs were examined using SYTOX green staining. Results showed that DPI reduced NETs formation by high glucose stimulation. Bar: 75 μ m **(c)** We calculated the percentage of NETs structures in the observed area. The result

showed that 35 mM glucose enhanced NETs production, while DPI reduced NETs formation after treated with high glucose. Data represent mean $\pm$ SD of triplicate experiments. **** $p < 0.0001$ by two-way ANOVA. **(d)** Neutrophils were stimulated with 35 mM high glucose for 2 h after pretreated with vehicle, TLR2 antagonist or TLR4 antagonist for 1 h, and ROS production was measured using the DCF-DA fluorescence assay. The result showed that HGNs-induced oxidative burst, with a slight inhibition by the TLR2 antagonist, while TLR4 antagonist treatment neither increased nor decreased this response. Data represent mean $\pm$ SD of triplicate experiments. * $p < 0.05$ by one-way ANOVA. Bar: 75 μ m

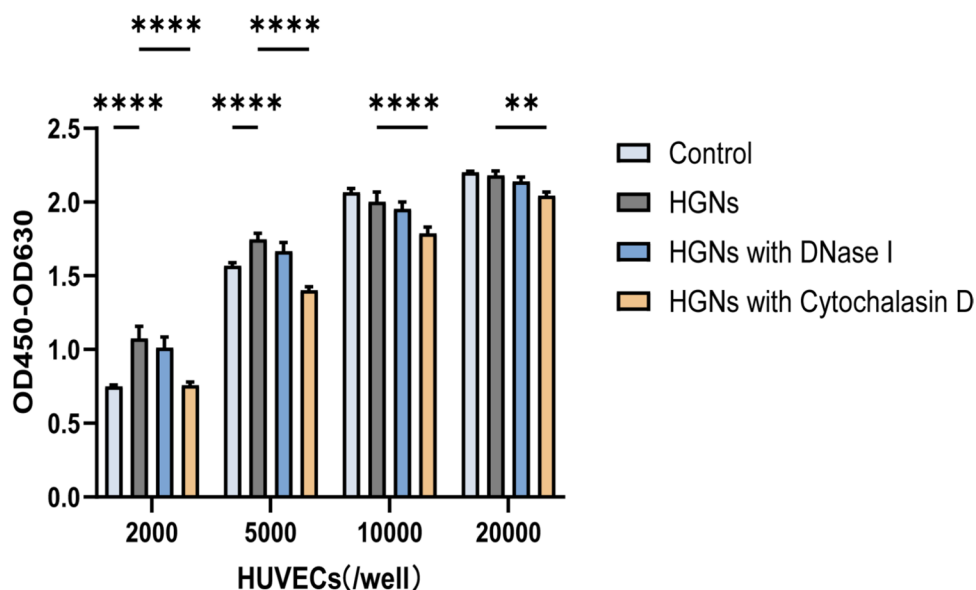
NETs formation by modulating ROS production. We found that the TLR2 antagonist reduced the high glucose-induced ROS production and that the TLR4 antagonist had no effect (Fig. 3d). These results indicated that ROS production is involved in TLR2- but not TLR4-regulated NETs formation.

HGNs Promoted HUVEC Proliferation Via Phagocytosis and not Via NETs

A WST-1 assay was used to assess HUVEC proliferation. To distinguish the effects of NETs from those of

neutrophil phagocytosis, cytochalasin D was used to inhibit neutrophil phagocytosis and DNase I was used to degrade NETs. When HUVECs are at low concentrations, high glucose-activated neutrophils (HGNs) promoted the proliferation of HUVECs, and this proliferative effect could be significantly inhibited by cytochalasin D (Fig. 4). However, DNase I neither enhanced nor inhibited the HGN-promoted HUVEC proliferation. These results indicated that HGNs promoted HUVEC proliferation, possibly via neutrophil phagocytosis but not via NETs formation.

Fig. 4 HGNs promote HUVEC proliferation via phagocytosis. The HUVEC proliferation exposed to vehicle, HGNs, HGNs with DNase I or HGNs with Cytochalasin D were accessed by WST-1 test. HGNs promoted HUVEC proliferation, which was inhibited by cytochalasin D, but not by DNase I. Data represent mean $\pm$ SD of triplicate experiments. ** $p < 0.01$, **** $p < 0.0001$ by two-way ANOVA



NETs were Involved in High Glucose-Induced Neutrophil–HUVEC Adhesion, Which Could be Inhibited by a TLR2 Antagonist and a TLR4 Antagonist

The results of the adhesion assay showed that high glucose promoted neutrophil–HUVEC adhesion (Fig. 5). DNase I, which degrades and thus eliminates NETs, was found to significantly inhibit neutrophil–HUVEC adhesion, while cytochalasin D had no effect. The TLR2 and TLR4 antagonists both reduced the high glucose-induced neutrophil–HUVEC adhesion. These results suggest that NETs promote high glucose-induced neutrophil–HUVEC adhesion and that this process can be modulated by TLR2 and TLR4.

HGNs Inhibited HUVEC Migration by Generating NETs, and the Migration Could be Partially Rescued by a TLR2 Antagonist and a TLR4 Antagonist

As shown in Fig. 6, HUVEC migration was determined using a wound-healing assay. The results showed that HGNs significantly inhibited HUVEC migration. DNase I significantly alleviated this inhibition of HUVEC migration, while cytochalasin D had no effect. These results indicated that HGNs may inhibit HUVEC migration through the formation of NETs. Furthermore, the TLR2 and TLR4 antagonists could attenuate the HGN-inhibited HUVEC migration, which suggested that TLR2 and TLR4 may participate in HGN-induced HUVEC migration inhibition by regulating NETs formation.

HGNs Disrupted the Barrier Function of HUVECs Through NETs Formation, and TLRs may Have Modulated this Process

We evaluated the leakage of horseradish peroxidase (HRP) to assess the barrier function of the HUVECs. The results showed that HGNs significantly increased HUVEC permeability (Fig. 7). DNase I significantly reduced the HGN-induced HUVEC permeability, while cytochalasin D had no significant impact. The TLR2 and TLR4 antagonists significantly reduced the HGN-induced HUVEC permeability. When the HUVECs were subjected to immunofluorescence staining, zonula occludens-1 (ZO-1) was observed in the HUVECs. The expression of ZO-1 was significantly lower in cells exposed to HGNs. DNase I rescued the HGN-reduced ZO-1 expression, while cytochalasin D had no effect. It was also observed that the TLR2 and TLR4 antagonists attenuated the HGN-reduced ZO-1 expression in the HUVECs. These results indicated that NETs are involved in HGN-mediated HUVEC barrier function damage and that TLR2 and TLR4 may enhance this damage by regulating NETs formation (Fig. 8a, b).

Discussion

DR is a common diabetic microvascular complication and a major cause of blindness worldwide. Currently, the main treatment options for DR include laser treatment, vitreous surgery, and intravitreal injections [35]. However, these treatments are invasive and may have side effects. Therefore,

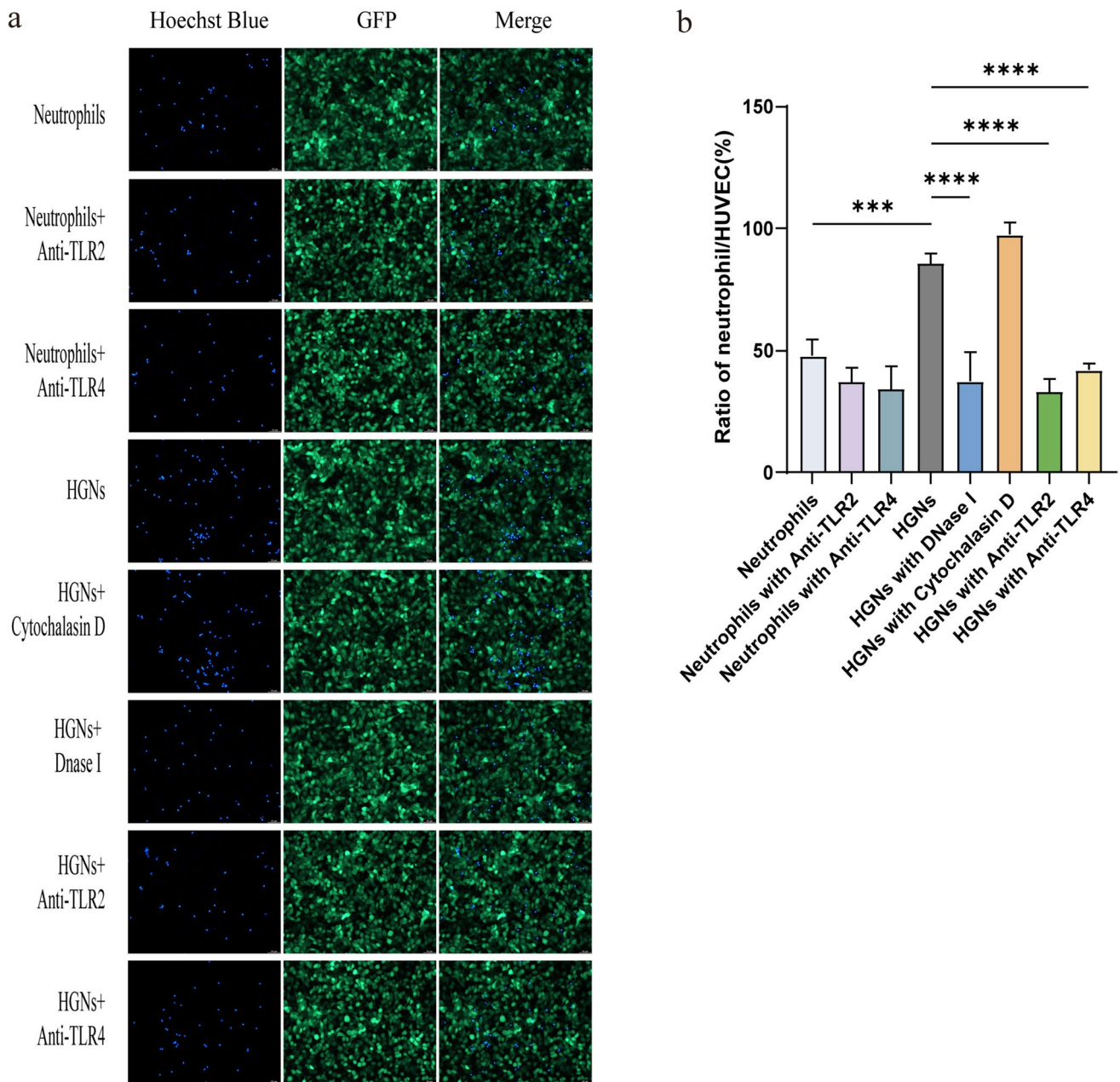


Fig. 5 NETs are involved in HG-induced neutrophil-HUVEC adhesion, which could be inhibited by a TLR2 antagonist and a TLR4 antagonist. **(a)** Images presented showed the adhesion of Hoechst 33,342 (blue) stained live neutrophils to HUVEC exposed to neutrophils, TLR2 antagonist, TLR4 antagonist, neutrophils with TLR2 antagonist, neutrophils with TLR4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist

or HGNs with TLR4 antagonist. **(b)** Quantification of the mean ratio of neutrophil/HUVEC showed that HGNs significantly increased the neutrophil-HUVEC adhesion as compared with control cells. Moreover, this response could be significantly inhibited by DNase I, TLR2 antagonist, TLR4 antagonist. Data represent mean $\pm$ SD of triplicate experiments, ***p < 0.001, ****p < 0.0001 by one-way ANOVA. Bar: 50 μ m

there is an urgent need to develop more effective therapeutic approaches.

Mounting evidence suggests that inflammation may cause DR. For example, results obtained from studies involving patient and animal models indicate that DR is a chronic inflammatory disease, with increased leukocyte levels

playing a critical role in the initial phases of DR [11]. Neutrophils, which are the predominant leukocytes, have been implicated in the pathogenesis of DR, with elevated neutrophil levels observed in diabetic rats [36] and an increased blood neutrophil count found to be highly related to the presence and severity of both DR and DM [16]. Moreover,

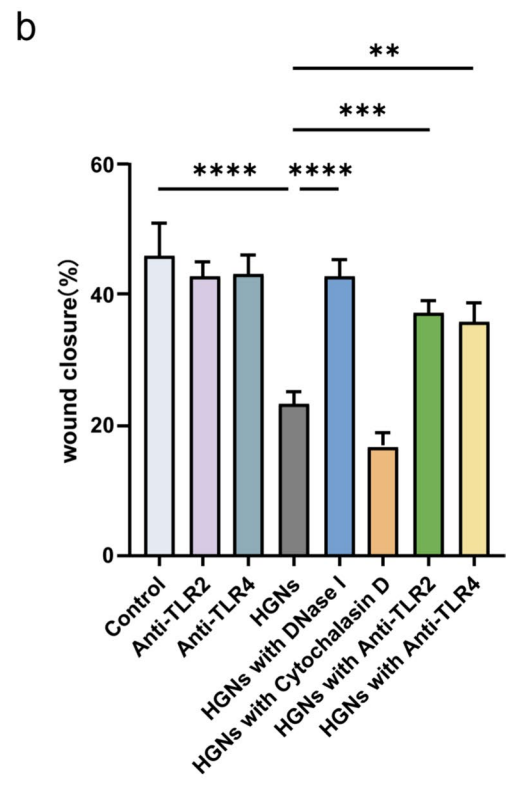
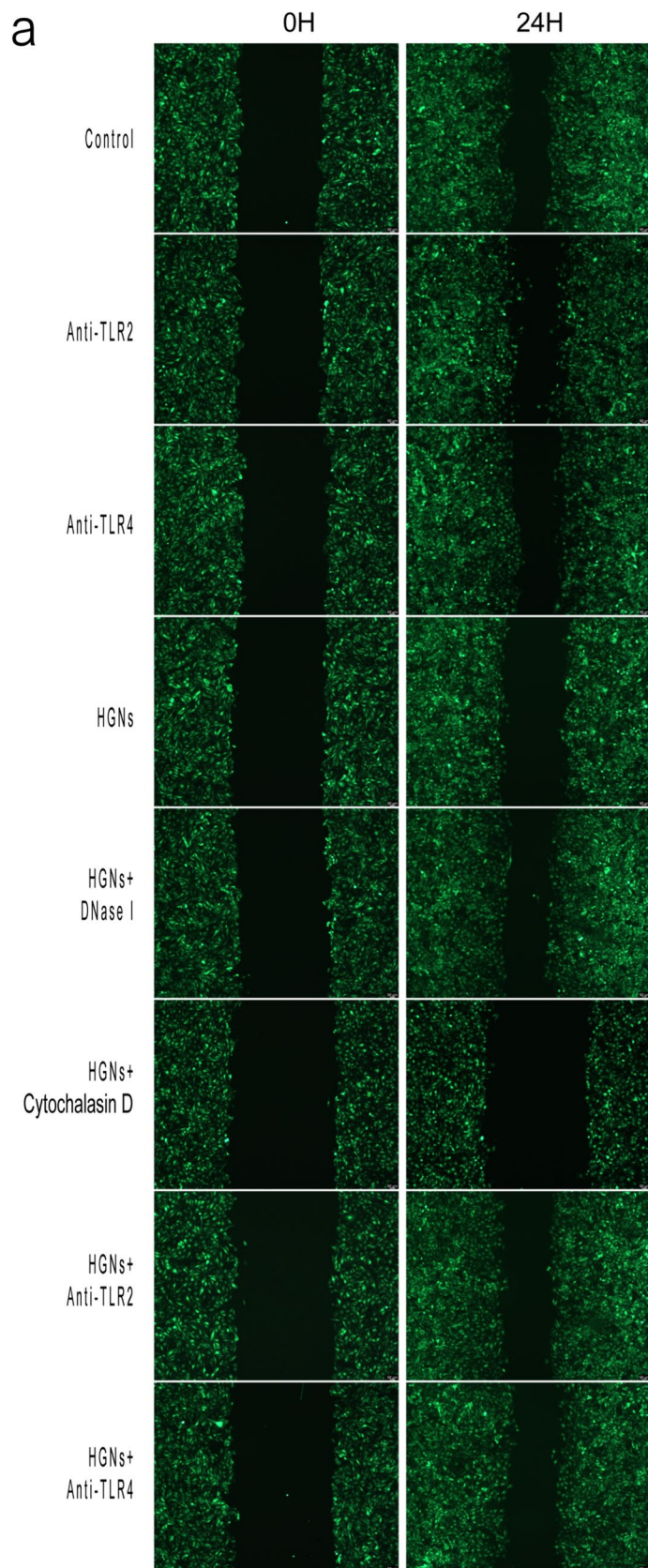


Fig. 6 HGNs inhibit HUVEC migration through NETs formation, which could be partially rescued by TLR2 antagonist and TLR4 antagonist. **(a)** The HUVEC migration exposed to vehicle, TLR2 antagonist, TLR4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist were assessed by making a scratch on confluent monolayer of HUVEC. **(b)** Quantification showed that HGNs inhibited HUVEC migration. Meanwhile, DNase I, TLR2 antagonist and TLR4 antagonist rescued HGNs-inhibited HUVEC migration ($p < 0.05$), but cytochalasin D exacerbated it. Data represent mean $\pm$ SD of triplicate experiments, * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA. Bar: 50 μ m

neutrophils participate in DR development [37], mainly by inducing vascular leakage and vascular endothelial cell damage [38]. Recently, it was found that neutrophils can release NETs, which are considered to be important in maintaining inflammation in chronic inflammatory diseases and to be associated with hyperglycemia and DR development. Menegazzo et al. reported that patients with type 2 DM had increased levels of plasma NET components compared with non-diabetic control individuals [18]. They also reported that high blood glucose levels increased NET formation both in vivo and in vitro [18]. Several other studies have shown that patients with DR have higher concentrations of NET-related molecules in the vitreous and plasma than diabetic patients without DR [19, 39, 40]. The results of our study confirmed that the expression of MPO and PADI4 was upregulated in DR patients and in vitro glucose stimulation promotes NETs formation with the amount of NETs increases with the glucose concentration, consistent with the findings of previous studies [19, 41]. These results suggest that NETs, which are important for maintaining the inflammatory environment, are involved in the development of not only DM but also DR.

TLRs recognize pathogen-associated molecular patterns and participate in inflammatory responses [42]. Among the TLR subtypes, TLR2 and TLR4 are closely related to the development of DM. Our study found that the expression of TLR2/4 was upregulated in the DR population, which is consistent with previous studies. For example, Zhu and Devaraj et al. found that TLR2 and TLR4 expression was upregulated in the peripheral blood of diabetic patients [43, 44]. TLR4 in bone marrow-derived cells has been proven to contribute to the development of DR [45]. It has also been reported that TLR2 is involved in both the pathogenesis of type 2 DM and the development of related microvascular complications [42, 46]. We first investigated the correlation between TLR2/4 and PADI4, which were significantly upregulated in the DR population. We found a significant positive correlation between TLR2/4 and PADI4, and both were enriched in the NETs formation pathway. Next, we investigated the effects of TLR2 and TLR4 on high glucose-induced NETs formation. We found that a TLR2 antagonist and a TLR4 antagonist both significantly inhibited high glucose-induced

NETosis. Consistent with our observations, TLR2/TLR4 have been shown to participate in NETs formation induced by *T. brucei* lipophosphoglycan and gut-derived lipopolysaccharide [25, 26]. Similarly, Oklu et al. reported that TLR4 mutant mice had obviously lower levels of NETs [47]. In addition, in a study with septic patients, Clark et al. demonstrated that TLR4 on platelets could activate neutrophils to release NETs and capture bacteria in the bloodstream [48]. We have also previously shown that TLRs promote the formation of NETs induced by *S. aureus* [27]. Together, these results indicate that TLR2/4 may modulate inflammatory reactions by regulating the formation of NETs under high glucose circumstance.

The formation of NETs can be ROS dependent or independent. In this study, an obvious oxidative burst was observed in neutrophils after high glucose stimulation. A ROS-scavenging agent (DPI) was used to verify the effect that ROS had on the observed high glucose-stimulated NETs formation, and DPI significantly inhibited the high glucose-stimulated NETs formation. These results indicated that ROS were involved in the high glucose-induced NETs formation, a finding that aligns with Wang's results [19]. Consistent with our predictions, the TLR2 antagonist hindered the observed high glucose-induced NETs formation and reduced the ROS levels. However, the TLR4 antagonist had no significant effect. These results indicate that TLR2, but not TLR4, promotes high glucose-induced NETs formation by regulating ROS signaling. However, several other studies have reported conflicting results. Zhan et al. demonstrated that hepatitis B virus-induced S100A9 stimulates abundant NETs generation through TLR4–ROS signaling [49], and Dong et al. found that TLR4 partially mediates *Streptococcus pneumoniae*-induced NETs generation in vitro and in vivo [50]. The discrepancy between our results and those of previous studies may be due to the use of different stimuli: we used high glucose, whereas pathogens were used in the abovementioned studies. Therefore, it is likely that the ROS signaling pathway is involved in TLR2-regulated high glucose-induced NETs formation but not in TLR4-regulated high glucose-induced NETs formation. These findings indicate that there are other important signaling pathways involved in TLR4-mediated high glucose-induced NETs formation that must be elucidated. Additionally, our results showed that neutrophils exhibit an oxidative burst after treatment with TLR4 antagonist, but TLR4 antagonist can reduce NETs formation induced by high glucose stimulation, which presents a contradiction. Regarding the promotion of ROS formation by TLR4 antagonists, we hypothesize that this could be due to the following reasons: TLR4 activation typically promotes immune responses through pathways like MAPK (mitogen-activated protein kinase) and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) [51]. When Anti-TLR4 antibodies are used,

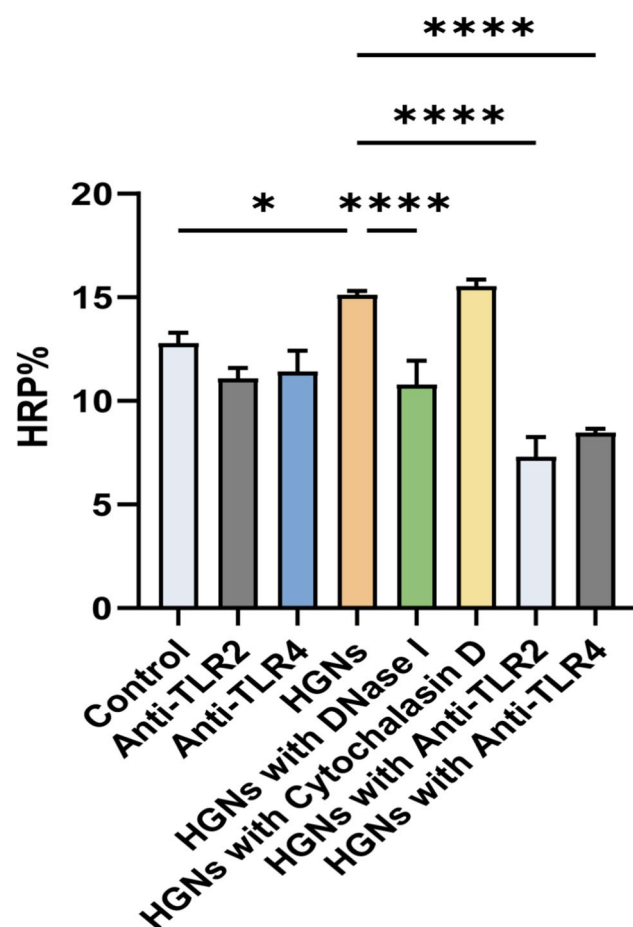


Fig. 7 HGNs disrupt the barrier function of HUVEC and increase permeability through NETs formation, which could be modulated by TLRs. The HUVEC permeability exposed to vehicle, TLR2 antagonist, TLR4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist were evaluated by the spillage rate of HRP by measuring absorbance at 450 nm wavelength. DNase I, TLR2 antagonist and TLR4 antagonist significantly rescued the increased permeability of HUVECs induced by HGNs, while Cytochalasin D had no impact on that. Data represents the mean $\pm$ SD for three independent monolayers. ** $p < 0.01$, **** $p < 0.0001$ by one-way ANOVA

they can partially or completely block the TLR4 receptor, disrupting normal receptor regulation. Given that TLR4 is closely involved in immune responses and ROS generation, blocking the receptor may trigger compensatory activation of other related pathways (such as $\text{TNF-}\alpha$ or $\text{IL-1}\beta$), which may, through negative feedback mechanisms or compensatory enhancement, promote ROS accumulation. Regarding the inhibition of NETs formation by TLR4 antagonists, we hypothesize that it is possible that TLR2 promotes NETs formation through ROS, whereas TLR4 may facilitate NETs formation independently of ROS. Some researches found the formation of NETs can be classified into ROS-dependent and ROS-independent pathways [52, 53]. Previous studies have shown that the formation of NETs requires autophagy

and ROS generation and inhibition of autophagy or NADPH oxidase activity can block NET formation [54, 55]. Interestingly, TLR4 is believed to induce autophagy [56]. Additionally, a study has shown that ROS production does not compensate for the defects in NETs formation in full-term neonatal polymorphonuclear leukocytes. The mechanism by which human PMNs form NETs involves distal and/or parallel events related to ROS production [57]. This provides an opportunity to independently dissect the molecular events behind these two pathways. In summary, we propose the hypothesis that TLR4 antagonists can inhibit NET formation by suppressing autophagy, which is unrelated to the increase in ROS. The phenomenon of TLR4 antagonists promoting ROS generation may be due to triggering a negative feedback mechanism to compensate for the inhibition of the TLR4 signaling pathway. Specifically, TLR4 antagonists may induce cellular stress by inhibiting the autophagy pathway, thereby activating ROS production. Autophagy plays a crucial role in maintaining intracellular ROS levels and removing harmful substances, such as damaged mitochondria. Therefore, inhibition of autophagy could lead to an increase in ROS [58]. In conclusion, although ROS levels increase, this does not necessarily mean that TLR4 antagonists directly promotes NETs formation through the ROS pathway. The elevation of ROS is more likely associated with changes in other regulatory mechanisms within the cell. In addition, considering that our results show that Anti-TLR4 promotes ROS formation, the CCK-8 assay was performed to assess the impact of Anti-TLR4 on the survival of HUVECs. The results indicated that Anti-TLR4 treatment does not significantly affect the cell viability or survival of HUVECs (Fig. S3). The viability of HUVEC cells treated with Anti-TLR4 was comparable to that of the control group, suggesting that the inhibition of TLR4 signaling does not cause substantial cytotoxic effects in this particular cell line under the experimental conditions. This observation implies that the effects of Anti-TLR4 on ROS generation and NETs formation may not be associated with any major alterations in cell survival. These findings point to the possibility that TLR4 inhibition may influence cellular processes such as ROS production without causing significant damage to the endothelial cells.

Neutrophils are the major cells responsible for the disruption of vascular barrier function, and increased neutrophil–endothelial cell adhesion has been proved to contribute to the progression of DR [59, 60]. As a newly discovered neutrophil action form, NETs were initially found to be critical for pathogen clearance. However, a growing number of studies have shown that an excessive amount of NETs is deleterious to the function vascular endothelial cells [61–63], possibly by upregulating adhesion molecule expression. To investigate the role that neutrophils play in the function of the vascular endothelium under high-glucose conditions

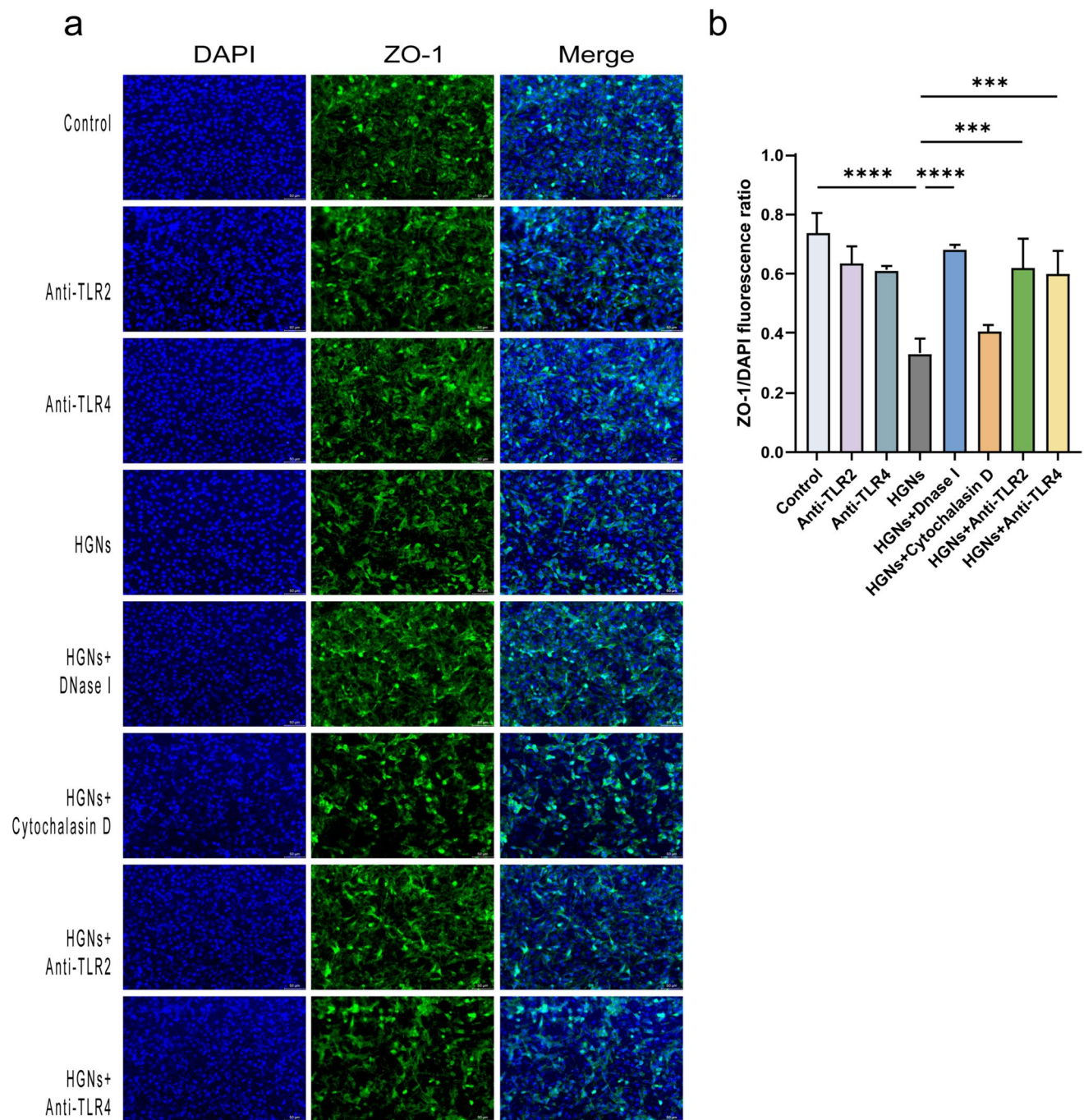


Fig. 8 HUVEC were treated with vehicle, TLR2 antagonist, TLR4 antagonist, HGNs, HGNs with DNase I, HGNs with Cytochalasin D, HGNs with TLR2 antagonist or HGNs with TLR4 antagonist. **(a)** Fluorescent images after different stimulation were shown. Nucleus were labeled with DAPI to identify DNA (blue) and with antibodies to identify tight junction protein (green). Bar: 50 µm. **(b)** The expression level of ZO-1 was quantified by calculating the mean fluores-

cence intensity ratio of ZO-1 to DAPI. The expression of ZO-1 was significantly reduced by neutrophils after high glucose stimulation, while HGNs with DNase I, HGNs with TLR2 antagonist and HGNs with TLR4 antagonist treatment rescued the decreased expression of ZO-1 caused by high glucose stimulation in neutrophils. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA

and the underlying mechanisms, we explored not only the effect of high glucose on neutrophil–HUVEC adhesion but also the influence that HGNs have on HUVEC proliferation,

migration, and permeability. We utilized DNase I to degrade NETs and cytochalasin D to inhibit neutrophil phagocytosis so that the effects of NETs and neutrophil phagocytosis

could be distinguished. We found that HGNs enhanced neutrophil–HUVEC adhesion, inhibited the migration of endothelial cells, and increased the permeability of the endothelial cell barrier, which could be alleviated by DNase I but not cytochalasin D. These results indicate that HGNs mainly interact with endothelial cells via NETs formation and not phagocytosis. Consistent with our results, Desilles et al. [64] and Omi et al. [65] demonstrated that high glucose levels increased neutrophil–endothelial cell adhesion. Moreover, Gupta et al. [66] found that in a high-glucose environment, NETs damage the glomerular endothelial filtration barrier.

Previously, we found that alkali-activated neutrophils promote human corneal epithelial cell proliferation via phagocytosis [67]. Similarly, in this study, we found that glucose-activated neutrophils promote endothelial cell proliferation, which was inhibited by cytochalasin D but not DNase I. However, the proliferative effect induced by HGNs only occurs when HUVECs are at low concentrations. When HUVECs are at high concentrations, HGNs do not significantly promote their proliferation. We speculate that this may be due to contact inhibition at higher cell densities, which slows down cell proliferation. These findings suggest that glucose-activated neutrophils promote HUVEC proliferation via phagocytosis and not NETs. Based on existing studies, one possible explanation is that neutrophil phagocytosis aids in the clearance of cellular debris or pathogens, which may lead to the release of pro-inflammatory cytokines and growth factors that could promote cell proliferation in the surrounding microenvironment [68]. Contrary to our results, Aldabbous et al. [69] found that phorbol-12-myristate-13-acetate induced NETs promoted endothelial proliferation. Our results may differ from those of Aldabbous due to differences in the stimuli used. Furthermore, Qiu et al. [70] found that high glucose reduced tumor necrosis factor- α -induced endothelial cell proliferation *in vitro*, which may lead to delayed wound repair in patients with DM. The discrepancy between our results and the abovementioned results may have been due to the fact that Qiu used TNF- α and glucose, whereas we used HGNs in this study. Nevertheless, our results indicate that HGNs may exert different effects through different pathways; specifically, they may promote endothelial cell proliferation through phagocytosis, while also enhance neutrophil–HUVEC adhesion, inhibit endothelial cell migration, and damage the endothelial barrier function by generating NETs. This may explain why patients with DR can experience inflammation-related angiogenesis as well as vascular leakage and related vision issues due to poor endothelial barrier function.

It is known that TLR2 and TLR4 are positively associated with the development of type 2 DM, as hyperglycemia leads to TLR2 upregulation, and insulin inhibits TLR2 expression [71, 72]. It has also been shown that inhibiting

TLR4 prevents autoimmune DM in non-obese diabetic mice [73]. Furthermore, TLR2 and TLR4 have been implicated in the pathogenesis of diabetic vascular complications, including DR [42]. Fu et al. observed that the DR present in diabetic TLR4 $^{-/-}$ mice was less severe than that in diabetic wild type mice [31], and Bayan et al. reported that hyperglycemia contributed to the pathogenesis of DR by promoting TLR4 expression [28]. Moreover, deletion of TLR2/4 has been shown to inhibit not only leukostasis but also endothelial death induced by DM [74]. In the present study, TLR2 and TLR4 were found to be involved in high glucose-induced endothelial cell dysfunction, because both a TLR2 antagonist and a TLR4 antagonist could inhibit HGN-induced neutrophil–HUVEC adhesion, reduction in HUVEC migration, and endothelial barrier function damage. Thus, we postulated that the TLR2 and TLR4 antagonists modulated the HGN-induced endothelial cell dysfunction by regulating NETs formation. Consequently, the use of TLR2 and TLR4 antagonists may be a new strategy for preventing hyperglycemia-induced vascular endothelial cell dysfunction.

In summary, our results indicate that high glucose can cause an oxidative burst in neutrophils and subsequent NETosis. TLR2 antagonists may inhibit high glucose-induced NETs formation through an ROS-dependent pathway. Furthermore, high glucose-activated neutrophils enhance HUVEC proliferation through phagocytosis and lead to HUVEC-neutrophil adhesion, HUVEC migration inhibition, and HUVEC barrier destruction by generating NETs. While TLR2 and TLR4 antagonists show potential in alleviating HGN-induced damage to HUVECs, the therapeutic application of Anti-TLR4 in delaying DR progression requires further investigation, particularly given its association with ROS production. Additional studies are warranted to elucidate the precise mechanisms and assess the long-term safety and efficacy of these antagonists in the context of DR.

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Author Contributions Material preparation was performed by Shirou Wu, Yahui Chen. The data collection was performed by Shirou Wu, Yahui Chen, Xiuming Jin, Jiayun Yu, Xueping Chen and Ting Wan. The data analysis was performed by Shirou Wu, Yahui Chen and Ting Wan. The first draft of the manuscript was written by Shirou Wu. The final draft of the manuscript was written by Shirou Wu and Ting Wan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability Data is provided within the manuscript or supplementary information files.

Declarations

Ethics Approval The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (protocol code 20210011; 2 January 2021).” for studies involving humans.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Competing Interests The authors declare no competing interests.

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