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Lychee seed polyphenol protects blood–retinal barrier by increasing tight joint proteins and inhibiting the activation of TLR4/MYD88/NF- κ B-mediated NLRP3 inflammasome

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ABSTRACT

Emerging evidence indicates that blood–retinal barrier (BRB) damage is the main cause of diabetic retinopathy (DR), and the downregulation of tight proteins (TJs) located between endothelial cells and the activation of NLRP3 inflammasome-mediated inflammation in Müller cells result in BRB injury. In this study, we aimed to study the protective effect of lychee seed polyphenol (LSP) on BRB integrity and the mechanism of action. By establishing a cellular model of BRB injury by using high glucose (HG)-induced hRECs or co-culture system of hRECs with MIO-M1 cells, we found that LSP significantly maintained the integrity of monolayer of hRECs as revealed by the decreased PD40 permeability and increased TJs, including ZO-1, Occludin, and Claudin 5. In addition, LSP inhibited the NLRP3 inflammasome activation and the levels of inflammatory cytokines via the TLR4/MYD88/NF- κ B pathway. Collectively, this study provides valuable insight into the utilization of LSP in the treatment of DR.

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Lychee seed polyphenol; blood–retinal barrier; tight proteins; NLRP3 inflammasome; TLR4/MYD88/NF- κ B

Introduction

Diabetes, a complex systemic metabolic disease, is characterized by both hyperglycemia and insulin dysfunction. Chronic hyperglycemia damages the normal functions of the eye, kidney, blood vessel and other organs in the human body (Monami et al., 2013).

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Emerging evidence indicates that diabetic retinopathy (DR) is the biggest cause of blinding eye disease among the working population (Fong et al., 2004). The epidemiological data indicate that almost 93 million people worldwide suffer from DR and 28 million people have vision problems (Chan et al., 2014). DR is pathologically divided into non-proliferative DR (NPDR) and proliferative DR (PDR). Among them, NPDR begins with the occurrence of microaneurysms and microhemorrhages, increased vessel permeability, and the formation of soft and hard exudates (Lechner et al., 2017). Therefore, NPDR is recognized to be the main cause of vision loss in diabetic patients. PDR is induced by the progressive damage of the retinal microvascular network, which is diagnosed by the presence of neovascularization or preretinal/vitreous hemorrhages (Lechner et al., 2017). Blood–retinal barrier (BRB) is a physiological barrier that is necessary to maintain normal visual function. The inner barrier (iBRB) is composed of endothelial cells, pericytes, Müller cells and astrocytes, and the tight junction proteins (TJs) located between endothelial cells play important roles in maintaining the integrity of BRB (Rizzolo et al., 2011). BRB is a natural barrier that protects retinal cells and optic nerves from cytotoxic substances in the blood and adjacent tissue fluids, thus provides a stable metabolic environment for the visual system. To date, there is a growing body of evidence showing BRB injury is closely accompanied with the pathological process of DR (Cunha-Vaz et al., 2011). Therefore, targeting protection of BRB integrity has been a promising strategy for the treatment of DR.

Most clinical studies showed that a large number of inflammatory cytokines are detected in the vitreous and aqueous humour of patients with DR. The increased inflammatory cytokines result in the high permeability of retinal vessels, which is an important hallmark of BRB injury. Recently, the role of inflammasomes in the BRB injury has been well illustrated over the past decade, and the inhibition of inflammasomes activation is generally helpful to reduce the inflammation of DR (Yerramothu et al., 2018). In addition, the pro-inflammatory cytokines, such as IL-1 β and TNF- α , were reported to reduce the expression of TJs and damage the normal function of BRB (Mesquida et al., 2019). The NLRP3 inflammasome is a critical component of the innate immune system that mediates caspase-1 activation and the secretion of proinflammatory cytokines including IL-1 β and IL-18 in response to microbial infection and cellular damage. The aberrant activation of NLRP3 inflammasome has been linked with several inflammatory disorders, including Alzheimer's disease, diabetes and atherosclerosis (Kelley et al., 2019). In addition, the activation of NLRP3 inflammasome in cerebral microvascular endothelial bEnd.3 cells plays an important role in BBB injury (Cao et al., 2016; Carrano et al., 2012; Hoegen et al., 2011), while knockdown of NLRP3 reduces the cell death and the loss of TJs in bEnd.3 cells (Xu et al., 2019). Therefore, targeting inhibition of NLRP3 inflammasome-mediated inflammatory responses can maintain BRB integrity and normal function.

To date, the therapies of DR mainly include laser photocoagulation, injections of anti-vascular endothelial growth factor (VEGF) drugs and steroids, surgery and so on. However, the surgery is reported to result in the loss of vision and bring permanent damage to patients. Therefore, the early drug intervention of DR via the inhibition of neovascularization has been a critical way for the treatment of DR (Wang & Lo, 2018). Compared to the chemical synthetic drugs, traditional Chinese medicines (TCMs) with side effects have been proved to be safe and effective in the clinic for almost 2000

years. Lychee seed from the popular fruit Litchi is commonly prescribed in the Chinese medicine formula in the clinic. Its traditional efficacies are promoting qi and dispelling stagnation. Our previous studies have found that the polyphenols are the main components in the purified fraction named LSP (lychee seed polyphenol) (Tang et al., 2018; Xiong et al., 2020; Zhao et al., 2018). In addition, microglia in the brain are the macrophages-like cells that regulate the inflammatory response and secretion of cytokines (Li & Barres, 2018; Zhang et al., 2021). While LSP was demonstrated to inhibit the NLRP3-mediated neuroinflammation and protect blood–brain barrier (BBB) against NLRP3 inflammasome-mediated injury in the cell and animal models of AD (Xiong et al., 2021). In this study, we further found that LSP protected against high glucose (HG)-induced BRB injury in human retinal microvascular endothelial cells (hRECs). The mechanistic study revealed that HG-induced activation of NLRP3 inflammasome via the TLR4/MYD88/NF- κ B pathway. While LSP significantly inhibited the TLR4/MYD88/NF- κ B pathway and the activation of NLRP3 inflammasome. Overall, our current results suggested that LSP protected BRB normal function via increasing the expression of TJs in hRECs and inhibiting the NLRP3 inflammasome-mediated inflammatory responses through the TLR4/MYD88/NF- κ B pathway in human Müller cell line (MIO-M1) cells, which provides valuable insight for the utilization of LSP in the prevention and treatment of DR.

Materials and methods

Materials

LSP was prepared and the quality control of LSP was performed according to the previous methods (see Supplementary Figure S1) (Wang, Wu, et al., 2017; Wang, Zhang, et al., 2017). LSP was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 2 g/L and stored at -20°C before utilization. High glucose was purchased from Chongqing Heping Pharmaceutical Co., Ltd. (Chongqing, China). The primary antibodies used in this study include ZO-1 (ab96587), Occludin (ab167161) and Claudin 5 (ab15106) from Abcam (Cambridge, MA, USA); IL-1 β (#12242), Acetyl-NF- κ B p65 (12629), p-I κ B(2859), I- κ B (9242) and TNF- α (3707) from Cell Signaling Technology, Inc. (CST, MA, USA); IL-18 (D044-3) from Medical & Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan); Caspase-1 (sc-56036), ASC (sc-514414) and β -actin (sc-47778) from Santa Cruz Biotechnology, Inc. (CA, USA); GSDMD (20770-1-AP), MYD88 (23230-1-AP), TLR4(19811-1-AP) from Proteintech Group, Inc. (Wuhan, China). The plasmids, including pEGFP-N1-NLRP3 (PPL00151-2a), pEGFP-N1-caspase-1 (PPL00392-2e) and mCherry-C1-ASC (PPL01752-2b), were bought from Public Protein/Plasmid Library (PPL, Nanjing, China). Human IL-1 β ELISA Kit (E-EL-H0149c), human TNF- α ELISA Kit (E-EL-H0109c) and Human IL-18 ELISA Kit (E-EL-H0253c) were purchased from 4A Biotech Co., Ltd. (Beijing, China).

Cell culture

hREC cells (BNCC340909) and MIO-M1 cells (BNCC341652) were purchased from Beijing Beina Chuanglian Biotechnology Research Institute (City, China). All cells

were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% (v/v) penicillin/streptomycin (PS; Beyotime, Shanghai, China) in a 5% CO₂ humidified incubator at 37°C.

Establishment of the co-culture system of hRECs with MIO-M1 cells

The cell model of BRB was simulated by establishing the co-culture system of hRECs with MIO-M1 cells using a trans-well plate (Jet Bio-Filtration Co., Ltd., Guangzhou, Guangdong, China). In brief, MIO-M1 cells with a density of 4×10^4 cells/cm² were seeded into the lower chamber of the trans-well plate, and hRECs were seeded into the upper chamber of the insert with a cell density of 4×10^4 cells/cm². Then, 0.5 mL of DMEM was added into the upper chamber and 1.5 mL of DMEM was added into the lower chamber for cell culture. After the hRECs reached a tight monolayer, the co-culture system for BRB was successfully established and could be used for the followed experiments.

MTT assay

The cytotoxicity of the test drugs on hRECs was measured by MTT assay. In brief, hRECs were seeded onto 96-well plates at a density of 5×10^3 cells/well and treated with the test drugs for 24 h. After treatment, 10 µL of MTT solution (5 mg/mL) was added to each well and followed by incubation for 4 h. Then, the medium was removed and 150 µL of DMSO was added to dissolve the formazan in cells. After shaking at a low speed for 10 min at room temperature, the colorimetric reading of each well was then obtained by a spectrophotometer (BioTek, VT Lab, USA) at OD 570 nm. The cytotoxicity was measured by calculating the cell viability according to the following formula: Cell viability (%) = $100 \times \text{OD Value}_{\text{Cells treated}} / \text{OD Value}_{\text{Cells control}}$. The data were from three separated experiments.

Permeability assay

The measurement of the monolayer permeability of hRECs was performed according to a previously described method (Xiong et al., 2021). In brief, hRECs were seeded onto the 0.4 µm pore polycarbonate membrane in a 6.5-mm trans-well plate at a density of 6×10^4 cells/well (Jet Bio-Filtration Co., Ltd., Guangzhou, Guangdong, China). After incubation for 3 days, hRECs were treated with LSP in the presence or absence of HG for 24 h. Then, 0.1 mg/mL fluorescein isothiocyanate-dextran (FD-40, Sigma-Aldrich) in phenol red-free DMEM (Gibco, Waltham, MA) was added to the apical (AP) compartment, and the medium in the basolateral (BL) compartment was replaced by the phenol red-free DMEM. After incubation for 30 min, 100 µL of solution in the BL compartment was collected and transferred into a new 96-well plate (Jet Bio-Filtration Co., Ltd., Guangzhou, China). The fluorescence intensity (A.U.) reflecting the amount of penetrated PD-40 was measured by a spectrophotometer (BioTek, VT Lab, USA) with an excitation wavelength at 490 nm and an emission wavelength at 520 nm.

Flow cytometry analysis

Cell pyroptosis was measured by flow cytometer using the Annexin V-FITC/PI Detection Kit (BD Biosciences, CA, USA). In general, the cells undergoing pyroptosis were stained with PI reagent and the apoptotic cells were stained by the Annexin V-FITC reagent. After treatment, MIO-M1 cells were collected and stained with Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. After incubation in the dark at room temperature for 15 min, cells were analysed by a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA). The data was analysed by the Flowjo software (BD Biosciences, San Jose, CA, USA).

Cell transfection

The expressions of NLRP3, caspase-1 and ASC were detected by determining the GFP intensity in pEGFP-N1-NLRP3, pEGFP-N1-caspase-1 or mCherry-C1-ASC transiently transfected MIO-M1 cells. In brief, MIO-M1 cells were seeded on the glass coverslips in a 6-well plate. The second day, cells reached approximately 70% confluent were used for the transfection of pEGFP-N1-NLRP3, pEGFP-N1-caspase-1, or mCherry-C1-ASC plasmid using the Perfect[®] Transfection Reagent (V azyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. After 18–24 h of transfection, the MIO-M1 cells were treated with the test drugs for 24 h. Then, cells were fixed with 4% PFA and stained with 4'-6-diamino-2-phenylindole (DAPI) (1:100; Shanghai, China, Beijing) solution for 30 min. The representative images of cells with GFP and DAPI fluorescence were captured and analysed under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan). Then, the GFP or RFP intensity indicating the expressions of NLRP3, caspase-1 or ASC were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

hRECs were seeded on glass coverslips in a 6-well plate and treated with LSP at the indicated concentrations in the presence or absence of 100 mM HG for 24 h. After treatment, the cells were washed 3 times with PBS and fixed with 4% PFA at 4°C for 20 min, following by the infiltration with 0.2% Triton X-100 (ZSGB-BIO, Beijing, China) for 10 min. After blocking with 10% goat serum at room temperature for 30 min, the cells were incubated with the primary antibodies, including Claudin 5 (1:50), Occludin (1:100) or ZO-1 (1:300), overnight at 4°C. Then, the cells were washed 3 times with PBS and incubated with the secondary antibody (1:1000) for 2 h in dark. After incubation, the cells were stained with DAPI solution at room temperature for 15 min. The coverslips were taken out for air-drying and mounted by Fluor-SaveTM media (Calbiochem, San Diego, CA, USA). The representative images were captured and analysed under a fluorescence microscope (Nikon, Japan). The GFP intensity indicating the protein expression was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

ELISA

The secretion of pro-inflammatory cytokines was determined by ELISA. In brief, after treatment, the cell supernatant of MIO-M1 cells was collected and centrifuged at a speed of 1000×g for 10 min. Then, the supernatant was used for the detections of IL-1 β , IL-18 and TNF- α using the Human IL-1 β ELISA kit, Human IL-18 ELISA kit and Human TNF- α ELISA kit according to the manufacturer's instructions. The absorbance of the solution was measured by a spectrophotometer at 450 nm (BioTek, VT Lab, USA). The standard curves for IL-1 β , IL-18 and TNF- α were respectively calculated using the responding standard sample, and the concentrations of IL-1 β , IL-1 and TNF- α were then measured (Wu et al., 2019).

Western blot

After treatment, cells were collected and lysed by 1× RIPA lysis buffer (CST, MA, USA) containing protease inhibitor cocktail for 15 min on ice. The solution was centrifuged at a speed of 12,000 rpm for 15 min at 4°C. The supernatant was subjected to the measurement of protein concentration using Quick Start™ Bradford 1× dye reagent (Bio Rad, California, USA). Equal amount of protein of each sample was then loaded and separated on SDS-PAGE gels. After electrophoresis, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% fat-free milk for 1 h at room temperature. After wash for 3 times using PBST, the membrane was incubated with the primary antibodies overnight at 4°C, following by incubation with the secondary antibodies at 37°C for 1 h. The bands were then revealed by the UltraSignal Hypersensitive ECL Chemiluminescent Substrate (4A Biotech Co., Ltd., Beijing, China), and detected by the ChemiDoc MP Imaging System (Bio-Rad, California, USA). The quantification of the bandy intensity was performed by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Hoechst/PI staining assay

The cell pyroptosis of MIO-M1 cells was detected by the Hoechst/PI staining assay as described previously (Teng et al., 2020). In short, after treatment, MIO-M1 cells were washed with PBS for 3 times. After that, the cells were stained with 5 mg/L Hoechst 33,342 and 5 mg/L PI solution for 5 min. After incubation and fixation using 4% PFA, the slides were air-dried and mounted with FluorSave™ medium. Representative images were captured and analysed under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan). The representative images with blue and red fluorescence at the same field were merged. The cell pyroptosis was measured by calculating the percentage of cells with PI signal (red fluorescence) in cells with Hoechst signal (blue fluorescence).

Quantitative reverse transcription PCR (qRT-PCR)

After treatment, the total RNA of MIO-M1 cells was extracted by TRIZOL reagent. Then, the reverse transcription into cDNA was performed using the Prime Script RT reagent kit according to the manufacture's protocol (Trans Serum, Beijing, China). The primers of

TLR4 used were as follows: forward: AGTGAGGATGATGCCAGGATG reverse: TTAGGAACCACCTCCACGC.

Statistics

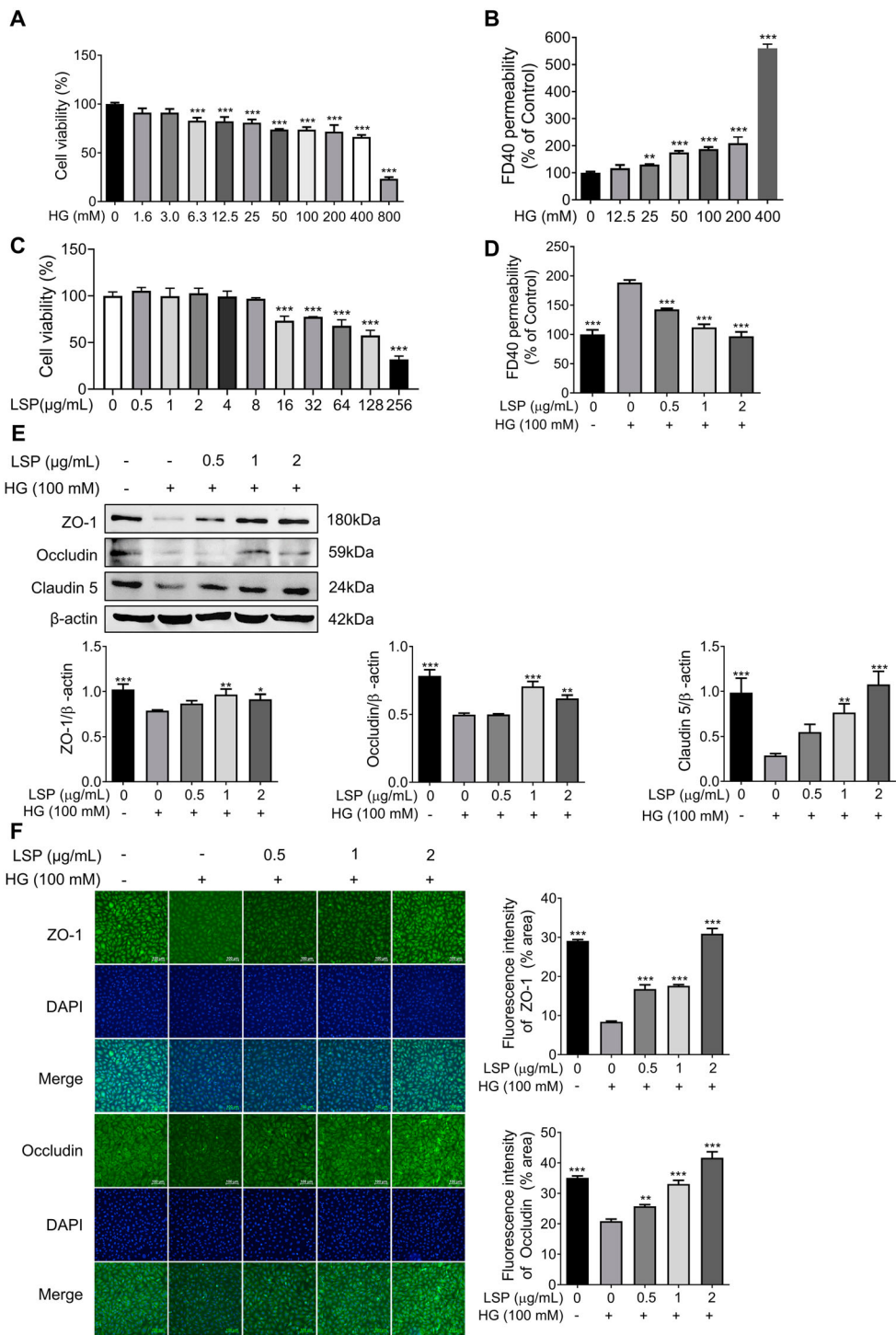
All data in this study were from at least three independent experiments. Data presented as mean $\pm$ standard deviation (SD) were analysed by the GraphPad Prism 5.0 software (San Diego, CA, USA) using one-way ANOVA followed by the post-Tukey test. $p \leq 0.05$ was considered to be statistically significant among the groups.

Results

LSP improves HG-induced BRB dysfunction in hRECs

Emerging evidence indicates that the increased glucose content is the hallmark of DR and is also the main cause of BRB injury (Cunha-Vaz et al., 2011). Therefore, we employed HG to induce hRECs for the establishment of the cellular model of BRB injury. At first, we investigated the effect of glucose on the cell viability of hRECs. As shown in Figure 1(A), 100 mM of glucose significantly decreased the cell viability to 73%, and the effect of glucose on the permeability of cellular monolayer was evaluated by detecting the amount of FD40 in hRECs. Figure 1(B) showed that glucose dose-dependently increased the amount of penetrated FD40, and 100 mM of glucose resulted in 1.8 fold of PD40 across the cellular monolayer compared to the untreated hRECs. Therefore, 100 mM of glucose was used to induce BRB injury in hRECs. The cell viability of hRECs under the treatment of LSP showed that LSP with the concentrations between 0.5 and 8 $\mu\text{g/mL}$ has no significant cytotoxicity on hRECs (Figure 1C). After

Figure 1. LSP improves HG-induced BRB dysfunction in hRECs. (A) hRECs were treated with glucose at the indicated concentrations of 0–800 μM for 24 h. After treatments, the cell viability was measured by MTT assay. The bar chart indicates the cell viability (%) of hRECs; bars, S.D., *** $p \leq 0.001$. (B) hRECs seeded onto the permeable filter were treated with glucose at the indicated concentration of 12.5–400 μM for 24 h. The monolayer permeability of hRECs was measured by detecting the amount of penetrated FD40 using the spectrophotometer. The bar chart indicates the relative percentage of FD-40 permeability; bars, S.D., *** $p \leq 0.001$. (C) hRECs seeded in 6-well plates were treated with LSP under the indicated concentrations (0.5–256 μM) for 24 h. Then, the cell viability was measured by the MTT assay. The bar chart indicates the cell viability (%) of hRECs; bars, S.D., *** $p \leq 0.001$. (D) hRECs seeded onto the permeable filter were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, the monolayer permeability of hRECs was measured by detecting the amount of penetrated FD40 using the spectrophotometer. The bar chart indicates the relative percentage of FD-40 permeability; bars, S.D., *** $p \leq 0.001$. (E) hRECs were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates were then harvested for the analysis of TJs including ZO-1, Occludin and Claudin 5 by Western blot. The bar chart indicates the relative expressions of ZO-1/ β -actin, Occludin/ β -actin and Claudin 5/ β -actin; bars, S.D., * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The full images of Western blotting were showed in Supplementary Figure S2. (F) hRECs were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, the expressions of ZO-1 and Occludin were detected by the immunofluorescence method. Representative images were captured by a fluorescence microscope (Nikon, Japan). Magnification: $\times 20$, Scale bar: 100 μm . The bar chart indicates the relative GFP intensity; bars, S.D., ** $p \leq 0.01$, *** $p \leq 0.001$.



the treatment of LSP (0.5–2 µg/mL), the amount of penetrated FD40 was significantly decreased in 100 mM glucose-treated hRECs (Figure 1D), suggesting that LSP could protect the integrity of cellular monolayer of hRECs. Then, we further detected the expressions of TJs associated with BRB integrity. The Western blotting results in Figure 1(E) showed that 100 mM of glucose significantly decreased the protein expression of TJs, including ZO-1, Occludin and Claudin 5, which were increased by LSP. Similarly, the immunofluorescence results in Figure 1(F) displayed that LSP remarkably increased the GFP intensity representing the expressions of ZO-1 and Occludin in HG-induced hRECs. Taken together, the above data indicate that LSP protects BRB integrity in HG-induced hRECs, an established in vitro model of BRB.

LSP inhibits the pro-inflammatory responses in HG-induced MIO-M1 cells

Müller cells are a type of retinal glial cells that locate in the vertebrate retina, which are found to be activated and accompanied by a number of pro-inflammatory cytokines secreted in DR. Emerging evidence indicates that the pro-inflammatory responses in Müller cells aggravate BRB damage (Rübsam et al., 2018). In this study, we employed 100 mM of glucose to treat individually cultured MIO-M1 cells, and then investigated the inhibitive effect of LSP on the inflammatory responses. The ELISA results in Figure 2(A–C) showed that 100 mM of glucose significantly promoted the secretions of IL-1 β , IL-18 and TNF- α in the supernatants of MIO-M1 cells, which was dose-dependently inhibited by LSP. In addition, the protein expressions of IL-1 β , IL-18 and TNF- α were determined by the Western blotting method. Figure 2(D) showed that LSP significantly inhibited the protein expressions of IL-1 β , IL-18 and TNF- α in 100 mM of glucose-induced MIO-M1 cells in a dosed manner. Taken together, LSP can inhibit HG-induced pro-inflammatory responses in MIO-M1 cells.

LSP improves BRB dysfunction in HG-induced co-culture system of hRECs with MIO-M1 cells

To better simulate the BRB in vitro, we established the co-culture system by seeding hRECs and MIO-M1 onto the trans-well plate. Then, we further evaluated the protective effect of LSP against HG-induced MIO-M1 secretions of inflammatory cytokines to the monolayer disruption of hRECs. The ELISA results in Figure 3(A–C) showed that 100 mM of glucose significantly promoted the secretions of IL-1 β , IL-18 and TNF- α in the supernatants of MIO-M1 cells in the co-culture system, which was inhibited by LSP. In addition, we detected the permeability of monolayer of hRECs and the expressions of TJs in hRECs. As shown in Figure 3(D), LSP significantly decreased the amount of penetrated FD40 across hRECs in the co-culture system. The Western blotting results showed that 100 mM of glucose significantly decreased the protein expression of TJs, including ZO-1, Occludin and Claudin 5, which were increased by LSP (Figure 3E). Similarly, the immunofluorescence results in Figure 3(F) displayed that LSP remarkably decreased the GFP intensity representing the expressions of ZO-1 and Claudin 5 in hRECs of the HG-induced co-culture system. Therefore, LSP protects the integrity of cellular monolayer of hRECs may via inhibiting the inflammatory responses of MIO-M1 cells in the co-culture system.

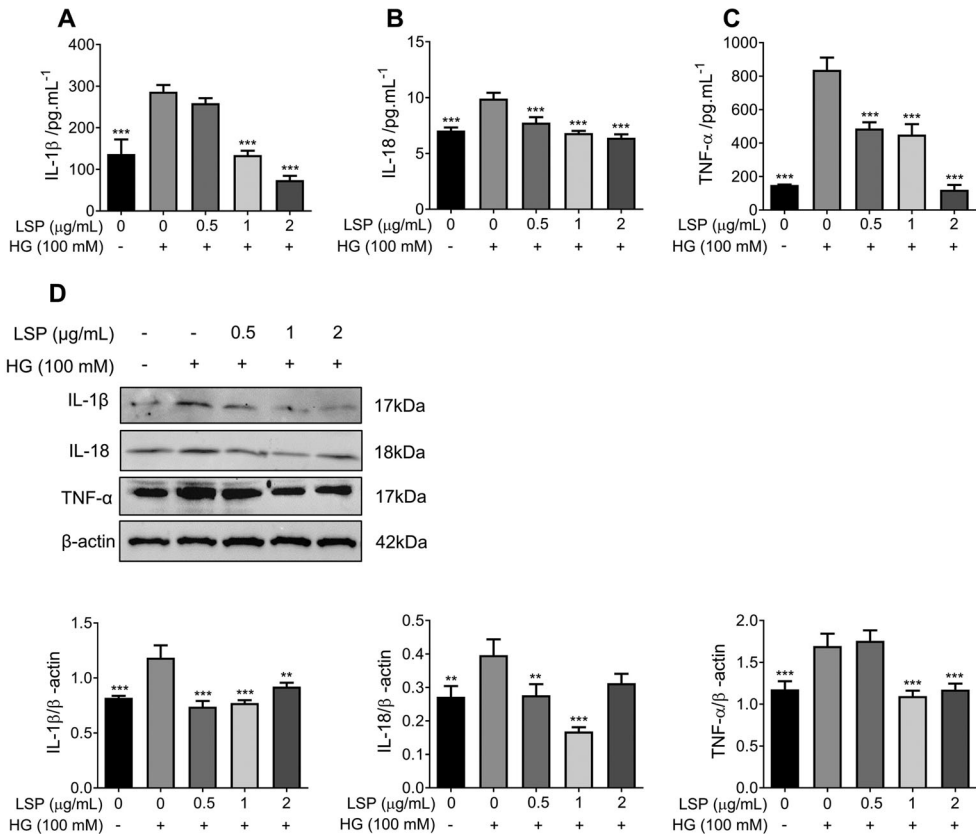


Figure 2. LSP inhibits the pro-inflammatory responses in HG-induced MIO-M1 cells. MIO-M1 cells were treated with LSP as the indicated concentrations in the presence or absence of 100 mM of glucose for 24 h. After treatment, the culture medium was collected for centrifugation and the supernatant was used for the detection of the levels of IL-1β (A), IL-18 (B), and TNF-α (C) using a Human IL-1β ELISA Kit, a Human IL-18 ELISA Kit, and a Human TNF-α ELISA Kit. The bar chart indicates the levels of IL-1β, IL-18 and TNF-α in the cell supernatant of MIO-M1cells; bars, S.D., *** $p \leq 0.001$. (D) MIO-M1 cells were treated with LSP as the indicated concentrations in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates were harvested for the analysis of IL-1β, IL-18, and TNF-α by Western blot. The bar chart indicates the relative protein expressions; bars, S.D., ** $p \leq 0.01$, *** $p \leq 0.001$. The full images of Western blotting were shown in Supplementary Figure S2.

LSP suppresses the activation of NLRP3 inflammasome in HG-induced MIO-M1 cells

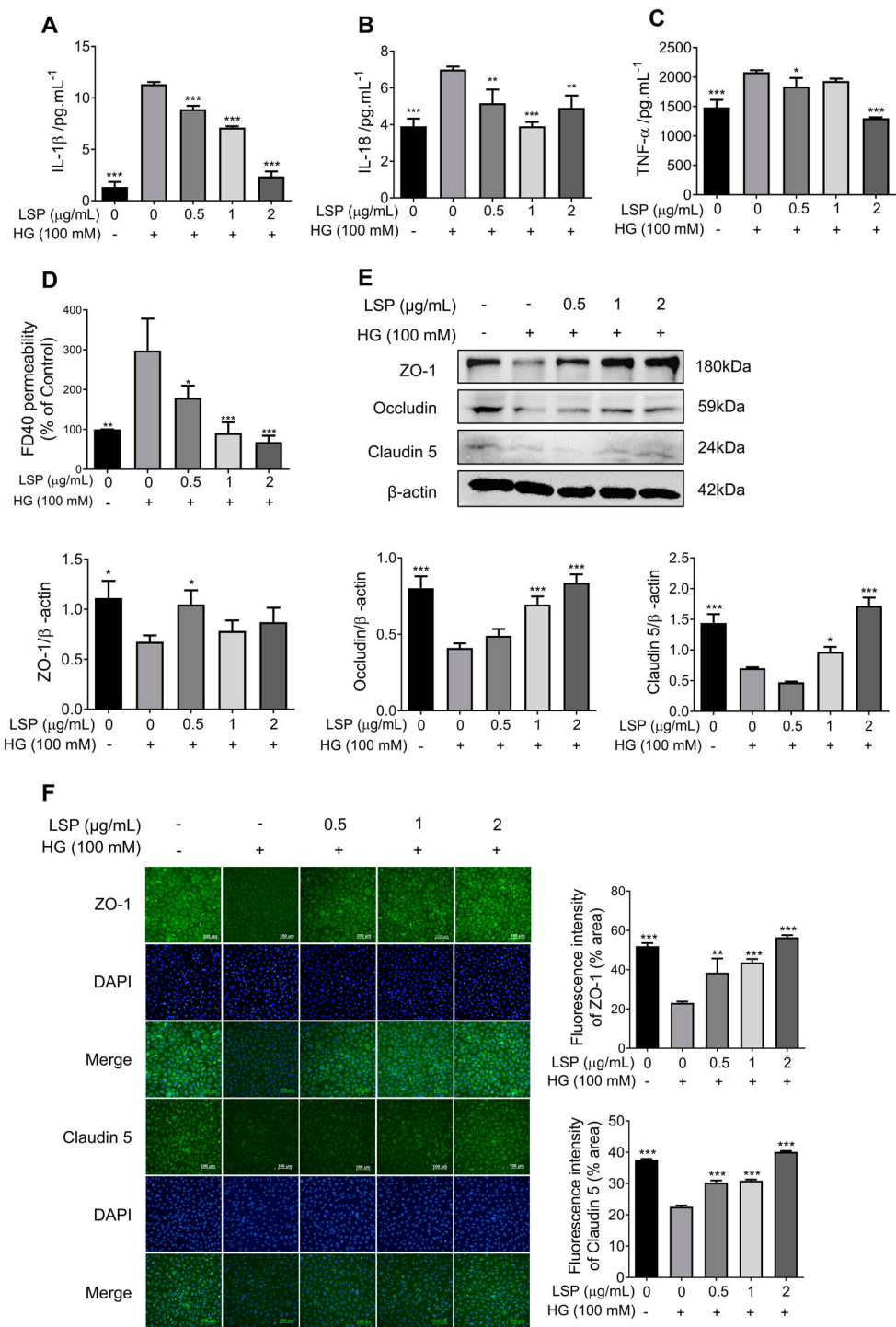
NLRP3, the most commonly studied inflammasome, is reported to regulate the inflammatory responses in DR. Increasing evidence indicates that NLRP3 is activated and participates in the occurrence and development of NPDR. The generation of IL-1β, an important indicator of the activation of NLRP3 inflammasome, was decreased by LSP in HG-induced MIO-M1cells and co-culture system. In this experiment, we further investigated whether LSP inhibited the activation of NLRP3 inflammasome in HG-induced MIO-M1 cells. As shown in Figure 4(A), 100 mM of glucose significantly increased the protein expressions of NLRP3, ASC and the cleaved form of

caspase-1. While LSP remarkably inhibited the activation of NLRP3 inflammasome as revealed by the decreased protein expressions of NLRP3, ASC and caspase-1 (p20). In addition, we transiently transfected MIO-M1 cells with pEGFP-NLRP3, pEGFP-caspase-1 or mCherry-ASC plasmid, then investigated the inhibitive effect of LSP on the fluorescence intensity. Figure 4(B) showed that LSP significantly decreased the GFP and RFP intensity indicating the expressions of NLRP3, caspase-1 and ASC specks. Taken together, LSP inhibits the activation of NLRP3 inflammasome in HG-induced MIO-M1 cells.

LSP inhibits cell pyroptosis in HG-induced MIO-M1 cells

Pyroptosis is a pro-inflammatory form of cell death as demonstrated by the over-activation of NLRP3 inflammasome and secretion of inflammatory cytokines (Sharma & Kanneganti, 2021). In addition, increasing studies are indicating that pyroptosis is closely related to the occurrence and development of metabolic diseases such as DR (Hu et al., 2020). Here, we further investigated whether LSP inhibited HG-induced pyroptosis in MIO-M1 cells by flow cytometry using the Annexin V-FITC/PI apoptotic detection kit and Hoechst/PI staining methods. As shown in Figure 5(A), 100 mM of glucose significantly increased the percentage of cells in PE channel indicating the pyroptotic cells, which was decreased by LSP in a dosed manner. In addition, the Hoechst/PI staining result showed that LSP significantly decreased the ratio of cells with PI signal (Figure 5B). Moreover, we further detected the protein expression of GSDMD, a key protein for the regulation of cell pyroptosis. As shown in Figure 5(C), LSP significantly inhibited the protein expression of the cleaved form of GSDMD (p43). Taken together, LSP inhibits cell pyroptosis and maintains the cell viability of HG-induced MIO-M1 cells.

Figure 3. LSP improves BRB dysfunction in HG-induced co-culture system of hRECs with MIO-M1 cells. hRECs were co-cultured with MIO-M1 cells in the trans-well plate to establish the in vitro BRB model. They were treated with LSP as the indicated concentrations in the presence or absence of HG for 24 h. After treatment, culture medium was collected for centrifugation and the supernatant was used for the detection of the levels of IL-1 β (A), IL-18 (B) and TNF- α (C) using a Human IL-1 β ELISA Kit, a Human IL-18 ELISA Kit, and a Human TNF- α ELISA Kit. The bar chart indicates the levels of IL-1 β , IL-18 and TNF- α in the cell supernatant of the co-culture system.; bars, S.D., * $p \leq 0.01$, ** $p \leq 0.01$, *** $p \leq 0.001$. (D) After treatment, the monolayer permeability of hRECs was measured by detecting the amount of penetrated FD40. The bar chart indicates the relative percentage of FD-40 permeability; bars, S.D., * $p \leq 0.01$, ** $p \leq 0.01$, *** $p \leq 0.001$. (E) In the co-culture system, cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates of hRECs were harvested for the analysis of ZO-1, Occludin and Claudin 5 by Western blot. The bar chart indicates the relative expressions of ZO-1/ β -actin, Occludin / β -actin and Claudin 5/ β -actin; bars, S.D., * $p \leq 0.01$, *** $p \leq 0.001$. The full images of Western blotting were shown in Supplementary Figure S2. (F) In the co-culture system, cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, the expressions of ZO-1 and Claudin 5 in hRECs were detected by the immunofluorescence method. The representative images were captured by a fluorescence microscope (Nikon, Japan). Magnification: $\times 20$, Scale bar: 100 μ m. The bar chart indicates the relative GFP intensity; bars, S.D., * $p \leq 0.01$, *** $p \leq 0.001$.



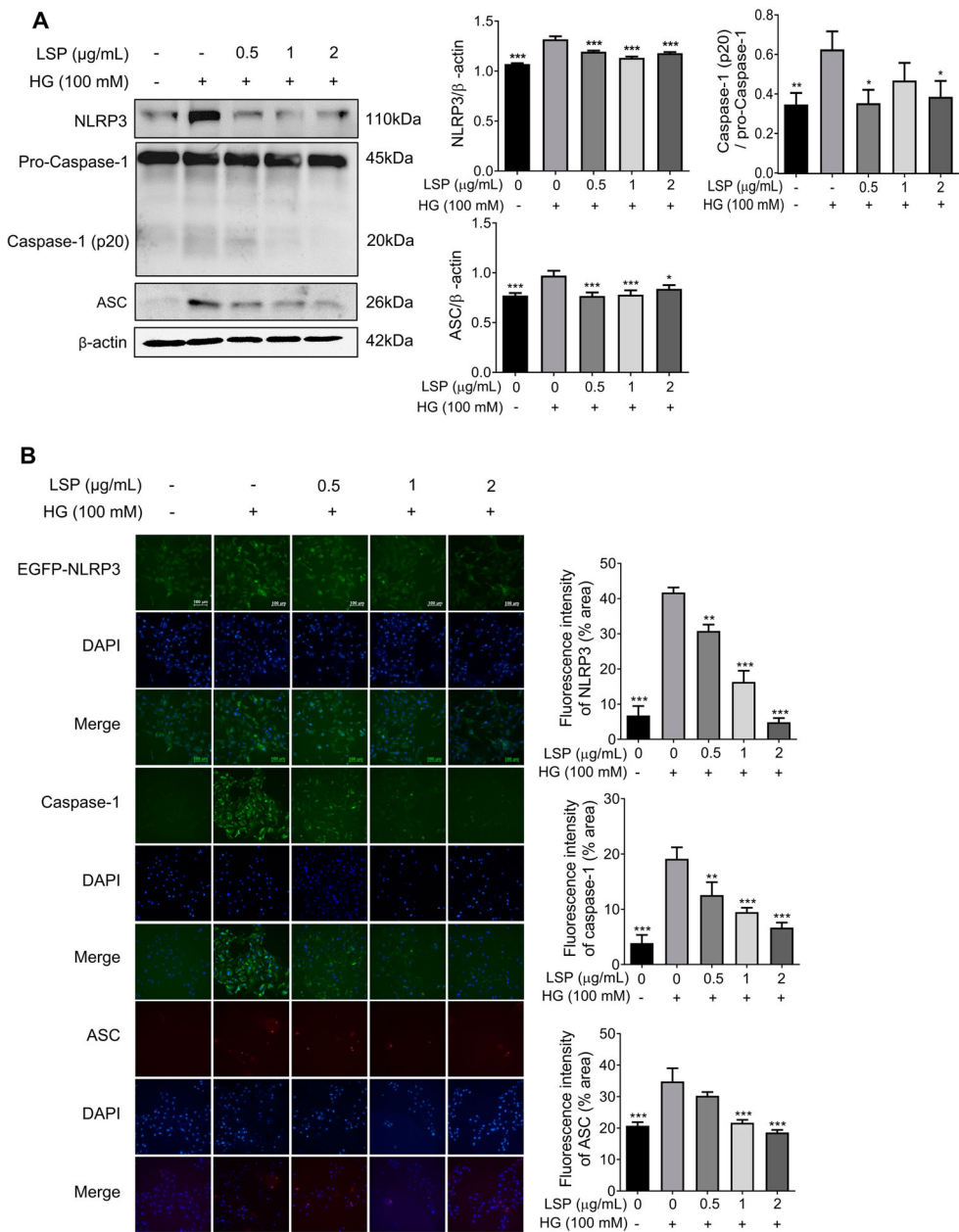


Figure 4. LSP suppresses the activation of NLRP3 inflammasome in HG-induced MIO-M1 cells. MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates were harvested for the protein detections of NLRP3, caspase-1 and ASC by Western blot. The bar chart indicates the relative expressions of NLRP3/β-actin, caspase-1 (p20)/pro-caspase-1, and ASC/β-actin; bars, S.D., ** $p \leq 0.01$, *** $p \leq 0.001$. The full images of Western blotting were shown in Supplementary Figure S2. (B) MIO-M1 cells were transiently transfected with pEGFP-NLRP3, pEGFP-caspase-1, or mCherry-ASC plasmid. After 18–24 h, cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cells were fixed for the capture of the representative images by a fluorescence microscope (Nikon, Japan). Magnification: $\times 20$, Scale bar: 100 μm. The bar chart indicates the percentage of cells with GFP signal or RFP-labelled ASC specks; bars, S.D., ** $p \leq 0.01$, *** $p \leq 0.001$.

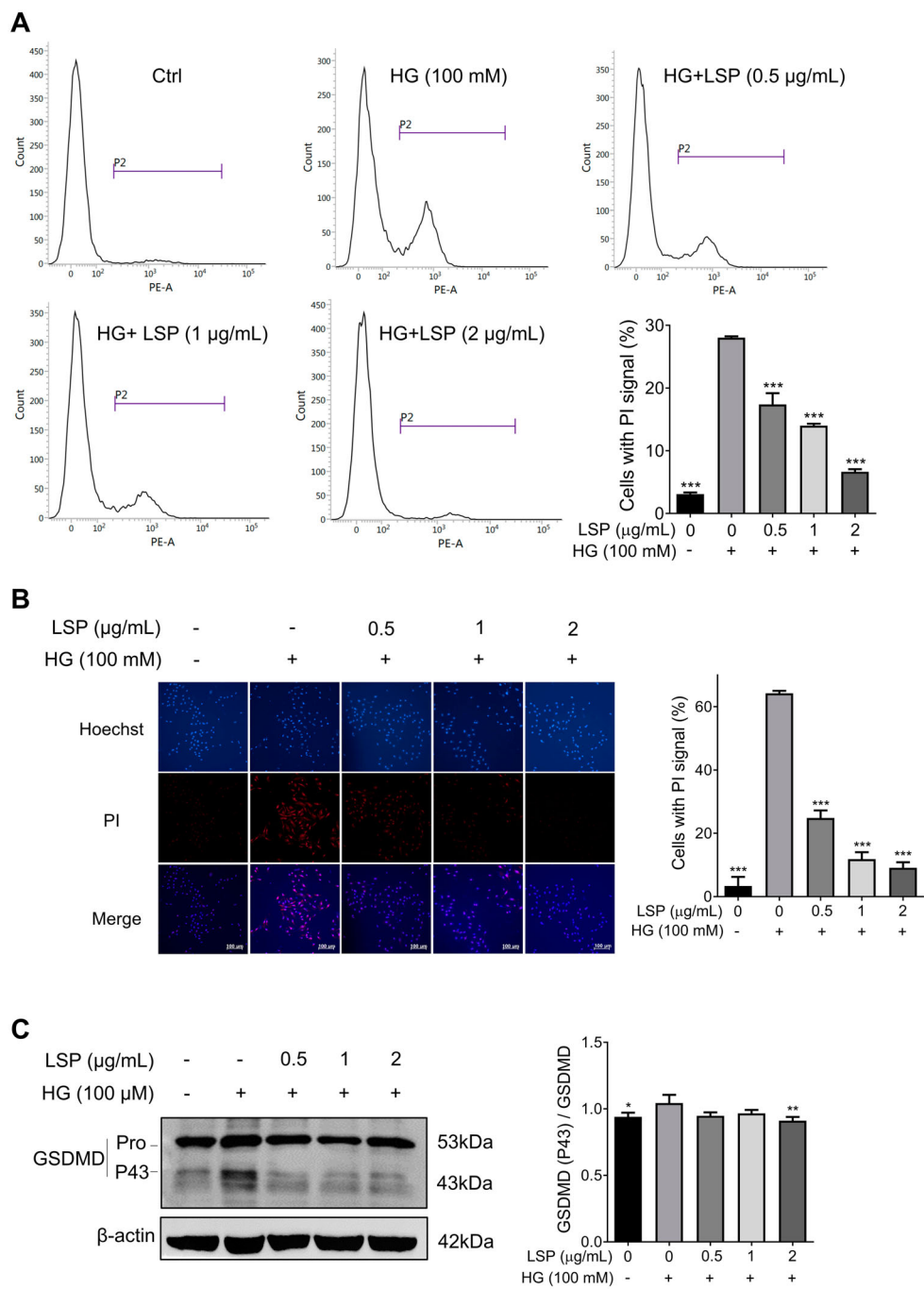
LSP inhibits the TLR4/MYD88/NF- κ B pathway

Emerging evidence indicates that the inflammatory responses are regulated via TLRs or other immune receptor-signalling pathways (Takeda & Akira, 2004). TLR4 is one of the important members of the TLR family, which combines with the molecular patterns to identify the pathogens, then activates the innate immune system of the body (Kim et al., 2016). The MYD88-dependent pathway is the main intracellular signalling pathway in responding to TLR4, which activates the downstream NF- κ B cascade and releases a large number of inflammatory cytokines (Kawai & Akira, 2007). Moreover, NLRP3 inflammasome is reported to be activated via the TLR4/MYD88/NF- κ B pathway. Thus we here investigated the modulation effect of LSP on the TLR4/MYD88/NF- κ B pathway by detecting the protein expressions of TLR4, MYD88, I κ B and NF- κ B. The Western blot results showed that 100 mM of glucose significantly activated the TLR4/MYD88/NF- κ B pathway as evidenced by the increased expressions of TLR4, MYD88, p-I κ B and NF- κ B. While LSP dose-dependently inhibited the activation of TLR4/MYD88/NF- κ B pathway (Figure 6A). In addition, the RT-PCR results showed that LSP significantly decreased the mRNA level of TLR4 in HG-induced MIO-M1 cells (Figure 6B). Collectively, LSP inhibits the NLRP3 inflammasome-mediated inflammatory response via the TLR4/MYD88/NF- κ B pathway.

Discussion

DR has become one of the important complications of diabetes that seriously affects people's life and vision, which is characterized by progressive changes in retinal microvessels. The pathologies of DR mainly include hemodynamic changes, inflammatory responses, activation of the polyol pathway, thickening of capillary basement membrane, activation of DAG-PKC pathway and excessive formation of advanced glycation end products (AGEs). Emerging evidence indicates that the main causes for DR are the oxidative stress, activation of the renin-angiotensin-aldosterone system (RAAS), increased generation of inflammatory cytokines (Brownlee, 2005; Cheung et al., 2010). BRB is an important tissue structure that consists of retinal capillary endothelial cells, pericytes and Müller cells, which plays an important role in maintaining the stability of the retinal

Figure 5. LSP inhibits cell pyroptosis in HG-induced MIO-M1 cells. MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, necrotic cells, pyroptotic cells, apoptotic cells or living cells were analysed by flow cytometry using the Annexin V-FITC/PI Apoptosis detection Kit. FITC⁻/PI⁺ indicates the necrotic cells, FITC⁺/PI⁺ indicates the pyroptotic cells, FITC⁺/PI⁻ indicates the apoptotic cells and FITC⁻/PI⁻ indicates the living cells. The bar chart indicates the percentage of pyroptotic cells; bars, S.D., *** $p \leq 0.001$. (B) MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, the cells were stained with 2 μ g/mL PI and 2 μ g/mL Hoechst 33,342 solutions for 10 min, and then subjected to the observation by fluorescent microscopy. Magnification: 10 $\times$, Scale bar: 100 μ m. The bar chart indicates the percentage of cells with PI signal of cells with blue signal; bars, S.D., *** $p \leq 0.001$. (C) MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates were harvested for the protein detection of GSDMD by Western blot. The bar chart indicates the relative protein expression of GSDMD (p43)/GSDMD; bars, S.D., * $p \leq 0.05$, ** $p \leq 0.01$. The full images of Western blotting were shown in Supplementary Figure S2.



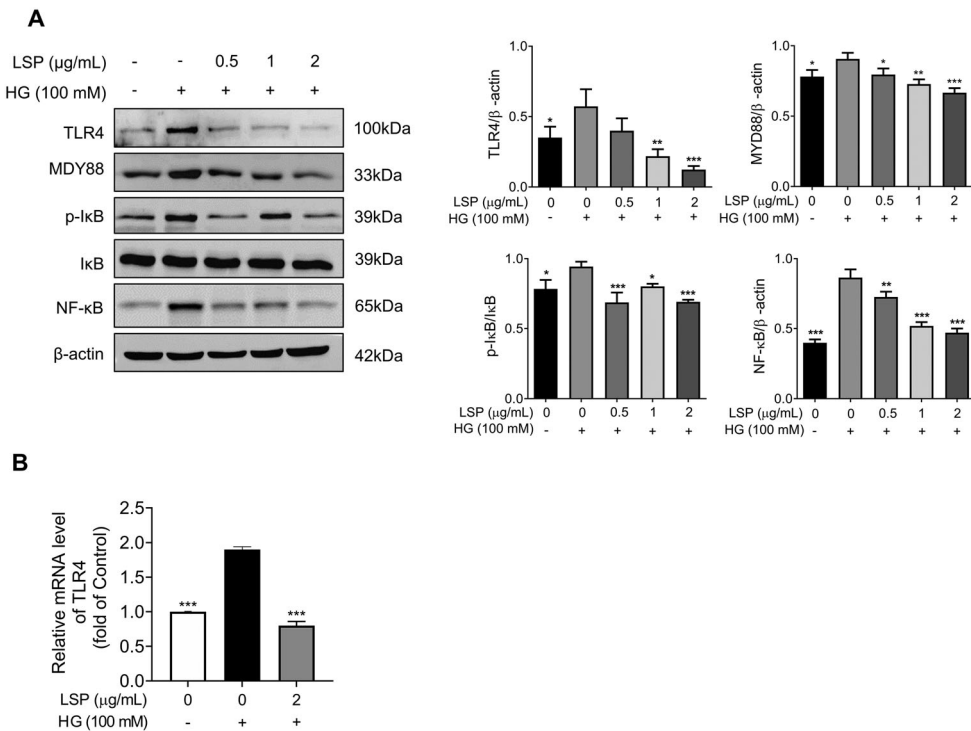


Figure 6. LSP inhibits the TLR4/MYD88/NF-κB pathway. MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, cell lysates were harvested for the protein detections of TLR4, MYD88, p-IκB and NF-κB by Western blot. The bar chart indicates TLR4/β-actin, MYD88/β-actin, p-IκB/IκB and NF-κB/β-actin; bars, S.D., * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The full images of Western blotting were shown in Supplementary Figure S2. (B) MIO-M1 cells were treated with LSP in the presence or absence of 100 mM of glucose for 24 h. After treatment, total mRNA were extracted and subsequently employed for the measurement of TLR4 mRNA level using qRT-PCR. Bar chart indicated the relative mRNA level of TLR4; bars, S.D., *** $p \leq 0.001$.

environment (Rizzolo et al., 2011). Among them, the inflammatory responses in Müller cells result in the change in the morphology of endothelial cells and BRB injury. The damaged BRB with destructed structure and increased permeability is an important pathological hallmark of DR (Raman & Matsubara, 2020). With the development of diabetes, the risk of retinal ischemia and hypoxia increases, which is accompanied by the progressive destroyed BRB structure and function, as well as increased capillary permeability, thickened basement membrane and capillary occlusion. In diabetes, HG is proved to not only induce the injury of hRECs but also the pro-inflammatory responses in MIO-M1 cells, which results in the dysfunction of endothelial cells and BRB injury (Glenn & Stitt, 2009). Therefore, we employed 100 mM of glucose to induce hRECs or the co-culture system of hRECs with MIO-M1 cells as the in vitro model of BRB injury. By measuring the amount of penetrated FD-40 across hRECs, we found that 100 mM of glucose significantly increased the permeability of hRECs. To date, almost over 40 proteins associated with TJs are found, which mainly include transmembrane proteins and cytoplasmic-scaffold proteins (Balda & Matter, 2016; Bazzoni &

Dejana, 2004). Among these TJs, Occludin, Claudin 5 and ZO-1 are considered to be the key proteins for BRB integrity (Díaz-Coránguez et al., 2017). Emerging evidence indicates that TJs restrict the penetration of molecules across the paracellular space, and the movements of lipids and proteins between plasma membrane (Mandel et al., 1993; van Meer & Simons, 1986). Therefore, TJs in hRECs play an important role in the tight control of solute and fluid flux across the endothelium in BRB. In this study, we have detected the expressions of TJs in HG-induced hRECs using Western blot and immunofluorescence methods. The results showed that 100 mM of glucose significantly down-regulated the expressions of TJs, including Claudin 5, Occludin and ZO-1 in hRECs. In our previous study, LSP, the active fraction enriching polyphenol from lychee seed was found to protect against β -amyloid ($A\beta$)-induced BBB damage in the cellular and animal models of AD. Thus we here further investigated whether LSP exerted similar effect that maintains the integrity of monolayer in HG-induced hRECs. As we expected, LSP dose-dependently decreased the amount of penetrated FD40 and increased the protein expressions of TJs.

Inflammation has become a key player in DR by guarding the neuronal and vascular components of retina against the deleterious materials. However, the chronic retinal inflammation is harmful and has been found from early phase to the sight-threatening advanced stage of DR (Rübsam et al., 2018). In addition, the inflammation destroys the BRB and impairs the normal visual function (Mesquida et al., 2019). Numerous in vitro and in vivo studies have shown that the pro-inflammatory cytokines not only increase the adhesion of leukocyte to retinal endothelium but also damage the morphology and function of endothelial cells and increase BRB permeability (Behl et al., 2008; Joussen et al., 2002; Sagara et al., 1994). Müller cells acting as one of important components of BRB are the main immune cells that monitor the microenvironment change of entire retina. There are a growing of studies showing hyperglycemia, retinal ischemia and hypoxia, dyslipidemia and endoplasmic reticulum stress activate Müller cells and ultimately induce the secretion of a large number of pro-inflammatory cytokines, including IL-1 β , IL-18, TNF- α and VEGF. Numerous in vitro and in vivo studies indicate that TNF- α is secreted via the PKC ζ /NF- κ B pathway in Müller cells. Then, TNF- α reduces the expression and alters the distribution of Claudin 5 and ZO-1 in bovine retinal endothelial cells (BRECs) (Aveleira et al., 2010). In addition, HG is reported to increase the production of IL-1 β in retinal Müller cells (Busik et al., 2008; Kowluru & Odenbach, 2004; Liu et al., 2012; Yego et al., 2009), which leads to BRB injury, including leukocyte recruitment, increased permeability, and impairment in the morphology and function of endothelial cells (Claudio et al., 1994; Kern, 2007; Klaassen et al., 2013; Kowluru & Odenbach, 2004; Vincent & Mohr, 2007). In our previous study, LSP was found to inhibit $A\beta$ -induced neuroinflammation in BV-2 cells (Tang et al., 2018; Zhang & Zeng, 2019). Therefore, we here further investigated whether LSP protected BRB via inhibiting the inflammatory responses in MIO-M1 cells. By employing ELISA and Western blot methods, the expressions of IL-1 β , IL-18 and TNF- α in HG-induced MIO-M1 cells or the co-culture system were detected, the results showed that LSP significantly reduced the expression and secretion of pro-inflammatory cytokines.

NOD-like receptors (NLRs) are the well-known receptors that are composed of protein complexes participating in IL-1 β and IL-18 maturations and secretions (Kowluru & Odenbach, 2004). Increasing evidence indicates that the activation of the

NLRP3 inflammasome is found in a variety of diseases, such as atherosclerosis, ischemic stroke, metabolic disease and neurodegenerative diseases (Nakanishi et al., 2018; Yang et al., 2014). When the NLRP3 inflammasome is activated, pro-caspase-1 is cleaved into the active form of caspase-1, following by the cleavages of pro-IL-1 β and pro-IL-18 into mature forms of IL-1 β and IL-18 (Li et al., 2015; Liu et al., 2018). Recent studies have found that NLRP3 inflammasome is closely related to DR, especially the formation of retinal neovascularization in PDR (Chaurasia et al., 2018; Loukovaara et al., 2017). In addition, the NLRP3 inflammasome complex cleaves GSDMD to form GSDMD-N-terminal and GSDMD-C-terminal. GSDMD-N-terminal then binds to phosphatidylinositol phosphate on eukaryotic cells to form pores, which facilitates the secretion of pro-inflammatory cytokines and induces cell pyroptosis (Shi et al., 2015). In this experiment, we detected the activation of NLRP3 inflammasome and cell pyroptosis in HG-induced MIO-M1 cells, and found that LSP significantly suppressed HG-induced activation of NLRP3 inflammasomes and cell pyroptosis in MIO-M1 cells.

TLR4, an important member of the TLR family, combines with specific pathogen-related molecular patterns (PRMPs) to recognize the pathogens and activate the innate immune system. Its signal transduction pathways include MYD88-dependent and independent pathways. In the MYD88-dependent signal pathway, the extracellular signal stimulation activates TLR4 and transduces into intracellular signal through the downstream IL-1R associated kinases, then transform growth factor- β activated kinase 1 (TAK1) of the MYD88 molecule to cause phosphorylation and degradation of NF- κ B protein. In addition, MYD88 induces the transduction of NF- κ B from cytoplasm to nucleus, and initiates gene transcription and protein synthesis of various inflammatory cytokines to regulate the inflammatory responses (Wang et al., 2015). Emerging studies have shown that the TLR4/MYD88/NF- κ B signalling pathway is one of the important pathways that regulate inflammatory response, and the activation of this pathway leads to the release of pro-inflammatory factors, including TNF- α , IL-1 β , IL-6, IL-10 and IL-12 (Dolinay et al., 2012; Liu, Zhao, et al., 2020; Montravers et al., 1995). Therefore, the downregulation of TLR4/MYD88/NF- κ B signal inhibits the inflammatory responses (Liu et al., 2012; Peng et al., 2015; Wang et al., 2016). In this study, we investigated whether LSP inhibited HG-induced activation of TLR4/MYD88/NF- κ B pathway. The results showed that HG significantly increased the expression of TLR4 and MYD88 and the phosphorylation of I κ B and NF- κ B in MIO-M1 cells, while LSP dose-dependently downregulated the TLR4/MYD88/NF- κ B signalling pathway. There is a growing body of studies showing that the NLRP3 inflammasome, the most extensively studied inflammasome, is activated by the TLR4/MYD88/NF- κ B pathway in various cellular and animal models of diseases (Chen et al., 2018; Tsutsui et al., 2010; Wu et al., 2021). In addition, TAK-242 and TLR4-IN-C34, the inhibitors of TLR4 are reported to inhibit the expression of NLRP3 inflammasome and inflammatory response via the TLR4/MYD88/NF- κ B pathway (Liu, Wu, et al., 2020; Su et al., 2018). Therefore, LSP used in the present study may show similar effect as TLR4-IN-C34 to inhibit the TLR4/MYD88/NF- κ B pathway, which then contributes to the inhibition of HG-induced activation of NLRP3 inflammasome.

In summary, the current study here demonstrates that LSP protects BRB integrity and maintains its normal function via increasing TJs expressions in HG-induced hRECs and inhibiting the TLR4/I κ B/NF- κ B-mediated activation of NLRP3 inflammasome in HG-

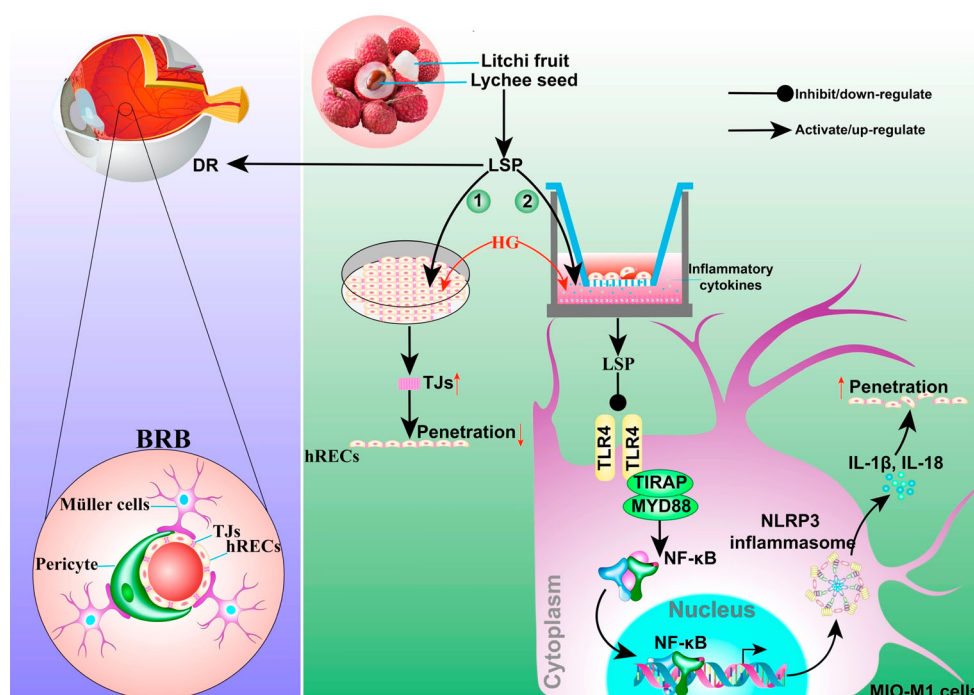


Figure 7. A schematic diagram of this study. Left panel: Eye of patients with DR and the magnification of BRB. Right panel: The molecular mechanism by which LSP protects blood–retinal barrier via increasing tight joint proteins (1) and inhibiting TLR4/I κ B/NF- κ B-mediated NLRP3 inflammasome activation (2).

induced co-culture system of hRECs with MIO-M1 cells. Therefore, the findings in this study provide evidence for the use of LSP in the prevention and treatment of DR in the future (Figure 7).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available upon reasonable request.

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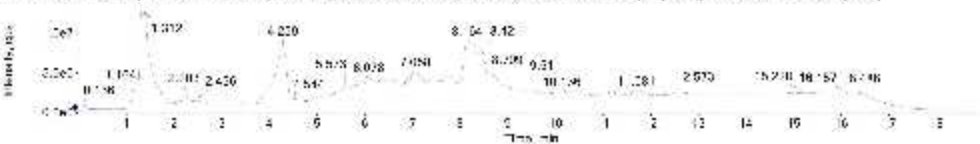
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0.1 mm L2-13112 example 21 - L2H, 1 CH, 35, 100 - 150°C, 240000 rpm (100 - 150 rpm), 1.0 sec (4), 50 - 60 sec (40 sec) (10 sec).



0.1 mm L2-13112 example 21 - L2H, 1 CH, 35, 100 - 150°C, 240000 rpm (100 - 150 rpm), 1.0 sec (4), 50 - 60 sec (40 sec) (10 sec).

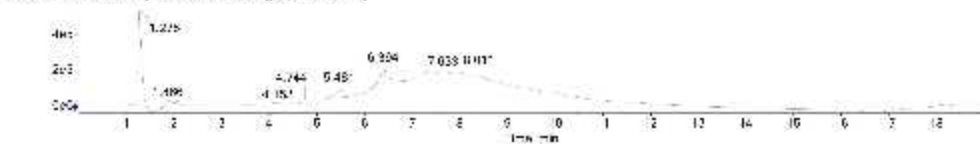


Fig.1D

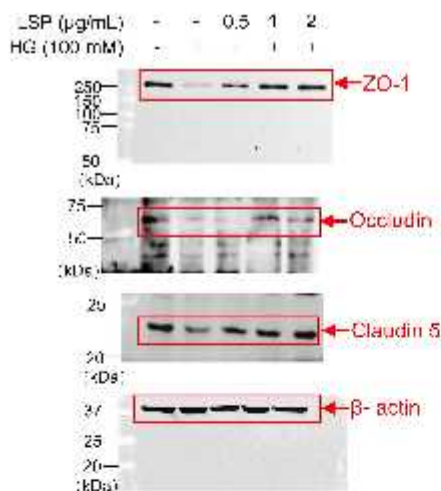


Fig.4A

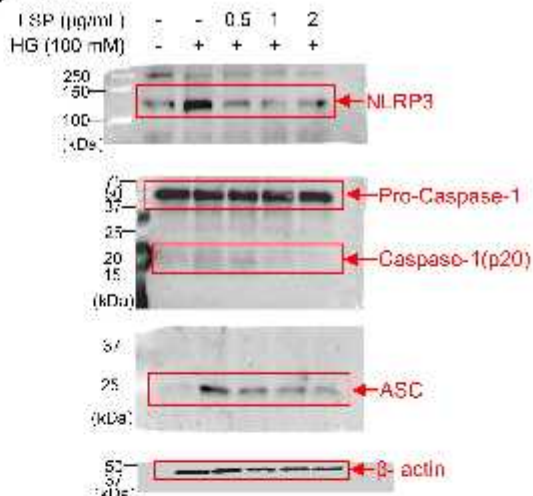


Fig.2D

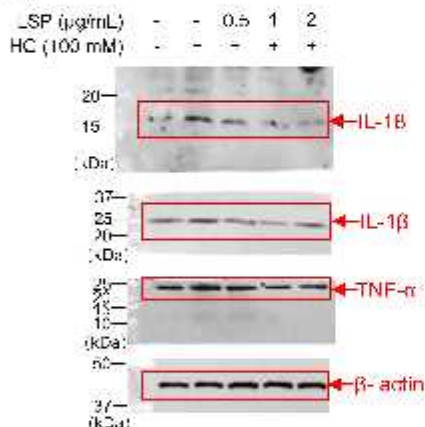


Fig.5C

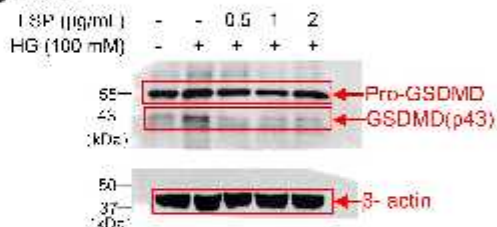


Fig.6A

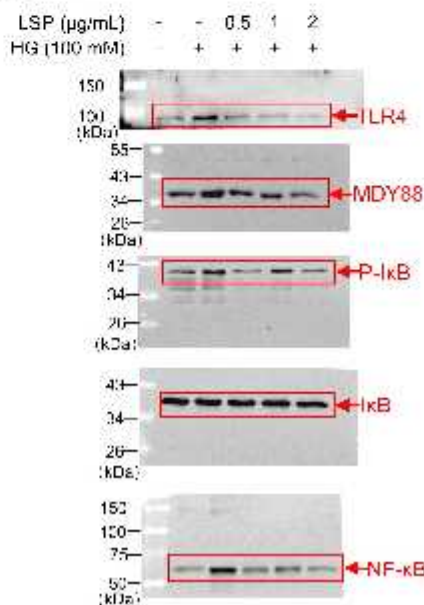
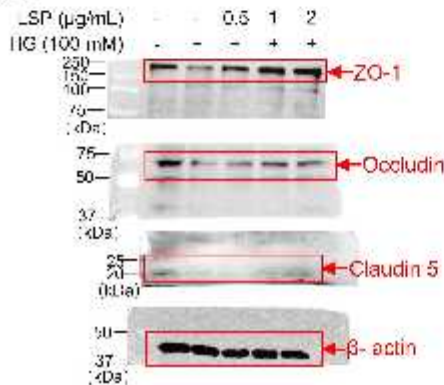


Fig.3E

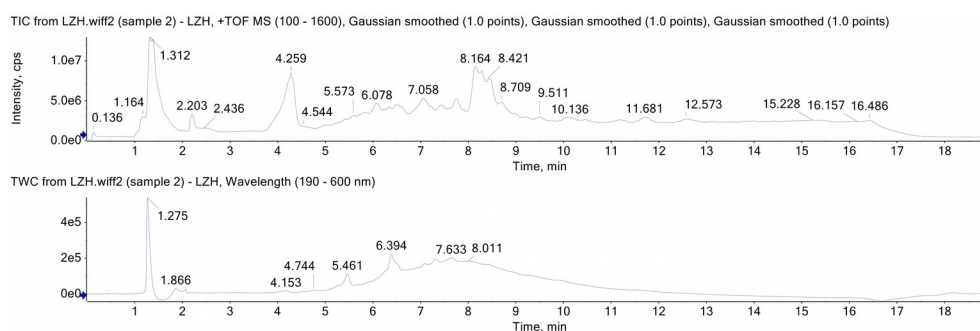


Supplementary Information

**Lychee seed polyphenol protects blood-retinal barrier by
increasing tight joint proteins and inhibiting the activation of
TLR4/MYD88/NF- κ B-mediated NLRP3 inflammasome**

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Supplementary Figures



Supplementary Figure 1. The quality control of LSP was monitored by UHPLC-DAD-TOF-MS. Upper: The total ion chromatogram (TIC) of LSP; Below: The diode-array detection (DAD) chromatogram of LSP. The analysis was performed by an ultra-high performance liquid chromatography system equipped with a DAD and a SCIEX X500R QTOF detection System. The samples were separated on a C18 column (Kinetex, 100 mm×2.1mm, particle size: 2.6 μ m) with a mobile phase composed of methanol-formic acid (99.9:0.1, v/v) and water-formic acid (99.9:0.1, v/v) as the mobile phase A and mobile phase B, respectively. The delivered rate of mobile phase was 0.3 mL/min with the injection volume of 5 μ L. For the gradient separation, the process was set as follows: 0-15 min, 95%-50% (B); 15-19 min, 95% (B).

Fig.1D

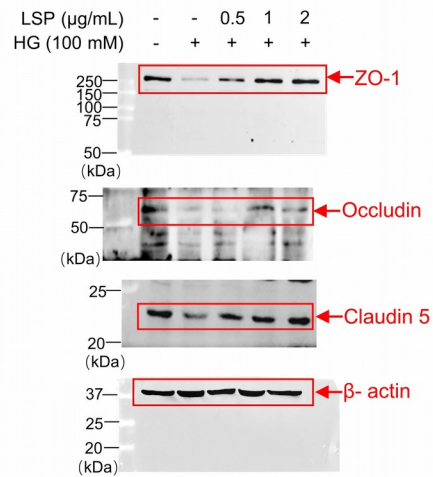


Fig.4A

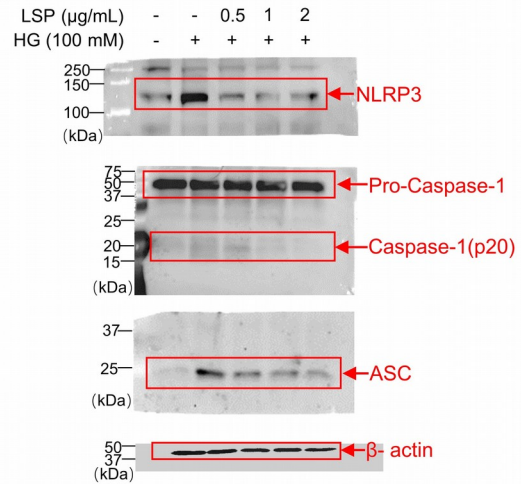


Fig.2D

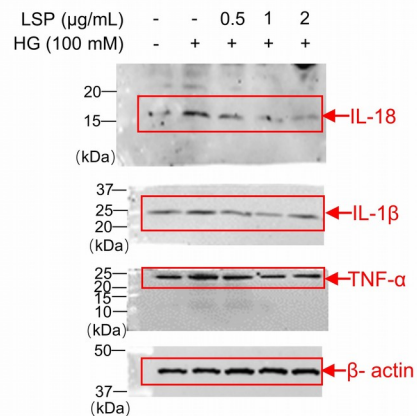


Fig.5C

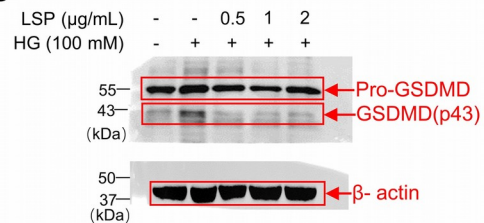


Fig.6A

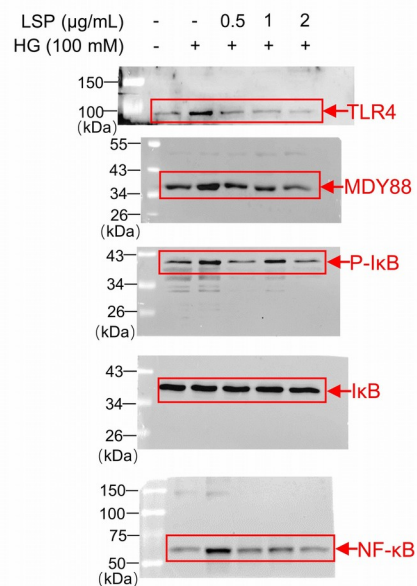
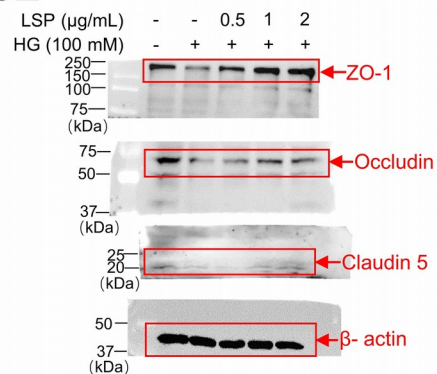


Fig.3E

**Supplementary Figure 2. Full images of Western blotting**

All blots derive from the same experiment and that they were processed in parallel