

Enhanced Expression of NLRP3 Inflammasome-Related Inflammation in Diabetic Retinopathy

Hui Chen, Xiongze Zhang, Nanying Liao, Lan Mi, Yuting Peng, Bing Liu, Shaochong Zhang, and Feng Wen

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China

Correspondence: Feng Wen, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 South Xianlie Road, Guangzhou 510060, China; wenfeng208@foxmail.com.

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PURPOSE. The aim of this study was to determine the association between nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) family, pyrin domain-containing 3 (NLRP3) inflammasome-induced inflammation and disease severity in diabetic retinopathy (DR).

METHODS. Blood samples were collected from 64 patients with diabetes (DR, 43; without DR, 21) and 25 healthy controls. The protein and mRNA expression levels of NLRP3 inflammasomes in peripheral blood mononuclear cells were determined using western blotting and quantitative real-time reverse transcription-PCR. A total of 82 vitreous samples were obtained from patients with DR ($n = 60$) and nondiabetic controls ($n = 22$). All patients were candidates for vitrectomy. Interleukin (IL)-1 β and IL-18 in the peripheral blood mononuclear cell culture medium and vitreous fluid were detected by enzyme-linked immunosorbent assay (ELISA). Immunofluorescence staining for apoptosis-associated speck-like protein with a caspase recruitment domain (ASC) and NLRP3 was performed in fibrovascular membranes from 21 proliferative DR patients and 22 controls with idiopathic epiretinal membranes.

RESULTS. We observed increased gene and protein expression of NLRP3, ASC, and caspase-1 in peripheral blood mononuclear cells of adults with DR compared with that in normal controls. Furthermore, the elevated expressions of NLRP3 and ASC were observed in the fibrovascular membranes from 21 adults with proliferative DR when compared with the 22 controls. IL-1 β and IL-18 in the peripheral blood mononuclear cells and vitreous fluid were elevated in the DR patients when compared with controls.

CONCLUSIONS. These outcomes suggested that NLRP3 inflammasomes are upregulated in adults with DR and may play a key role in the pathogenesis and progression of DR.

Keywords: NLRP3 inflammasomes, diabetic retinopathy, peripheral blood mononuclear cell, vitreous fluid

Diabetic retinopathy (DR), a frequent microvascular problem associated with diabetes mellitus, is the main cause of blindness worldwide.^{1,2} Timely interference for those at high risk of sight-threatening problems linked to DR, including diabetic macular edema and proliferative DR (PDR), is critical to preventing vision loss; however, the molecular mechanisms underlying DR pathogenesis are poorly understood.³

Innate immunity and the dysregulation of inflammatory processes are currently thought to be important in the induction and advancement of DR,⁴⁻⁶ but the mechanism by which chronic hyperglycemia induces an immune response and DR is unclear. Recent studies have examined the roles of inflammasomes in different systemic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus.⁷ In addition, inflammasomes appear to be associated with the pathogenesis of type 2 diabetes mellitus (T2DM).⁸⁻¹¹

Inflammasomes are part of a large family of multiprotein complexes stimulated by pathogen-linked or damage-linked molecular patterns, including reactive oxygen species (ROS), cholesterol crystals, and high glucose levels. In the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasomes are the most widely examined inflammasomes. NLRP3

inflammasomes are composed of the sensor protein NLRP3, caspase recruitment domain (CARD)-containing adaptor protein (ASC), and caspase-1.^{12,13} The stimulation of NLRP3 inflammasomes causes accumulation of the multiprotein complex, which cleaves and stimulates caspase-1, resulting in cleavage of prointerleukin-1 β (pro-IL-1 β) and pro-IL-18 into the active types.¹⁴ NLRP3 inflammasomes have been suggested to be associated with insulin resistance, circulating immune markers, macrophage function, immunogenetic susceptibility, and chronic inflammation.¹⁵ Genetic variations in NLRP3 and altered inflammasome production have been linked to various inflammatory diseases, including obesity, insulin resistance, and T2DM.^{8,16,17} Elevated mRNA and the protein expression of NLRP3, ASC, and proinflammatory cytokines have been used as controls in previous studies.⁸ In addition, a recent study revealed that thioredoxin-interacting protein (TXNIP) is substantially induced in diabetic rat retina in vivo and regulates IL-1 β expression by stimulating NLRP3 inflammasomes.¹⁸ Therefore, NLRP3 inflammasomes play a pivotal role in the pathogenesis of DR, but additional studies are required to determine the role of NLRP3 inflammasomes in DR.



TABLE 1. Clinical and Biochemical Characteristics of Type 2 Diabetic Patients and Healthy Controls

Parameter	Control, <i>n</i> = 25	NDR, <i>n</i> = 21	NPDR, <i>n</i> = 20	PDR, <i>n</i> = 23	<i>P</i> Value
Sex, m/f	15/10	12/9	9/11	12/11	0.770
Age, y	61.8 ± 11.5	57.2 ± 7.4	63.5 ± 6.2	58.7 ± 5.6	0.057
BMI, kg/m ²	21.8 ± 2.0	22.0 ± 2.9	23.8 ± 3.3	24.6 ± 4.9	0.018*
Diabetes duration, y	-	10.1 ± 4.9	12.0 ± 2.5	15.2 ± 1.4	<0.001*
FPG, mmol/l	-	8.0 ± 2.2	8.5 ± 1.7	10.2 ± 1.2	<0.001*
HbA1c, %	-	10.1 ± 4.9	12.0 ± 2.5	15.3 ± 1.1	<0.001*

Data are expressed as mean ± SD. BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin.

* *P* ≤ 0.05.

We evaluated the expression of NLRP3 inflammasomes in peripheral blood mononuclear cells (PBMCs) obtained from DR patients and biopsies from PDR patients. We also examined whether the NLRP3 expression and NLRP3-mediated yield of IL-1β and IL-18 by PBMCs and vitreous fluid correspond to elevated disease severity.

MATERIALS AND METHODS

Participants

Consecutive patients with T2DM and nondiabetic controls from the outpatient clinics at the Zhongshan Ophthalmic Centre, China, were recruited for this study between January 2017 and July 2017 (Table 1). The 2002 American Diabetes Association standards were used to confirm the diagnosis of T2DM.¹⁹

We excluded patients with infectious diseases or other diabetic complications such as nephropathy (defined as patients with stage 3 chronic kidney disease, macroalbuminuria, or proteinuria, and those undergoing hemodialysis). Chronic kidney disease stages were based on the National Kidney Foundation Disease Outcomes Quality Initiative clinical practice guidelines. Patients were also excluded if they had been subjected to intraocular procedures, intravitreal treatments, photocoagulation in the prior 3 months, uveitis, trauma, vitreous hemorrhage, retinal detachment, or immunosuppressive drug administration.

DR was assessed by fluorescein fundus angiography (FF450 fundus camera; Carl Zeiss Meditec AG, Jena, Germany). Body mass index was determined using the following formula: weight (kg)/height (m²). Based on the Diabetic Retinopathy Disease Severity Scale, diabetics were placed into one of the following three groups: no apparent retinopathy (NDR), nonproliferative diabetic retinopathy (NPDR), and PDR.²⁰

The study followed the guidelines of the Declaration of Helsinki, and all experimental protocols were performed with approval from the Human Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University. Written informed consent was obtained from the enrolled participants.

Patient Demographics

Age- and sex-paired specimens obtained from 64 T2DM patients and 25 healthy controls were used in this investigation. The mean age of the T2DM patients (33 men and 31 women) and controls (15 men and 10 women) was 59.7 ± 6.8 years and 61.8 ± 11.5 years, respectively. Among the 64 T2DM patients, 21 had NDR, 20 had NPDR, and 23 had PDR. The

male/female ratio and mean ± SD age was 12/9 and 57.2 ± 7.4 years for the NDR group, 9/11 and 63.5 ± 6.2 years for the NPDR group, and 12/11 and 58.7 ± 5.6 years for the PDR group.

Sample Preparation

A whole blood specimen (12 mL) was obtained from each participant and placed in a sterile tube containing the anticoagulant lithium heparin (Vacutainer; BD Biosciences, San Jose, CA, USA) prior to protein and mRNA quantification. Additional blood samples were also taken for fasting plasma glucose and glycated hemoglobin determination.

PBMC Isolation

PBMCs were isolated from heparinized blood by Ficoll-Hypaque density-gradient centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). PBMCs were incubated for 4 hours with 100 ng/mL lipopolysaccharide (Sigma-Aldrich Corp., St. Louis, MO, USA). After 4 hours, PBMCs were incubated for an additional 15 minutes with Roswell Park Memorial Institute (RPMI) containing 1 mM of adenosine 5-triphosphate (ATP; Sigma-Aldrich Corp. lipopolysaccharide/ATP).

Vitreous Fluid Sample Collection

Undiluted vitreous fluid samples were carefully obtained from 60 eyes of 60 diabetes patients (PDR, *n* = 21; NPDR, *n* = 20; NDR, *n* = 19) during pars plana vitrectomy from 2015 to 2017. The indications for vitrectomy were rhegmatogenous and tractional retinal detachment, nonclearing vitreous hemorrhage, epiretinal membrane peel, macular hole repair, or retained lens fragments. Vitreous fluid samples were also obtained from 22 participants with epiretinal membranes without diabetic mellitus who served as a control group. The clinical characteristics of the participants are shown in Table 2.

Samples of vitreous fluid were collected by manual suction into a syringe through the aspiration line of vitrectomy before opening the infusion line. The samples were transferred to sterile polypropylene screw cap conical bottom vials and rapidly frozen at -80°C.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from PBMCs was removed using TRIzol (Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed with a reverse transcription kit (Toyobo, Osaka, Japan) to a total volume of 20 μL. The expression of specific genes was determined by

TABLE 2. Clinical and Biochemical Characteristics of Patients With DR and Controls Undergoing Vitrectomy

Parameter	Control, n = 22	NDR, n = 19	NPDR, n = 20	PDR, n = 21	P Value
Sex, m/f	13/9	7/12	9/11	12/9	0.445
Age, y	60.7 ± 7.3	58.5 ± 8.3	62.8 ± 7.5	60.6 ± 5.5	0.336
BMI, kg/m ²	20.9 ± 2.2	21.8 ± 1.9	23.1 ± 3.4	23.9 ± 3.4	0.001*
Diabetes duration, y	-	7.8 ± 3.9	13.2 ± 4.7	15.2 ± 3.1	-
FPG, mg/dl	4.5 ± 0.3	7.7 ± 1.6	8.0 ± 1.7	10.1 ± 0.5	<0.001*
HbA1c, %	5.3 ± 0.7	7.8 ± 1.4	8.0 ± 0.7	12.8 ± 3.6	<0.001*

Data are expressed as mean ± SD.

* $P \leq 0.05$.

quantitative real-time PCR with a QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany). The primers for the investigated genes were as follows: NLRP3 forward 5'-GCA GCA AAC TGG AAA GGA AG-3' and reverse 5'-CTT CTC TGA TGA GGC CCA AG-3'; ASC forward 5'-GCACCTT TAT AGA CCA GCA CCG-3' and reverse 5'-GGC TGG TGT GAA ACTGAA GA-3'; caspase-1 forward 5'-CCG AAG GTG ATC ATC ATCC A-3' and reverse 5'-ATA GCA TCA TCC TCA AAC TCT TCT G-3'; and IL-1 β forward 5'-TTA CAG TGG CAA TGA GGA TGA C-3' and reverse 5'-GTC GGA GATTCG TAG CTG GAT-3'. Quantitative real-time PCR was conducted with a LightCycler CFX96 (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. β -actin was used as an internal control. Each specimen was examined in triplicate. PCR products were separated in an agarose gel and showed a single band of the expected size in all cases. Melting curve analysis was conducted to confirm primer specificity. Relative mRNA expression was measured using the comparative ($2^{-\Delta\Delta CT}$) method.

Western Blotting

Total protein was removed from the PBMCs of T2DM patients and controls and lysed in radioimmunoprecipitation buffer. A total of 60 μ g of cell lysate were fractionated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes by semidry electroblotting. The blots were probed with specific antibodies against NLRP3, ASC, and caspase-1 (Abcam, Cambridge, UK). β -actin expression was examined as an internal control with an anti- β -actin antibody (Abcam). Immunoreactive bands were measured using radiographic film and a SuperSignal West Pico Substrate Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Band intensity analysis was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to β -actin.

ELISA

IL-1 β and IL-18 levels in the culture supernatants and vitreous fluid were measured by ELISA following the manufacturer's instructions (IL-1 β and IL-18 ELISA kits; R&D Systems, Minneapolis, MN, USA). The minimal detectable concentrations of IL-1 β and IL-18 were 3.9 and 26.6 pg/mL, respectively. All samples were measured in duplicate.

Immunofluorescence Staining of Fibrovascular Membranes (FVMs)

During pars plana vitrectomy, FVMs were surgically removed via membrane peeling from the eyes of T2DM patients with

PDR (21 eyes). As controls, epiretinal membrane resection was performed in 22 patients with idiopathic epiretinal membranes. The variation in ages among the groups was not significant (Table 2).

In the laboratory, preretinal membranes were snap frozen within 1 hour of removal in an optimal cutting temperature compound and kept at -70°C . The 8- μ m sections were cut and stained by immunofluorescence staining.

Immunofluorescence staining was performed on frozen sections of the FVMs and control membranes by staining with rabbit anti-NLRP3 polyclonal IgG (1:300 dilution; ab214185; Abcam) or rabbit anti-ASC receptor polyclonal IgG (1:200 dilution; ab180799; Abcam). The samples were counterstained with DAPI (1:1000 dilution; D9542; Sigma-Aldrich). The sections were examined with a fluorescence microscope (DS-Ril-U2; Nikon, Tokyo, Japan) and photographed (DS-U2; Nikon).

Statistical Analysis

All evaluations were conducted with the Statistical Package for the Social Sciences Statistical Software for Windows (version 19.0, SPSS, Inc., Chicago, IL, USA). Group variations between diabetes patients and controls were evaluated by one-way analysis of variance or nonparametric Kruskal-Wallis tests according to normality assumptions and homogeneity of variances. The variations between all groups were examined by Mann-Whitney U tests or Student's t -tests. The relationships between study parameters were examined via Spearman's correlation test. Graphs were drawn using Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). For each test, P values < 0.05 were considered statistically significant.

RESULTS

Patient Clinical Characteristics

A summary of clinical manifestations and laboratory measurements of the studied patients is shown in Table 1. There was no significant variation among groups in age and sex ($P = 0.770$, $P = 0.057$, respectively). Body mass index distribution was significantly higher in patients with T2DM than in the healthy controls ($P < 0.001$). The mean extent of diabetes was significantly longer in the PDR group than in the NPDR and NDR groups ($P < 0.001$). HbA1c values ranging from 4.27% to 6.07% were considered normal. HbA1c and fasting glucose levels were also found to be significantly elevated in the PDR group than in the NPDR and NDR groups (both $P < 0.001$).

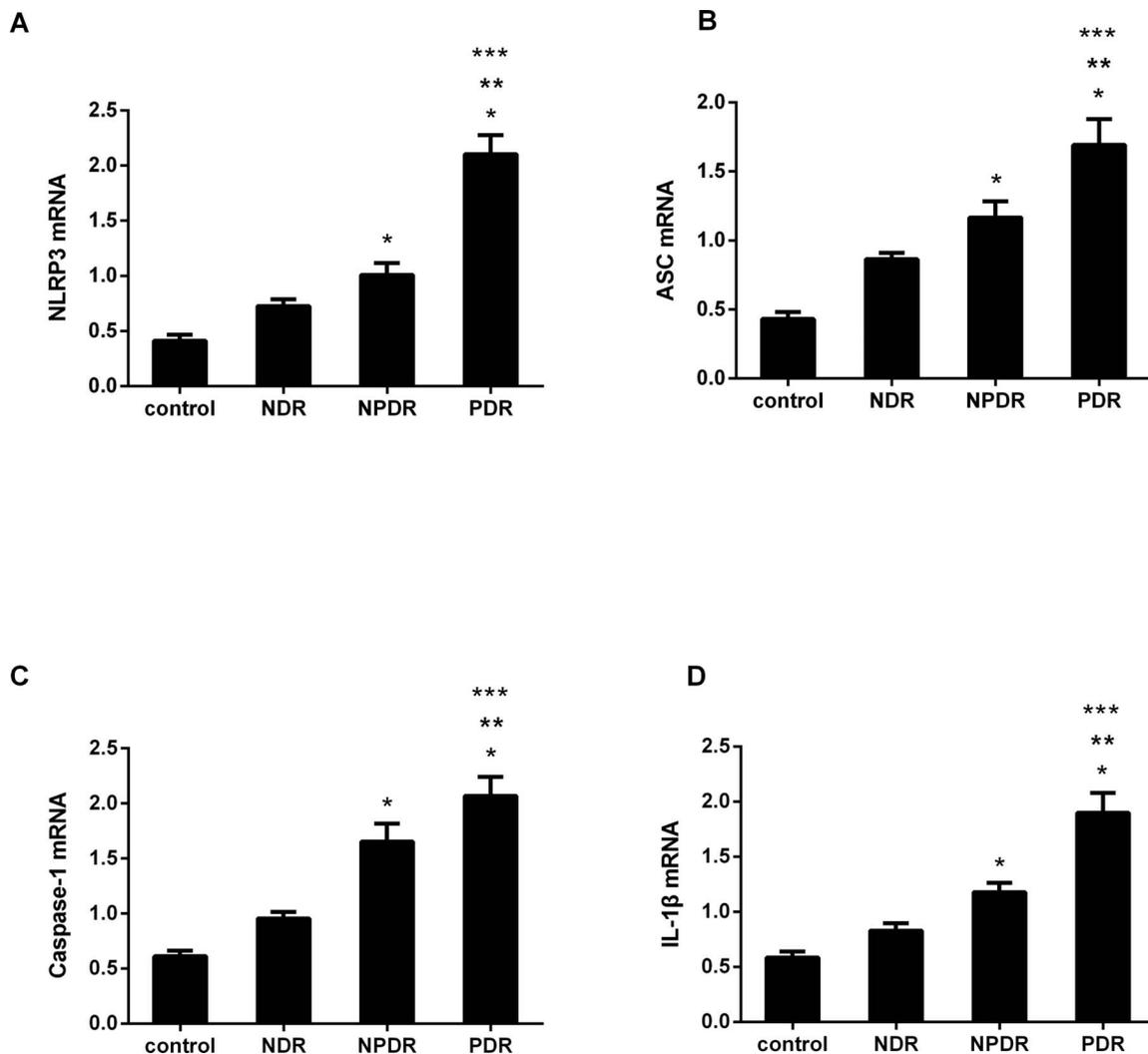


FIGURE 1. NLRP3, ASC, caspase-1, and IL-1 β mRNA expression is elevated in DR patients (PDR, $n = 23$; NPDR, $n = 20$; NDR, $n = 21$; control, $n = 25$). (A–D) The mRNA expression of NLRP3, ASC, caspase-1, and IL-1 β in freshly isolated PBMCs was quantified by real-time PCR and normalized to the expression levels of β -actin. The values are shown as the fold-change when compared with the control. * $P < 0.05$ versus control; ** $P < 0.05$ versus NDR; *** $P < 0.05$ versus NPDR.

Expression of NLRP3 Inflammasomes Was Significantly Upregulated in Patients With DR

We evaluated NLRP3, ASC, caspase-1, and IL-1 β levels in T2DM patients and controls by using quantitative real-time PCR. NLRP3 mRNA expression in PBMCs from NPDR and PDR patients was significantly elevated when compared with controls ($P = 0.001$ and $P < 0.001$, respectively; Fig. 1A). In addition, the expression of the key NLRP3 inflammasome molecules ASC and caspase-1 and IL-1 β mRNA expression levels were also significantly increased in patients with NPDR (all $P < 0.001$) and PDR (all $P < 0.001$) when compared with healthy controls (Figs. 1B–D).

To verify the upregulation of NLRP3 inflammasomes in patients with DR at the protein level, total protein was extracted from PBMCs from untreated patients and controls and was analyzed by western blotting. Our data showed that the protein expression of NLRP3, ASC, and caspase-1 was significantly elevated in participants with NPDR ($P < 0.001$, $P < 0.001$, and $P = 0.001$) and PDR (all $P < 0.001$) when

compared with controls (Fig. 2). Representative western blotting results from 6 patients and 2 controls are shown in Figure 2A. ELISA revealed that PBMC IL-1 β and IL-18 levels in patients with NPDR ($P < 0.001$ and $P = 0.010$) and PDR (both $P < 0.001$) were significantly increased when compared with those in healthy controls (Fig. 3). Our data revealed that the mRNA and protein levels of NLRP3 inflammasomes were significantly increased in patients with DR.

Concentrations of IL-1 β and IL-18 in Vitreous Fluid

IL-1 β and IL-18 secretions are tightly regulated by NLRP3 inflammasome activation; therefore, we compared the vitreous fluid levels of IL-1 β and IL-18 in the DR patients. A significantly higher expression of IL-1 β and IL-18 was observed in the patients with PDR ($n = 21$) than in the patients with NPDR ($n = 20$; both $P < 0.001$) and NDR ($n = 19$; both $P < 0.001$; Fig. 4).

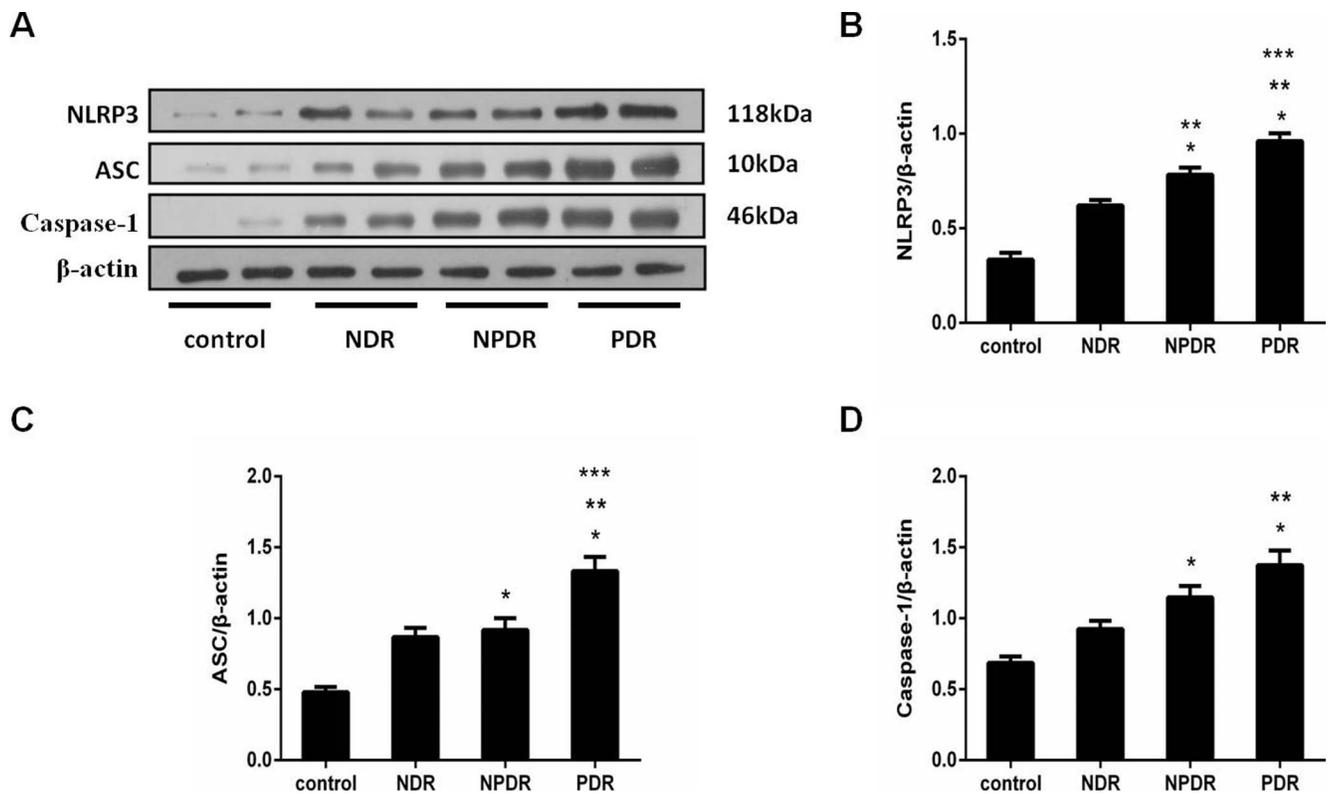


FIGURE 2. NLRP3, ASC, and caspase-1 protein expression is elevated in DR patients (PDR, $n = 23$; NPDR, $n = 20$; NDR, $n = 21$; control, $n = 25$). (A–D) Typical western blot analysis (lanes 1–2, healthy control; lanes 3–4, NDR; lanes 5–6, NPDR; and lanes 7–8, PDR) and quantitation of NLRP3, ASC, and caspase-1 from PBMCs. β -actin was implemented as the loading control. * $P < 0.05$ versus control; ** $P < 0.05$ versus NDR; *** $P < 0.05$ versus NPDR.

NLRP3 Expression in Epiretinal Membranes of DR Patients

The expression of NLRP3 and ASC was detected in samples from all FVMs of the study group with PDR with strong staining (Fig. 5). None of the membranes removed from the eyes of the controls showed specific staining of NLRP3 and ASC.

DISCUSSION

The initiation of NLRP3 inflammasomes promotes the pathogenesis of various inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and inflammatory bowel disease.^{21–23} In addition, previous studies showed that the NLRP3 inflammasome complex and cytokines IL-1 β and IL-18 play essential roles in the development of T2DM and its major complica-

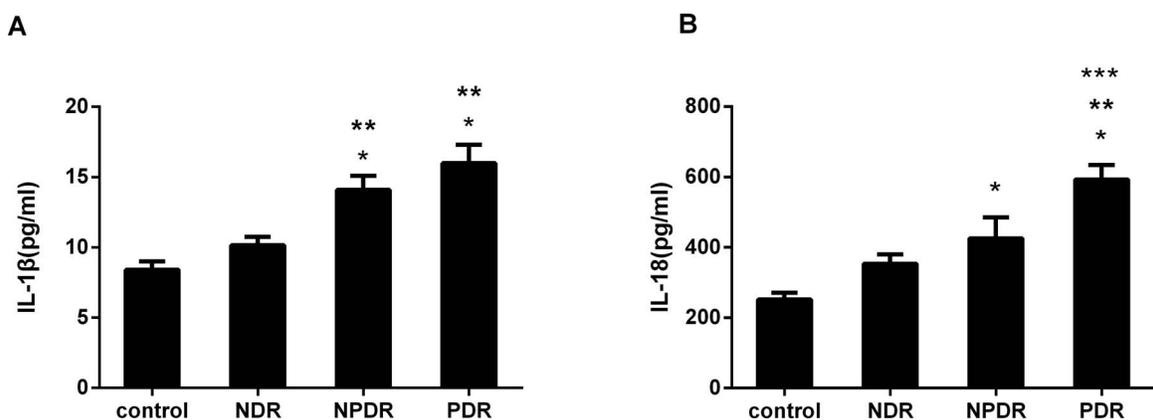


FIGURE 3. Levels of IL-1 β and IL-18 increased in PBMCs from DR patients (PDR, $n = 23$; NPDR, $n = 20$; NDR, $n = 21$; control, $n = 25$). (A) IL-1 β and (B) IL-18 cytokine levels in culture supernatants of PBMCs isolated from healthy controls ($n = 25$), patients with NDR ($n = 21$), and patients with DR ($n = 43$). Data represent means \pm SD. * $P < 0.05$, not significant. * $P < 0.05$ versus control; ** $P < 0.05$ versus NDR; *** $P < 0.05$ versus NPDR.

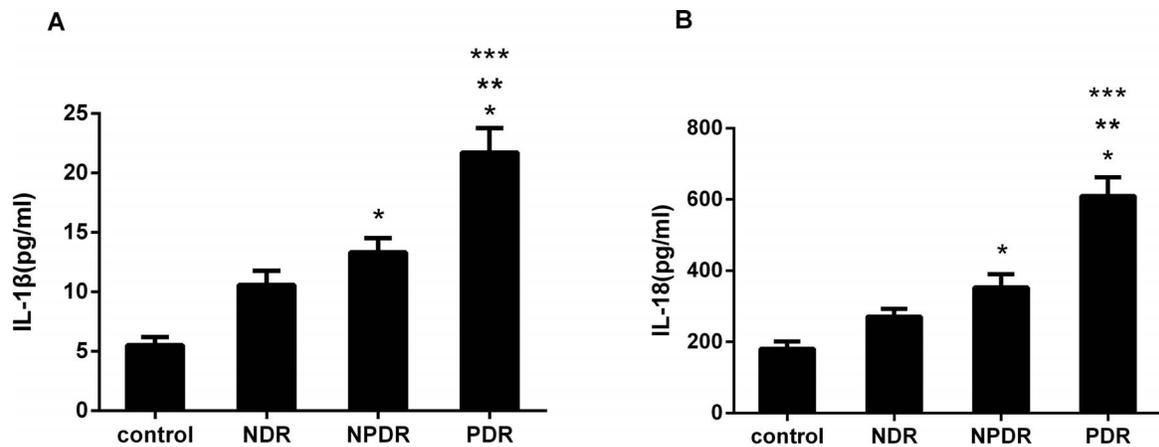


FIGURE 4. Levels of IL-1 β and IL-18 increased in vitreous fluid from DR patients (PDR, $n = 21$; NPDR, $n = 20$; NDR, $n = 19$; control, $n = 22$). (A) IL-1 β and (B) IL-18 cytokine levels in vitreous fluid from healthy controls ($n = 22$), patients with NDR ($n = 19$), and patients with DR ($n = 41$). Data represent means \pm SD. * $P < 0.05$, not significant. * $P < 0.05$ versus control; ** $P < 0.05$ versus NDR; *** $P < 0.05$ versus NPDR.

tions²⁴; however, the involvement of NLRP3 in DR remains unclear.

DR is considered a low-grade inflammatory condition. The inflammatory response of mononuclear cells is thought to be involved in all stages of the DR process, from the development of microaneurysms to neovascularization formation. Several mediators secreted by mononuclear cells modulate the inflammatory process in different stages of DR. Here, we studied the expression and activation of different NLRP3 inflammasome components in the PBMCs of patients with DR. Our data indicated that key NLRP3 inflammasome molecules are overexpressed in the PBMCs of DR patients, and activation of NLRP3 inflammasomes triggers an increase in the expression of inflammatory cytokines IL-1 β and IL-18 in these cells and vitreous fluid. The data also showed that NLRP3 inflammasomes were correlated with the inflammatory process in DR patients. This was further supported by immunofluorescence staining analysis of the corresponding epiretinal membranes from the PDR patients.

The mechanisms by which activation of NLRP3 inflammasomes is induced in DR are unknown, but we propose two alternative hypotheses. One hypothesis is that extracellular ATP release may be involved in NLRP3 inflammasome activation.²⁵ Recently, in vitro culture experiments showed that NLRP3, caspase-1, IL-1 β , IL-18, and ATP were significantly increased in human renal proximal tubule (HK-2) cells after high-glucose stimulation.²⁶ Similarly, the extracellular ATP concentration was also found to be significantly enhanced in the rat Müller cell line.¹⁸ The other hypothesis is that, in addition to endogenous danger signals such as ATP, ROS induce NLRP3 activation. DR pathology includes several ROS-producing processes, such as uncontrolled endoplasmic reticulum stress, glucose auto-oxidation, polyol pathway activation, protein glycation, and the development of complex glycation end-products that induce NLRP3 inflammasome pathway signaling.²⁷⁻³² Collectively, our data suggest that systemically elevated endogenous-dangerous molecules, such as ATP and ROS, lead to NLRP3 inflammasome activation, as characterized by elevated NLRP3, ASC, IL-1 β , and IL-18 expression in the PBMCs from the DR patients.

We also observed abnormal regulation of IL-18 and IL-1 β in the DR patients. PBMCs and vitreous fluid from the DR patients expressed higher levels of IL-18 and IL-1 β . IL-18 has been suggested to be involved in the progression of DR¹⁶⁻¹⁸;

however, its role and mechanism are unclear. As a potent proinflammatory cytokine, IL-1 β synthesis, maturation, and secretion are tightly regulated by NLRP3 inflammasome activation.³³ IL-1 β release can drive the cytokine cascade, such as IL-6, and induce all clinical and laboratory features, and we have previously noted high circulating levels of IL-6 in DR patients.³⁴ Furthermore, our findings agree with those of a clinical study showing that IL-18 and vascular endothelial growth factor levels were higher in the vitreous fluids of PDR patients than in those of nondiabetic controls.³⁵

In addition to detecting increased NLRP3 inflammasome levels, we also found elevated caspase-1 initiation in the PBMCs obtained from the DR patients. As a protease, caspase-1 plays a biological role in catalyzing its substrates. Caspase-1 was shown to regulate NLRP3-linked inflammation as an inflammatory caspase.^{36,37} Furthermore, clinical evidence showed that the use of Prannacasan, an oral caspase-1 inhibitor, to treat T2DM led to a good response and ameliorated disease symptoms in T2DM patients.³⁸ Furthermore, our data additionally revealed that caspase-1 levels in PBMCs positively correspond to the level of disease severity. In agreement with our data, Cascão et al.³⁹ showed that caspase-1 is initiated in moving leukocytes in early T2DM.³⁹

Finally, our data indicate that elevated expressions of NLRP3 ASC, IL-1 β , and IL-18 are linked to DR clinical progression of the condition. These outcomes agree with those of previous studies showing that serum levels of IL-18 were increased early after the initiation of DR and linked to disease severity in humans.⁴⁰ These findings indicate that NLRP3 inflammasome effectors are important determinants of inflammatory progress and disease severity in DR.

In this study, the expression of NLRP3 inflammasome elements was elevated in the PBMCs obtained from DR patients; elevated levels of proinflammatory cytokines, including IL-1 β and IL-18, were also observed in the PBMCs and vitreous fluids. Furthermore, the expression of the NLRP3 inflammasome signaling axis increased with disease progression. These data suggest that the initiation of NLRP3 inflammasomes may promote the progression of DR. These findings provide insight into the pathogenesis and control of DR, with NLRP3 as a new potential target for therapeutic treatment in patients with DR. Additional studies are needed to clarify the precise molecular mechanisms by which the NLRP3 inflammasome induces and aggravates DR.

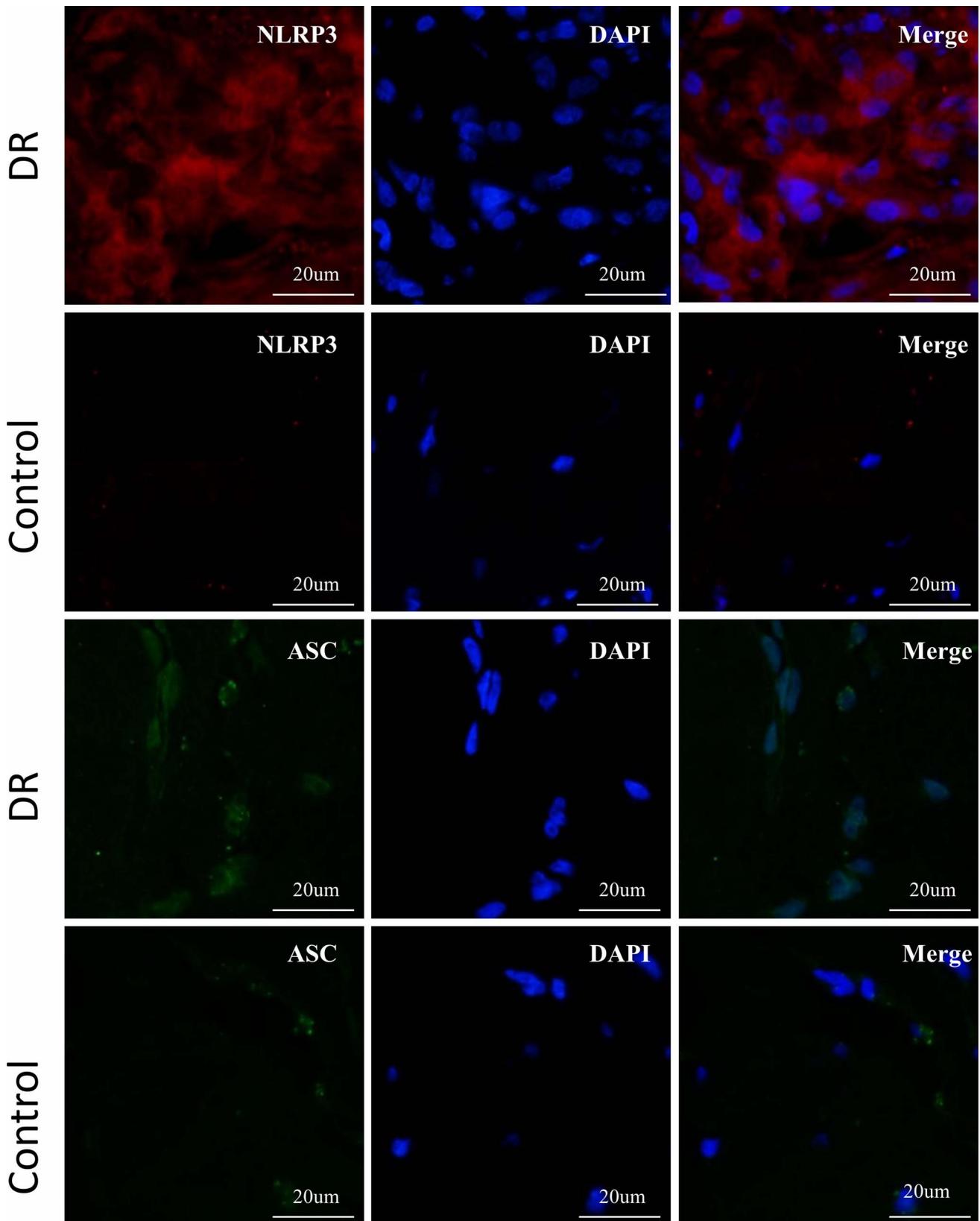


FIGURE 5. Immunofluorescence staining for NLRP3, ASC, and DAPI in fibrovascular membranes from eyes with PDR. The NLRP3 (red) and ASC (green) staining reaction is strongly positive. The DAPI stain (blue) shows many nuclei. Colocalization showing that the greatest positive staining was observed in the cytoplasm. Scale bar: 20 µm.

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