Interphotoreceptor Retinol-Binding Protein Ameliorates Diabetes-Induced Retinal Dysfunction and Neurodegeneration Through Rhodopsin

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Patients with diabetes often experience visual defects before any retinal pathologies are detected. The molecular mechanism for the visual defects in early diabetes has not been elucidated. Our previous study reported that in early diabetic retinopathy (DR), rhodopsin levels were reduced due to impaired 11-*cis*-retinal regeneration. Interphotoreceptor retinol-binding protein (IRBP) is a visual cycle protein and important for 11-*cis*-retinal generation. IRBP levels are decreased in the vitreous and retina of DR patients and animal models. To determine the role of IRBP downregulation in the visual defects in early DR, we induced diabetes in transgenic mice overexpressing IRBP in the retina. IRBP overexpression prevented diabetes-induced decline of retinal function. Furthermore, IRBP overexpression also prevented decreases of rhodopsin levels and 11-*cis*-retinal generation in diabetic mice. Diabetic IRBP transgenic mice also showed ameliorated retinal oxidative stress, inflammation, apoptosis, and retinal degeneration compared with diabetic wild-type mice. These findings suggest that diabetes-induced IRBP downregulation impairs the regeneration of 11-*cis*-retinal and rhodopsin, leading to retinal dysfunction in early DR. Furthermore, increased 11-*cis*-retinal–free opsin constitutively activates the phototransduction pathway, leading to increased oxidative stress and retinal neurodegeneration. Therefore, restored IRBP expression in the diabetic retina may confer a protective effect against retinal degeneration in DR.

Diabetic retinopathy (DR) is the leading cause of vision loss and disability in the working-age population (1). According to a 2017 report by the International Diabetic Federation, the worldwide prevalence of diabetes mellitus (DM) is 1 in 11 adults (425 million), and the number is skyrocketing accompanying the economic growth and increasing life span in developing countries.

DR was traditionally considered a microvascular complication of diabetes, and pathological angiogenesis and vascular dysfunction were regarded as the priority in clinical treatment (2). Emerging evidence suggests that dysfunction of the retinal neurons and retinal neurodegeneration play important roles in the pathogenesis of DR (3,4). Extensive reports have shown that oxidative stress, characterized by overproduction of reactive oxygen species (ROS), plays an important role in DR (5). ROS-induced chronic inflammation can lead to retinal degeneration and vascular injury in patients with diabetes (6). Anti-vascular endothelial growth factor drugs and laser photocoagulation have been used to ameliorate retinal vascular pathologies in DR, especially in proliferative DR (7). Nevertheless, there is no effective treatment for retinal degeneration in DR.

Photoreceptors are the most abundant cells in the retina and the most metabolically active neurons in the central nervous system (8). Under diabetic conditions, increased oxidative stress caused by mitochondrial dysfunction in photoreceptor cells subsequently results in retinal inflammation, leading to vascular dysfunction (9).

Interphotoreceptor retinol-binding protein (IRBP) was first identified as a visual cycle protein, which is a retinol-binding protein secreted from the photoreceptors and is

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present in the interphotoreceptor matrix of the retina (10). IRBP transports retinoids between the photoreceptors and the retinal pigment epithelium (RPE) (10,11) and plays an important role in the regeneration of 11-*cis*-retinal, the chromophore of visual pigments, and is thus essential for maintaining normal visual function. IRBP also plays a role in retinal development (12,13) and shows antioxidant activities via binding all-*trans*-retinal (14). Recently, IRBP levels were reported to be decreased in the vitreous of DR patients, and IRBP conferred protective effects against DR in rodent models (15,16).

Rhodopsin regeneration and homeostasis are critical for retinal function and health. The visual chromophore 11-*cis*-retinal binds to opsin to form rhodopsin in the dark, which locks the opsin in an inactive state. Unbound free opsin is known to be constitutively active, which can exhaust photoreceptor cells and promote retinal degeneration (17). In early diabetes, increased chromophore-free opsin due to insufficient 11-*cis*-retinal generation has been suggested to have deleterious effects on photoreceptor cells and accelerate DR progress (18).

To identify the effect of diabetes-induced reduction of IRBP on rhodopsin deficiency and retinal neurodegeneration, we induced diabetes in transgenic mice overexpressing IRBP in photoreceptors. We assessed the role of IRBP expression in diabetes-induced retinal function decline, retinal degeneration, and decreased 11-*cis*-retinal and rhodopsin levels. The potential antioxidation and anti-inflammation effects of IRBP on the diabetic retina were further investigated.

RESEARCH DESIGN AND METHODS

Animals

The animal experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (Oklahoma City, OK).

To induce diabetes, IRBP transgenic (IRBP-Tg) mice and wild-type (WT) littermates (12 weeks of age) received daily intraperitoneal injections of streptozotocin (STZ; Sigma-Aldrich) (55 mg/kg in 10 mmol/L of citrate buffer, pH 4.5) for five consecutive days. Blood glucose levels were measured 1 week after the last STZ injection and monthly thereafter. Animals with blood glucose levels >350 mg/dL were defined as diabetic animals.

Electroretinography Recording

Scotopic electroretinography (ERG) and photopic ERG were both recorded monthly using the Diagnosys Espion Visual Electrophysiology System (Lowell, MA), as described previously (18). Briefly, after dark adaptation for 16 h, mice were anesthetized with their pupils dilated. A series of flashes with intensities 0.002–600 candela (cd) · s/m^2 were applied to induce rod response under dark adaptation. For cone function, a series of flashes with 600 cd · s/m^2 intensity were applied to record the cone photoreceptor response after 10-min light adaptation under a background light of 50 cd/m^2. ERG responses of both eyes were simultaneously recorded and analyzed.

Optical Coherence Tomography

The retinal thickness was measured using a spectral domain optical coherence tomography (SD-OCT) device (Bioptigen, Durham, NC). Images were captured with the rectangular scan at 1,000 A-scans per B scan, and 100 B-scans per frame. Total retinal thickness was recorded and averaged automatically using the InVivoVue diver software (Bioptigen) by researchers blinded to the animal group information.

Immunohistochemistry and TUNEL of Eyecup Sections

Mouse eyes were carefully enucleated and fixed in Davidson's fixation solution for 48 h The eyecups were paraffin embedded, and 5-μm sections were collected. The sections were immunostained with antibodies for 3-nitrotyrosine (3-NT) (ab61392; Abcam), glial fibrillary acidic protein (GFAP) (G-3893; Sigma-Aldrich), superoxide dismutase 2 (SOD2) (06-984; Millipore), NADPH oxidase 4 (NOX4) (ab133303; Abcam), ionized calcium-binding adaptor molecule 1 (Iba1) (019-19741; Wako), arginase I (NBP1-32731; Novus), and inducible nitric oxide synthase (iNOS) (NBP1-97471; Novus) following the manufacturers' protocols. TUNEL staining was performed using the in situ cell-death detection kit, TMRed (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Histological staining was performed on three sections per animal, with five to six animals in each group of the same condition. Observation and imaging were performed under the same setting for each experiment using Olympus FluoView (Version 2.1a) (Olympus, Tokyo, Japan). The images were analyzed and semiquantified with ImageJ (National Institutes of Health) by researchers blinded to the specimen identifications.

Retinoid Profile Analysis

The retinoid profile was analyzed using high-performance liquid chromatography (HPLC), as described previously (18). Briefly, mice were sacrificed under dim red light after 16-h dark adaptation. The eyecup was homogenized individually in lysis buffer (10 mmol/L NH_2OH, 50% ethanol, 50% 2-[*N*-morpholino] ethanesulfonic acid, pH 6.5), and retinoids were extracted with hexane. The retinoids were dried under argon gas, resuspended in 200 μL of mobile phase (11.2% ethyl acetate, 2.0% dioxane, 1.4% octanol, 85.4% hexane), and injected into HPLC (515 HPLC pump; Waters, Milford, MA) for separation using a normal-phase column (LiChrosphere Si-60; 4.6 × 250 mm; 5 μm) (Alltech, Deerfield, IL) with isocratic elution (1 mL/min). Each retinoid isomer was quantified from the area of its corresponding absorption peak based on synthetic retinoid standards for calibration.

Rhodopsin Content Measurement

After 16-h dark adaptation, each retina was isolated individually and homogenized in 150 μL of 1× PBS containing 1% (w/v) dodecyl maltoside. The retinal homogenates were centrifuged at 70,000*g* for 1 h. The supernatant was transferred to clean spectrophotometer cuvettes and scanned from 250 to 700 nm of wavelength using a DU800 spectrophotometer (Beckman Coulter, Brea, CA). The difference of absorbance spectra between prebleached and postbleached samples at 500 nm was used to calculate rhodopsin content using a molar extinction coefficient of 42,000 mol/$L^{-1} \cdot cm^{-1}$. The data were normalized to the total volume of supernatant and presented as rhodopsin content per retina.

Western Blot Analysis

Eyecups were homogenized in the radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Thermo Fisher, Waltham, MA). Total protein concentrations were measured by bicinchoninic acid protein assay (Thermo Fisher). Western blotting used antibodies for IRBP (14352; Proteintech), caspase 3 (9661; Cell Signaling), 3-NT (10006966; Cayman), SOD2 (06-984; Millipore), NOX4 (ab133303; Abcam), nuclear factor (NF)-κB (P65) (ab16502; Abcam), and arginase I (NBP1-32731; Novus) and followed the conditions suggested by the manufacturers. Protein bands were visualized, examined, and quantified by densitometry using ChemiDoc Imaging System and Image Lab software (Bio-Rad, Hercules, CA).

Statistical Analysis

Data were entered and extracted from PRISM 7 (GraphPad Software, San Diego, CA). All of the experiments were performed at least three times. Quantitative data are presented as mean ± SEM and were analyzed by ANOVA. A P value of <0.05 was considered statistically significant.

Data and Resource Availability

The data sets and source data generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

IRBP-Tg mice on a C57BL/6J background overexpressing IRBP under the rhodopsin promoter were generated through a contracted service with Cyagen Biosciences (Santa Clara, CA) (Supplementary Fig. 1*A*).

Diabetes was induced in the IRBP-Tg mice and their WT littermates using injections of STZ. Blood glucose concentrations and body weights of the mice were monitored and recorded, before and after the onset of STZ-induced

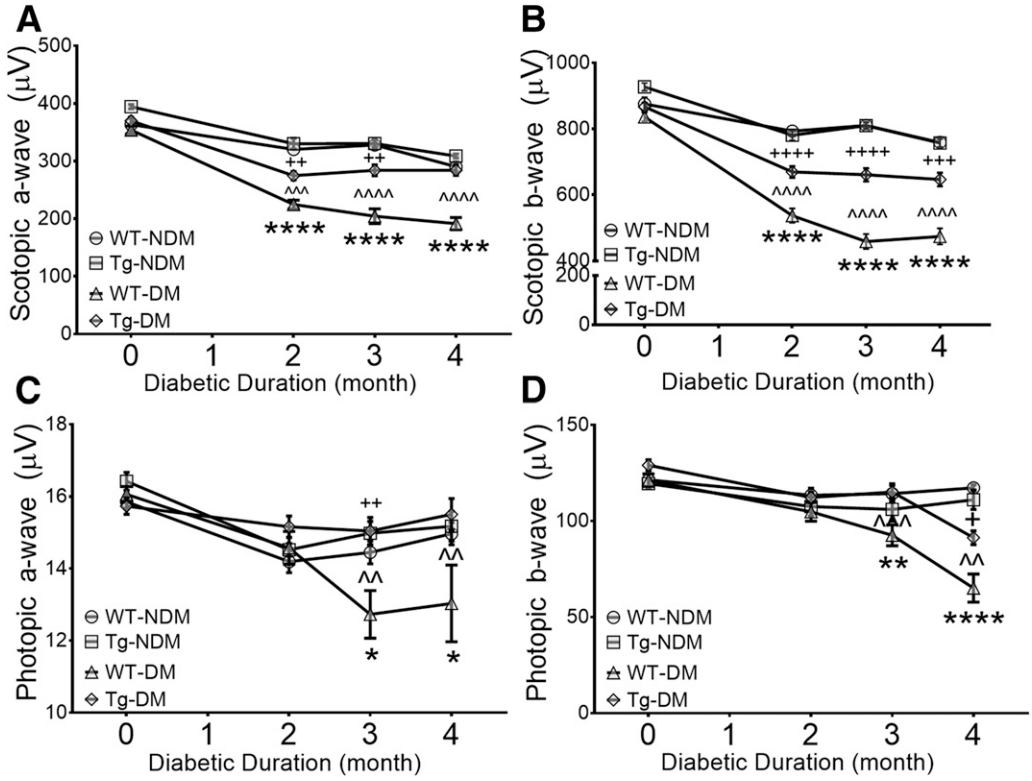

Figure 1—Overexpression of IRBP prevented retinal function decline in diabetic mice. Retinal function was evaluated using ERG in diabetic WT (WT-DM), diabetic IRBP-Tg (IRBP-Tg-DM), and their nondiabetic controls (NDM). Amplitudes of scotopic a-wave (*A*), scotopic b-wave (*B*), photopic a-wave (*C*), and photopic b-wave (*D*) at 2, 3, and 4 months after diabetes onset. *WT-NDM vs. WT-DM, +IRBP-Tg-NDM vs. IRBP-Tg-DM, ^WT-DM vs. IRBP-Tg-DM. Data are expressed as mean ± SEM ($N \geq 8$). */+/^$P < 0.05$, **/++/^^$P < 0.01$, ***/+++/^^^$P <$ 0.001, and ****/++++/^^^^$P < 0.0001$.

diabetes, together with nondiabetic controls (NDM). Diabetic groups (DM) showed lower body weights and higher blood glucose levels compared with age-matched NDM mice, with no significant difference between the IRBP-Tg and WT mice in both DM and NDM subgroups (Supplementary Fig. 2). Western blotting using an IRBP antibody showed that in the retinas of heterozygous IRBP-Tg mice, IRBP levels increased ~20% compared with that of WT littermates. As expected, IRBP levels were decreased by ~25% in the retinas of WT-DM mice compared with those of WT-NDM mice. Retinal IRBP levels of IRBP-Tg-DM mice were similar to those of the IRBP-Tg-NDM mice. As a result, the retinal IRBP level in IRBP-Tg-DM mice was ~40% higher than that of WT-DM mice (Supplementary Fig. 1*B* and *C*). This difference of IRBP levels between IRBP-Tg and WT mice under diabetic conditions were comparable to those between patients with DR and without DR (16).

IRBP Overexpression Prevents Diabetes-Induced Retinal Dysfunction

Electrophysiological analysis was performed before diabetes onset and at the 2-, 3-, and 4-month time points after diabetes onset. WT-DM mice showed continuous decline of scotopic a-wave and b-wave amplitudes at 2, 3, and 4 months compared with age-matched WT-NDM mice. Under the same diabetic conditions, the decline of ERG amplitudes was prevented completely (a-wave, Fig. 1*A*) or partially (b-wave, Fig. 1*B*) at 2 months after diabetic onset in IRBP-Tg-DM mice. Similarly, the decline of photopic a-wave and b-wave was observed after 3 months of diabetes in WT-DM mice, whereas the declines were partially prevented in IRBP-Tg-DM mice (Fig. 1*C* and *D*).

IRBP Overexpression Delays Neurodegeneration in the Retina of Diabetic Mice

OCT measurement demonstrated that the thickness of the photoreceptor layers (inner segment ellipsoid and outer nuclear layer) was significantly decreased in WT-DM mice compared with that of WT-NDM mice. In IRBP-Tg-DM mice, however, IRBP overexpression in the retina prevented the decrease of photoreceptor layer thickness (Fig. 2*A* and *B*). Consistently, TUNEL showed increased apoptosis in all of the retinal nuclear layers in WT-DM mice, which was attenuated in IRBP-Tg-DM mice (Fig. 2*C* and *D*). Moreover, Western blotting showed cleaved caspase 3 levels were increased in the retina of WT-DM mice while significantly decreased in the retina of IRBP-Tg-DM mice (Fig. 2*E* and *F*). Taken together, these observations suggested a critical role of IRBP in the prevention of diabetes-induced retinal neurodegeneration and retinal cell apoptosis.

IRBP Overexpression Improves Rhodopsin Regeneration in Diabetic Mice

Our previous study demonstrated that deficient regeneration of the visual pigment rhodopsin in diabetic rats contributed to the decreased ERG response in early DR (18). To determine whether IRBP downregulation in diabetes is responsible for the deficiency in 11-*cis*-retinal regeneration and decrease in rhodopsin, we measured rhodopsin levels at 3 months after diabetes onset in IRBP-Tg and WT mice. Rhodopsin was extracted after dark adaptation, and rhodopsin absorbance spectra were recorded before and after visual pigment bleach by light. The differential absorption spectra were used to quantify rhodopsin levels. As expected, rhodopsin levels were decreased in the retinas of WT-DM mice compared with those of WT-NDM mice. Overexpression of IRBP prevented the decline of rhodopsin content in IRBP-Tg-DM mice (Fig. 3*A*–*C*). Western blot analysis showed similar opsin levels in all of the groups (Fig. 3*D* and *E*), confirming the previous observation (18) that diabetes did not affect opsin expression or stability and suggesting that the decreased rhodopsin was likely caused by deficient chromophore regeneration. Taken together, these results suggest that overexpression of IRBP alleviates diabetes-induced deficiency of visual pigment formation.

IRBP Overexpression Normalizes 11-*cis*-Retinal Levels in Diabetic Mice

It has been well established that IRBP is important for 11-*cis*-retinal transport and subsequent rhodopsin regeneration (12,19). We also reported previously that 11-*cis*-retinal levels were decreased in diabetic rats compared with nondiabetic controls (18). In this study, 11-*cis*-retinal concentrations in the dark-adapted eyes of diabetic and nondiabetic IRBP-Tg and WT mice were measured by HPLC. The level of 11-*cis*-retinal in the eye of WT-DM mice was significantly lower than that of WT-NDM, consistent with our previous finding in diabetic rats (18). Overexpression of IRBP prevented the diabetes-induced decline of 11-*cis*-retinal levels in IRBP-Tg-DM mice (Fig. 4*A*–*E*), and as a result, 11-*cis*-retinal levels in IRBP-Tg-DM mice were similar to those of WT-NDM mice, supporting the notion that the increased rhodopsin levels are a result of restored 11-*cis*-retinal regeneration by IRBP overexpression in the retina of diabetic IRBP-Tg mice.

IRBP Protects the Retina From Oxidative Stress in Diabetic Mice

Because chromophore-free opsin is known to constitutively activate the phototransduction pathway in photoreceptor cells, resulting in excess oxidative stress, we measured 3-NT as a marker of oxidative stress in the retinas of diabetic mice. As shown by immunostaining, the 3-NT signal was significantly more intense in the WT-DM retina compared with that in WT-NDM mice. Diabetic IRBP-Tg mice showed lower 3-NT staining compared with diabetic WT mice with the same diabetic duration and similar glucose levels (Fig. 5*A* and *B*). Consistently, SOD2 was upregulated in the retinas of WT-DM mice, especially in the photoreceptor outer segment region, but not in the retinas of IRBP-Tg-DM mice, supporting that IRBP expression alleviated oxidative stress in the retina (Fig. 5*C* and *D*). Moreover, the increased immunostaining signal of NOX4, a major source

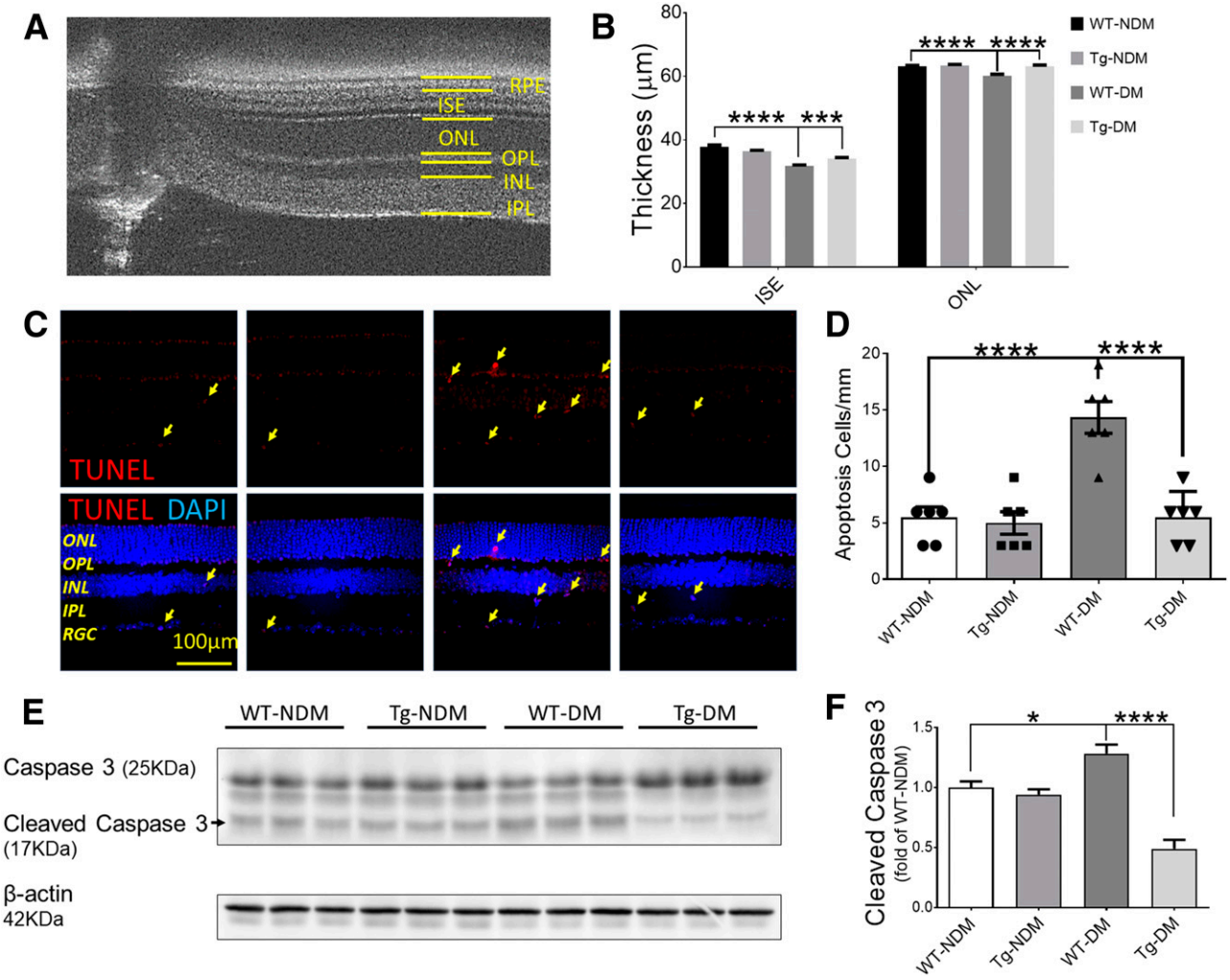

Figure 2—Overexpression of IRBP-attenuated retinal neurodegeneration and apoptosis in diabetic mice. *A*: A representative mouse retina OCT scan. *B*: The outer nuclear layer (ONL) and inner segment ellipsoid thicknesses were measured using OCT and compared among diabetic WT (WT-DM) and diabetic IRBP-Tg (IRBP-Tg-DM) mice at 3 months after diabetes onset, as well as their nondiabetic controls (NDM). *C*: Retinal sections were collected 3 months after diabetes onset for TUNEL staining (red), with nuclei counterstained by DAPI (blue). The apoptotic cells are indicated by arrows. *D*: Apoptotic cells were quantified and expressed as cells per mm of retinal length and compared. A representative Western blot of caspase 3 and cleaved caspase 3 in the retinas (*E*), and analysis by densitometry and normalization by β-actin (*F*). Data are expressed as mean ± SEM ($N \geq 6$). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

of oxidative stress, was observed in the retinas of WT-DM mice but not in those of IRBP-Tg-DM mice (Fig. 5*E* and *F*). Western blot analysis further confirmed that IRBP expression attenuated the upregulation of 3-NT, NOX4, and SOD2 in the retinas of diabetic mice, suggesting an antioxidant effect of IRBP in diabetic retinas (Fig. 5*G*–*P*). More interestingly, the activation of redox-sensitive NF-κB, a bridge connecting oxidative stress and inflammation in DR, was also ameliorated by overexpression of IRBP (Fig. 5*M* and *N*). Arginase I, a macrophage marker and mediator of DR (20), was increased in the retina of WT-DM mice and was normalized by IRBP overexpression in IRBP-Tg-DM mice (Fig. 5*O* and *P*). These observations indicated that IRBP overexpression protected the retina from diabetes-induced oxidative stress and resultant inflammation.

IRBP Protects the Retina Against Diabetes-Induced Retinal Inflammation

DM is known to induce chronic inflammation in the retina, leading to activation of microglia and leukocyte infiltration in the retina. Indeed, in the retina of diabetic WT mice, activated microglial cells, labeled by Iba1, were increased and migrated from the retinal ganglion cell (RGC) layer toward the photoreceptor layer. This increase and migration were not observed in the retinas of diabetic IRBP-Tg mice (Fig. 6*A* and *B*). Immunostaining of Iba1 on the flat-mounted retina showed increased numbers of activated and deformed microglia in the WT-DM mice, which was prevented in IRBP-Tg-DM mice (Fig. 6*C*–*E*). Immunostaining of arginase I, a marker of M2 macrophages, showed a decreased number of innate M2 in the RGC layer and an increased number of activated M2 in the outer retinal

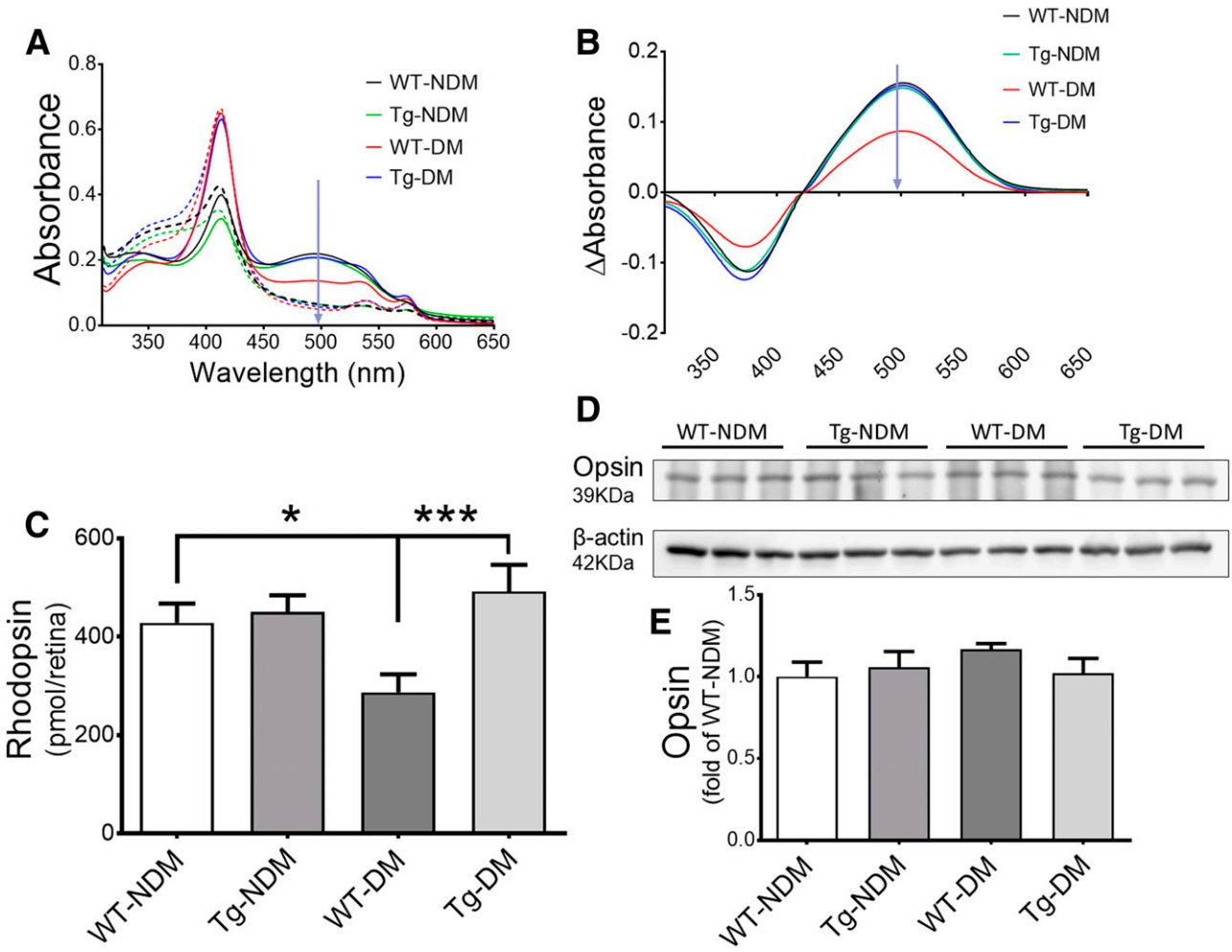

Figure 3—Overexpression of IRBP prevented the decline of rhodopsin levels in diabetic mice. After 3 months of STZ-induced diabetes, the eyeballs of IRBP-Tg mice and WT littermates were collected after 16-h dark adaptation. Rhodopsin levels were measured by absorption spectrophotometry before and after photobleaching. *A*: Representative spectra of rhodopsin absorbance recorded by spectrophotometry. An absorption peak with maximum absorption at ~500 nm is indicated by an arrow. The spectra before rhodopsin bleaching are shown in solid lines and after bleaching in dashed lines. *B*: Photobleaching difference (ΔAbsorbance) spectra acquired using the difference between the spectra before photobleaching and the spectra after photobleaching. *C*: Rhodopsin levels were calculated as pmol/eye and averaged within each group. *D*: Rod opsin in the retina was measured by Western blot analysis using an anti-rhodopsin (1D4) antibody. *E*: Opsin levels were analyzed by densitometry and normalized by β-actin levels. Data are expressed as mean ± SEM ($N = 6$). *$P < 0.05$ and ***$P < 0.001$.

layers of WT-DM mice compared with WT-NDM mice. Overexpression of IRBP attenuated the migration of M2 macrophages induced by diabetes (Fig. 6*F* and *H*). On the other hand, immunostaining of M1 macrophages with marker iNOS (Fig. 6*G*) showed augmented iNOS expression in the photoreceptor layer (Fig. 6*G* and *J*, white box) and an increase of M1 macrophages that had colocalized with GFAP (21) in the retinas of WT-DM mice (Fig. 6*G* and *I*, arrows), which was prevented in the retinas of IRBP-Tg-DM mice. All of these results suggested that IRBP overexpression alleviated diabetes-induced chronic inflammation in the retina.

DISCUSSION

DR was first defined as a microvascular disease, and later studies demonstrated that retinal inflammation and neurodegeneration also play important roles in DR pathogenesis (22). It is well known that patients with diabetes often experience visual defects, such as delayed dark adaptation in the early stages of DR, before any structural changes in the retina can be detected (23). Consistently, retinal function can be impaired before any sign of vascular changes (24). However, the exact underlying molecular mechanisms are not yet fully understood. Our previous study reported that visual pigment formation was deficient in a diabetic model due to the impaired regeneration of 11-*cis*-retinal, the chromophore for visual pigments (18), suggesting that the disturbance of the visual cycle or retinoid metabolism may contribute to DR. To identify the molecule that is responsible for this visual cycle defect in diabetes, the current study investigated the role of IRBP, which is decreased in the diabetic retina. Our results

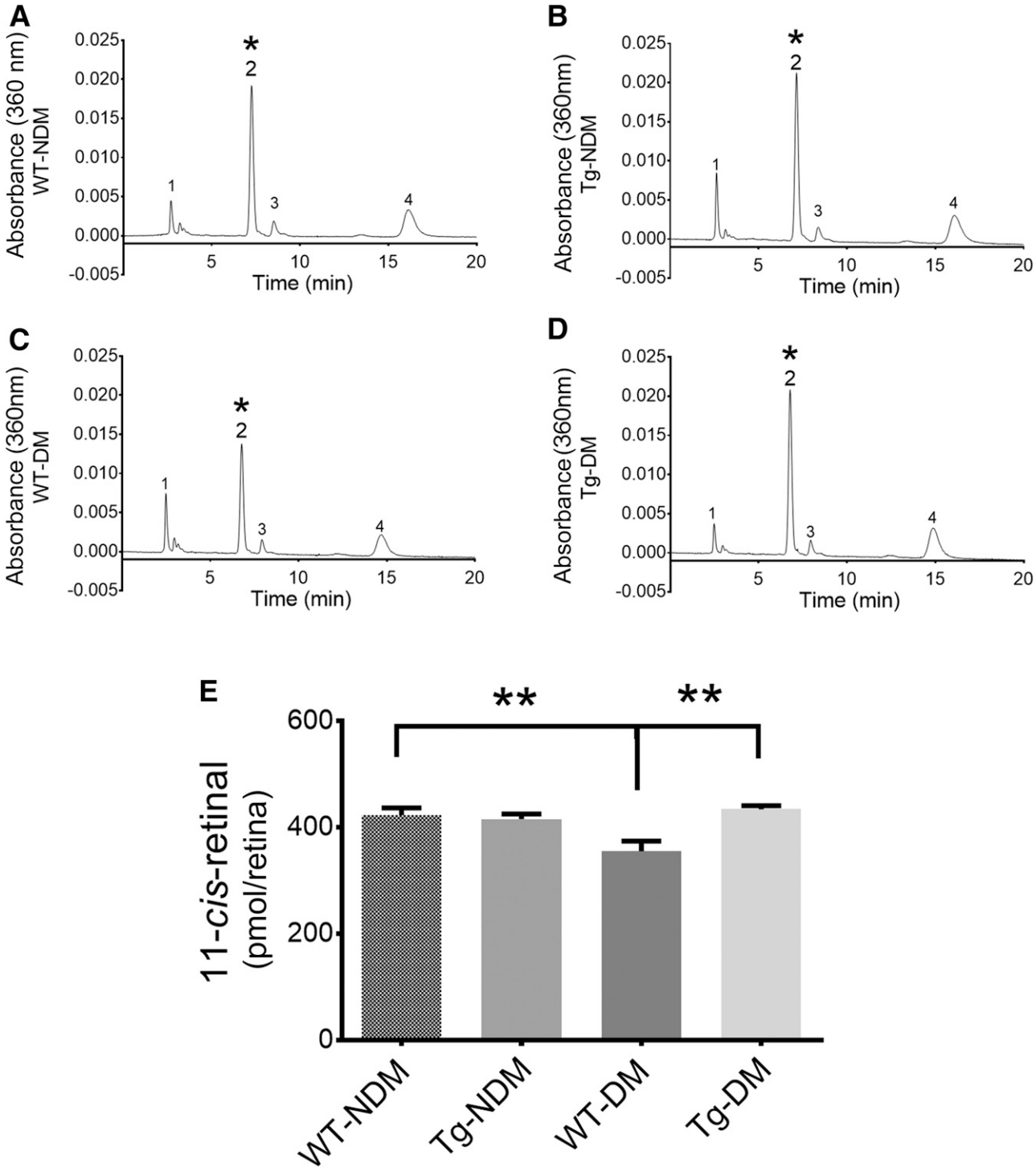

Figure 4— Overexpression of IRBP recovered 11-*cis*-retinal levels in diabetic mice. At 3 months after diabetes onset, eyeballs were collected in the dark after 16-h dark adaption, and retinoids were extracted and analyzed by HPLC. Representative HPLC chromatographs at absorbance of 360 nm are shown for WT-NDM (*A*), IRBP-Tg-NDM (*B*), WT-DM (*C*), and IRBP-Tg-DM (*D*). Peaks were identified according to retinoid standards as: *1*) retinyl esters; *2*) syn-11-*cis*-retinal oxime (marked by *); *3*) syn-all-*trans*-retinal oxime; *4*) anti–all-*trans*-retinal oxime. *E*: We quantified 11-*cis*-retinal by measuring the peak areas of the corresponding 11-*cis*-retinal oximes (mean ± SEM; $N = 5$ to 6). $^{**}P < 0.01$.

demonstrate that diabetes-induced IRBP downregulation may be responsible, at least in part, for the impaired regeneration of 11-*cis*-retinal, leading to reduced formation of visual pigments. As a result, the increased free opsin (without 11-*cis*-retinal) constitutively activates the phototransduction pathway and exhausts photoreceptors. The deficient visual pigment formation and exhausted photoreceptors can contribute to the declined ERG response and retinal degeneration in diabetes. These findings revealed the molecular basis for the functional defects

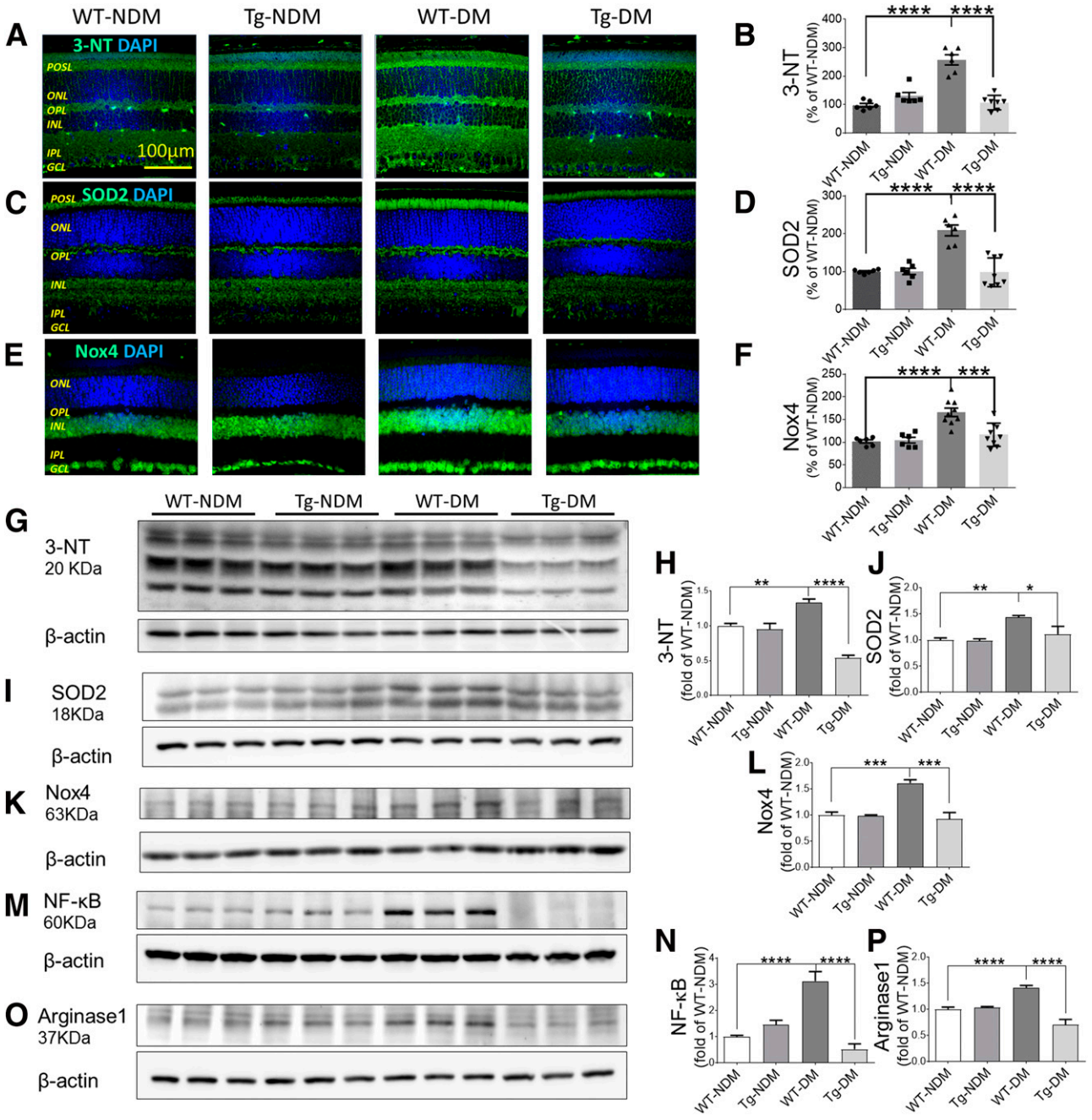

Figure 5—Overexpression of IRBP alleviated diabetes-induced oxidative stress in the retina. At 3 months after diabetes onset, 5-μm cross-sections of eyeballs were collected from diabetic WT (WT-DM) and diabetic IRBP-Tg (Tg-DM), with age-matched nondiabetic WT (WT-NDM) and nondiabetic IRBP-Tg (IRBP-Tg-NDM) mice as controls. *A–F*: Immunostaining of 3-NT (*A*), SOD2 (*C*), and Nox4 (*E*) (green), with the nuclei counterstained by DAPI (blue). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer; POSL photoreceptor outer segment layer. The expression levels of 3-NT (*B*), SOD2 (*D*), and Nox4 (*F*) were semiquantified by the fluorescence intensity. *G–P*: Representative Western blots of 3-NT, SOD2, Nox4, NF-κB, and arginase I in the retinas, and protein levels analyzed by densitometry and normalized by β-actin levels (mean ± SEM, $N \geq 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

in early DR and shed light on the development of a new therapeutic strategy for treating early DR and preventing its progression (25).

Our previous study reported that rhodopsin levels were decreased in the retinas of diabetic rats, which was attributed to the deficiency of 11-*cis*-retinal generation (18). The results from the current study further confirmed that 11-*cis*-retinal and rhodopsin levels were decreased in diabetic WT mice, supporting the notion that an impaired visual cycle or retinoid metabolism is associated with the functional decline of the retina in diabetes. To identify the molecular mechanism responsible for the visual cycle

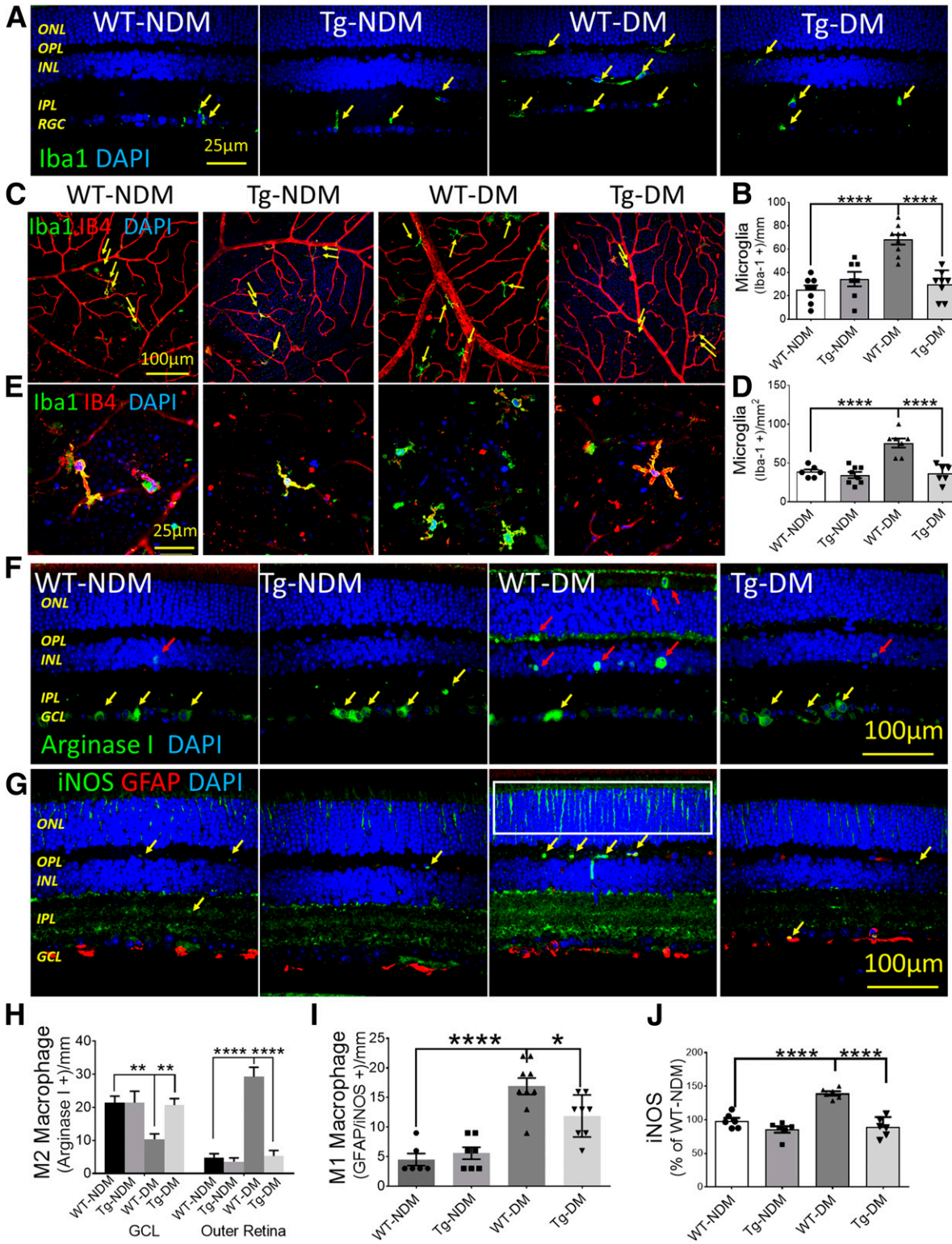

Figure 6—Overexpression of IRBP prevented diabetes-induced microglia activation in the retinas. After 3 months of diabetes induced by STZ, eyeball sections from diabetic WT (WT-DM), diabetic IRBP-Tg (IRBP-Tg-DM), and their nondiabetic controls (NDM) were collected for immunohistochemistry. *A*: Immunostaining of Iba-1 (green) with the nuclei counterstained by DAPI (blue). *B*: The activated microglia (Iba-1$^+$, indicated by arrows) were counted and compared. *C*: Coimmunostaining of Iba-1 (green) and isolectin B4 (IB4, red) on the flat-mounted retinas with the nuclei counterstained with DAPI (blue). *D*: Activated microglial cells (Iba-1$^+$, indicated by arrows) were counted in the retinal flat-mounts and compared. *E*: The morphology and number changes of activated microglia in the flat-mounted retinas of diabetic IRBP-Tg and WT mice (green). *F*: Immunostaining of arginase-I (green) with the nuclei counterstained with DAPI (blue). The microglial cells in the RGC layer are indicated by yellow arrows, and the migrating microglial cells are indicated by red arrows. GCL, ganglion cell layer; INL inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. *G*: Immunostaining of iNOS (green) and GFAP (red) with the nuclei counterstained with DAPI (blue). The activated M1 macrophages (GFAP/iNOS$^+$) are indicated by yellow arrows. The augmented

deficiency in diabetes, the current study investigated the role of IRBP. IRBP is a 135-kDa secreted protein that is almost exclusively produced by photoreceptor cells and is a major soluble protein in the interphotoreceptor matrix (26). IRBP binds, stabilizes, and transports retinoids (27) and facilitates the regeneration of 11-*cis*-retinal through the visual cycle, and thus, rhodopsin formation (27–29). To investigate the direct role of IRBP in visual cycle homeostasis in DR, we induced diabetes in IRBP-Tg mice in which the IRBP expression is under the control of a rhodopsin promoter and is thus not downregulated by diabetes. Our data showed that restored IRBP expression in the retina prevented the diabetes-induced decline of ERG response and partially restored both rhodopsin formation and 11-*cis*-retinal regeneration. These findings suggest that diabetes-induced IRBP downregulation is responsible, at least in part, for the deficient rhodopsin formation and declined ERG a-wave in early DR. This notion is supported by the observation that IRBP transgenic expression showed a more prominent protection against diabetes-induced a-wave decline, because a-wave is primarily from the photoreceptor response. These findings suggest that diabetes-induced IRBP downregulation prominently impairs photoreceptor function through reduced regeneration of chromophore and rhodopsin.

On the other hand, insufficient 11-*cis*-retinal regeneration in DR results in an increased level of chromophore-free opsin, which constitutively activates the phototransduction pathway in the photoreceptor, exhausts photoreceptor cells, and increases ROS production, further leading to retinal degeneration (17). However, the precise mechanism by which the visual cycle and its components modulate retinal function and integrity remains controversial (30,31). It is well known that DR development is closely associated with ROS overproduction and oxidative stress (5,32). The eye is one of the most vulnerable targets of ROS attack, especially under diabetic conditions (6,32). Increased oxidative stress in the diabetic retina can result in degeneration of photoreceptors (33), Müller cells (34), and other retinal neurons (5,6). Our results of apoptosis and OCT analyses confirmed the increased retinal cell death in diabetic mice, suggesting that the increased oxidative stress resulted in retinal degeneration, leading to ERG b-wave decline. Our data also demonstrated that IRBP overexpression prevented decline of the b-wave amplitudes in diabetic mice, reflecting the protective effects of IRBP on the functional integrity of the inner retina, which encouraged us to further investigate the other potential roles of IRBP. Indeed, our data showed that the diabetes-induced oxidative stress was ameliorated in diabetic IRBP-Tg mice (Fig. 5). Studies from other groups using different rodent models demonstrated that IRBP protected the photoreceptor cells from oxidative stress (14) and light-induced injury (35), also pointing to the protective role of IRBP against oxidative stress.

Western blot analysis and immunostaining both showed SOD2 accumulation in the photoreceptor outer segment region of WT-DM mice, indicating the activation of retinal intrinsic antioxidant defensive machinery and implying a local elevation of oxidative stress. As a scavenging enzyme of superoxide radicals, the activity of SOD2 has been reported to be decreased in the retinal vasculature in DR (36). The upregulated levels of SOD2 in photoreceptors may represent a compensatory response to counter the increased oxidative stress in DR.

DR is also a chronic inflammatory complication of diabetes (37,38) known to be driven by oxidative stress (5). In the retina, oxidative stress activates redox-sensitive NF-κB (39), which is a key regulator of inflammatory response (40). However, the increased levels of NF-κB and arginase I in diabetic retinas were attenuated by IRBP overexpression in diabetic IRBP-Tg mice (Fig. 5), further demonstrating the potential protective role of IRBP against oxidative stress and inflammation in the diabetic retina.

Through NF-κB activation, oxidative stress activated retinal microglia, the resident monocytes in the retina, resulting in neurotoxicity, tissue damage, and retinal angiogenesis (41,42). Activation of retinal microglia was characterized by microglial cell proliferation, migration (from ganglion cell layer, inner nuclear layer, and inner plexiform layer to inner nuclear layer and outer nuclear layer), and morphological changes (from ramified to amoeboid) in the diabetic retina (Fig. 6), all of which were inhibited by overexpression of IRBP in IRBP-Tg-DM mice. The microglia under diabetic inflammatory stress in the retina developed not only classic proinflammatory M1 microglia/macrophages but also anti-inflammatory M2 microglia/macrophages. The M2 microglia were activated and migrated from the RGC layer toward the outer retina region to address anti-inflammatory requirements at the early stage of DR; however, the M2 microglia decreased as the disease progressed (43).

In addition to the well-established function of binding and transporting 11-*cis*/*trans* retinols between photoreceptors and RPE, IRBP is also important in retinal development (13,44), and loss of IRBP leads to retinal degeneration (45–48). In addition, it has been shown that purified IRBP possesses thiol-dependent antioxidant activity (49). Recent reports demonstrated that decreased IRBP levels in the vitreous of DR patients were associated with the severity of DR (15,16). All of these reports demonstrated that IRBP, a photoreceptor protein, exerts its protective role spanning the entire retina.

expression of iNOS in the outer nuclear layer of WT-DM retinas is highlighted by the box. Numbers of residing and migrating microglia (*H*) and activated M1 macrophages (*I*) in the retinas of WT-DM and IRBP-Tg-DM mice. *J*: The expression level of iNOS was semiquantified by the fluorescence intensity. Data are expressed as mean ± SEM ($N \geq 6$). $^{**}P < 0.01$ and $^{****}P < 0.0001$.

The mechanism by which IRBP protects the retina from diabetes-induced oxidative stresses and inflammatory response warrants further investigation. Nevertheless, the current study provided clear evidence in support of the notion that IRBP attenuates diabetes-induced oxidative and inflammatory stresses, at least partially, via maintenance of homeostasis in visual pigment regeneration.

As one of the major soluble proteins of the interphotoreceptor matrix, IRBP plays an important role in maintaining a healthy environment for the photoreceptors, retina, and RPE (28,50). In addition to its retinoids-binding capacity, IRBP also contains a potential fatty acid-binding pocket (51), suggesting a probable role in lipid transport and metabolism. Recently, it was reported that IRBP inhibited glucose transport in retinal cells through binding GLUT1 and downregulating the vascular endothelial growth factor pathway (16). These previous studies suggest that IRBP may confer protective effects through multiple mechanisms, such as lipid oxidation, glucose metabolism, and retinoid metabolism. Considering that the retina is a complex tissue with intertwined cross talk among its many different resident cell types, in concordance with the multifactorial characteristic of DR pathogenesis, the multiple functions of IRBP warrant further investigation.

Taken together, our findings suggest that IRBP protects the retina from diabetes-induced oxidative stress, inflammation, and neurodegeneration at least partially through maintaining the homeostasis of 11-*cis*-retinal and rhodopsin regeneration, especially at the early stages of DR. These findings suggest that IRBP has therapeutic potential for early intervention in DR and could aid in slowing or preventing further disease progression.

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