[Frontiers in Bioscience, Landmark, 21, 958-972, June 1, 2016]

Photoreceptor IRBP prevents light induced injury

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1. ABSTRACT

Interphotoreceptor retinoid-binding protein (IRBP) is a classic inducer of experimental autoimmune uveoretinitis (EAU). Although IRBP causes neuronal loss in susceptible animals, resistant animals such as Sprague-Dawley (SPD) rats can benefit from the evoked protective autoimmune responses. The aim of the present study was to analyze the neuroprotective effects of IRBP against light-induced photoreceptor degeneration. We immunized 75 male SPD rats with IRBP and the rats were then exposed to blue light for 24 hours (IRBP group). Seventy five rats were included in the control group. We found that the number of apoptotic cells in the outer nuclear layer (ONL) peaked on 1 day after light exposure, and the ONL thickness decreased significantly on day 3. OX42-positive cells appeared in the ONL immediately after light exposure, and their number peaked on day 3, and changed from resting ramified cells to activated amoeboid cells. Compared with the control group (n=75), the IRBP group showed less apoptotic cells, a thicker ONL, and reduced expression of tumor necrosis factor-alpha. These outcomes indicate the IRPB might protect retinal photoreceptors against light-induced injury.

2. INTRODUCTION

Injuries to the neural tissues cause severe damage, not only to the primarily afflicted neurons, but also to the neighboring neurons undergoing secondary degeneration (1). Immunologic interventions mediated by circulatory T cells (2-4) and local macrophages and microglia (2, 5, 6) have proven to be protective against this damage. Substantial evidence suggests that antigen-specific immunotherapy is a promising treatment for nervous system diseases (2-4, 7), as well as for retinal and optic nerve diseases (8-11).

Microglia are the major antigen-presenting cells (APCs) in the neural tissues (12) and are regarded as the endogenous pathological factor contributing to many neurological diseases (13-15). However, activation of the microglia may also be beneficial; even if it is initially harmful, it can be rendered beneficial in particular contexts (16-18). Moreover, both *in vitro* (16,17) and *in vivo* (18) studies have shown that microglia readily upregulate the antigen-presenting molecule class II major histocompatibility complex (MHC-II) and could protect neurons against deleterious insults.

Interphotoreceptor retinoid-binding protein (IRBP) is a classic inducer of experimental autoimmune uveoretinitis (EAU) (19,20). Although IRBP causes neuronal loss in susceptible animals (e.g., the Lewis rat), resistant animals (e.g., Sprague-Dawley [SPD] rats) can benefit from the evoked protective autoimmune responses (21). Immunization with IRBP was also found to have a protective effect on retinal ganglion cells (RGCs) against various retinal and optic nerve challenges (21). The association between microglia activation and retinal photoreceptor degeneration in light-induced photoreceptor degeneration models has been established by other authors (22, 23), as well as in our previous work (24). The recombinant IRBP peptide is an immunodominant self-antigen (self-Ag) to the interphotoreceptor matrix (20, 25), and IRBP-induced uveoretinitis develops when IRBP-reactive lymphocytes are activated by recognition of MHC-II on local APCs (26). Therefore, we hypothesized that retinal microglia, the primary local APCs, actively participate in the local autoimmune response induced by photoreceptor degeneration.

Based on the evidence accumulated to date, the aim of the present study was to investigate whether immunization with IRBP could protect photoreceptors against photic injury. This study also focused on the influence of this immunotherapy on the antigen-presenting function of the local microglia.

3. MATERIALS AND METHODS

3.1. Animals

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Totally 150 specific-pathogen free adult male SPD rats, weighing 225–250 g were kept under a 12/12-hour light/dark cycle and were provided *ad libitum* access to a commercial diet and water. Animals were anesthetized by intramuscular injection of ketamine (120 mg/kg) and were humanely killed by an overdose of pentobarbitone.

3.2. Light exposure

Induction of photoreceptor apoptosis by excessive light exposure is a classic photoreceptor degeneration model (23, 27, 28). In this study, we followed the same procedures as described in our previous work (24). Briefly, SPD rats were maintained under a 12/12-hour light/dark cycle at 24 °C for 2 weeks. Before light exposure, they received 24-hour dark adaptation. Subsequently, the rats were randomly divided into several groups and were raised separately, with both eyes exposed to evenly distributed bright blue light of approximately 2500 lux. The rats were placed under these conditions at the same time of day, returned for another 24-hour dark adaptation period, and then placed back under a normal light/dark cycle.

3.3. Active immunization and animal grouping

The bovine IRBP R16 peptides (sequence 1177–1191, ADGSSWEGVGVVPDV) were purchased from the ChinaPeptides Co., Ltd, China. Protocols of immunization were conducted as reported previously (29, 30). Briefly, SPD rats were actively immunized subcutaneously in both footpads with 30 μg of IRBP R16 (10 mg/ml in phosphate buffer) that was emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 1.0. mg/ml *M. tuberculosis* 7 days before light exposure and were administered a booster of 30 μg of IRBP R16 emulsified in incomplete Freund's adjuvant (IFA, Sigma-Aldrich) immediately after light exposure. Controls were injected with phosphate-buffered solution (PBS) in CFA and boosted with PBS in IFA with or without light exposure. Animals were randomly divided into three groups: (a) the IRBP group was treated with IRBP R16 peptide and light exposure; (b) the PBS group was treated with PBS only and light exposure; and (c) the control group received no immunization or light exposure.

3.4. Antibodies

Mouse monoclonal antibodies specific to rat CD11b/c equivalent (mouse anti-rat CD11b/c; clone no. OX42) and to MHC class II (mouse anti-rat MHC class II; clone no. 3D6) were purchased from Abcam Ltd. (Hong Kong). Rabbit monoclonal antibody specific to rat MHC class II transactivator (CIITA) was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). A mouse monoclonal antibody specific to rat tumor necrosis factor-α (TNF-α) antibody was purchased from Sigma-Aldrich.

Cy3-conjugated donkey anti-mouse or anti-rabbit (with minimal cross-reaction to rat serum proteins) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit IgG (with minimal cross-reaction to rat serum proteins) were purchased from Invitrogen (Camarillo, CA, USA).

3.5. Histopathology and immunohistochemistry

Rats were anesthetized and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) solution in 0.1 M PBS (pH 7.4.). For hematoxylin and eosin (H-E) staining, the eyes were enucleated and fixed in 4% PFA for 1 hour, transferred to 10% neutral-buffered formalin for 24 hours, and then processed for routine paraffin-embedded sectioning (Shandon Pathcenter; Thermo Shandon Inc., Pittsburgh, PA, USA). The eyes were embedded sagittally, and 5-μm-thick serial sections were cut with a rotary microtome (Microm HM 330, McBain Instruments, Chatsworth, CA, USA) and were stained with H-E. Each section was inspected using an image analysis system (Leica Qwin QG2-32, Bensheim, Germany). All eyes were cut vertically, and only sections through the optic nerves were collected for subsequent analyses. According to our

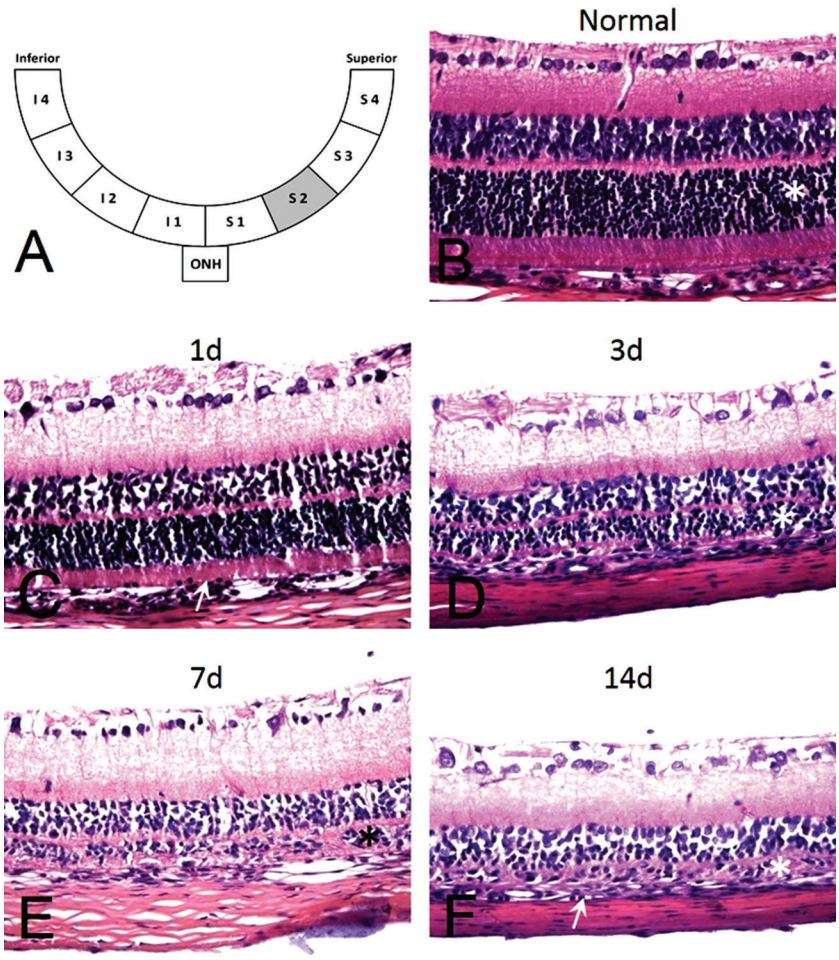

Figure 1. Photomicrographs of rat retinas in the light-induced photoreceptor degeneration model with hematoxylin-eosin staining. A: Schematic diagram of the retinal vertical sections through the optic nerve. S1–S4 indicates four measurements in the superior quadrant and I1–I4 for the inferior quadrant. All the photographs were taken in the S2 region. B: Normal retina shows fine arrangement of the retinal layers with intact ONL (white asterisk) and underlying IS/OS layer. C: One day after light exposure, a swelling and disorganized IS/OS layer appeared (white arrow). D: On day 3, ONL thinning was remarkable, indicating significant photoreceptor loss (white asterisk). E: On day 7, ONL cell loss was more severe (black asterisk). F: On day 14, only one to two rows of ONL remained (white asterisk), with overall disappearance of the IS/OS layer (white arrow). (ONL: Outer nuclear layer; IS/OS: photoreceptor inner and outer segment).

previous work (24), with simplification, each eyecup was divided into 8 equal regions (superior quadrant, S1–S4; inferior quadrant, I1–I4) (Figure 1A). The thickness of the outer nuclear layer (ONL) in the S2 region was observed using an image analysis system (Leica Qwin QG2–32).

For immunohistochemical analysis, the eyes were fixed in 4% PFA for 1 hour and cryoprotected in 20% and 30% sucrose solutions at 4 °C. The cornea and lens were removed and the eyecups were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Ted Pella, Inc., Redding, CA, USA), and then frozen in liquid nitrogen. Ten-micrometer-thick sections were cut using a cryostat (Bright Instruments Ltd., Huntingdon, UK). Sections were recovered onto gelatin-coated slides. Slides were incubated with blocking buffer (PBS containing 5% goat serum) at room temperature for 1 hour. After three washes with 0.0.1 M PBS, the sections were incubated with the primary antibody (OX42 and MHC-II) overnight at 4 °C; this was follows by rinsing with 0.0.1 M PBS three times and incubation with the secondary antibody (Cyk3-conjugated, 1:200, or FITC-conjugated, 1:200) at 37 °C for 45 min. Following three rinses with 0.0.1 M PBS, the sections were mounted with antifades mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and examined under a confocal laser-scanning microscope (TCS SP2; Leica Microsystems, Bensheim, Germany).

3.6. Terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) staining

Cell apoptosis was detected using a TdT-FragEL™ DNA fragmentation detection kit (TUNEL; Calbiochem, MERCK, UK) according to the manufacturer

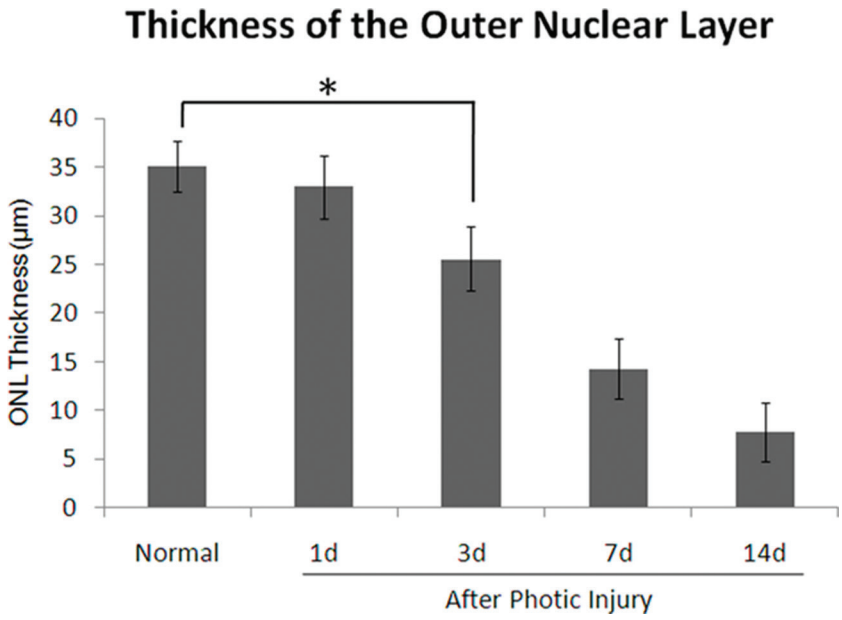

Figure 2. Quantitative analysis of the outer nuclear layer (ONL) thickness. ONL thickness in S2 region before and 1, 3, 7, and 14 days after 24-hour light exposure are 35.1.5 ± 2.6.1μm, 33.0.7 ± 3.2.3μm, 25.6.3 ± 3.3.2μm, 14.3.2 ± 3.1.2μm, and 7.8.5 ± 3.0.5μm, respectively. Compared to the normal retina, ONL thickness begins to reduce significantly on day 3. Student's t-test for two groups. (n = 10). *P < 0.0.01.

protocol. Only bodies of the apoptotic cells would be stained. Images were captured using a light microscope (DM IRB; Leica, Bensheim, Germany) with the Leica Qwin QG2-32 software. TUNEL-positive cells were counted in the S2 region of each retinal section.

3.7. Western blot analysis

The retinas were homogenized in RIPA buffer (1% Triton X-100, 5% sodium dodecyl sulfate, SDS, 5% deoxycholic acid, 0.5. M Tris–HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 × 10^{-12} μg/ml aprotinin, 1 × 10^{-12} μg/ml leupeptin, 1 × 10^{-12} μg/ml pepstatin, 200 mM sodium orthovanadate, and 200 mM sodium fluoride. Retinal extracts were incubated on ice for 10 min and centrifuged at 10,000 ×*g* for 25 min at 4 °C. Total protein concentration and the protein concentration in retinal extracts were measured using a standard BCA assay (Pierce, Rockford, IL, USA) and the Lowry method (Bio-Rad Life Science, Mississauga, ON, Canada), respectively. Retinal extracts were resuspended in 5× sample buffer (60 mM Tris–HCl pH 7.4, 25% glycerol, 2% SDS, 14.4. mM 2-mercaptoethanol, 0.1% bromophenol blue), boiled for 5 min, and resolved on a 12% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech, Germany), and blots were stained with Ponceau S (Sigma-Aldrich). After washing with TBST (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1.% Tween 20) and blocking with 5% non-fat dry milk in TBST for 45 min, the blots were probed with the primary antibody against MHC-II, CIITA, or TNF-α for 24 h, followed by the horseradish-peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:10,000 dilution). The bound antibodies were detected using an enhanced chemiluminescence system (ECL, Amersham, MA, USA) and X-ray film. The signal intensity was measured using an ImageMasterRVDS (Pharmacia Biotech, CA, USA), and the optical densities (mean ± SD) for each sample were obtained from three measurements on three separate blots.

3.8. Data analysis

The data on the mean ONL thickness of the entire retina and from each defined location across the retina, the mean numbers of TUNEL-positive cells in the ONL, and the protein levels of MHC-II, CIITA, and TNF-α in the retina were compared using the Student's t-test.

4. RESULTS

4.1. Light-induced photoreceptor degeneration

Photic injuries were located mainly in the outer retina, especially in the ONL and in the inner and outer segments (IS/OS) of the photoreceptor layer. Among the eight retinal regions, the S2 region exhibited the most remarkable changes. Figure 1B showed the S2 region of normal retina before light exposure. One day after light exposure, the S2 region presented a normal ONL but a swollen and disorganized IS/OS layer (Figure 1C). Three days after light exposure, the thickness of the ONL was markedly reduced, indicating significant photoreceptor cell loss (Figure 1D). On day 7, the ONL cell loss was more severe (Figure 1E), and by day 14,

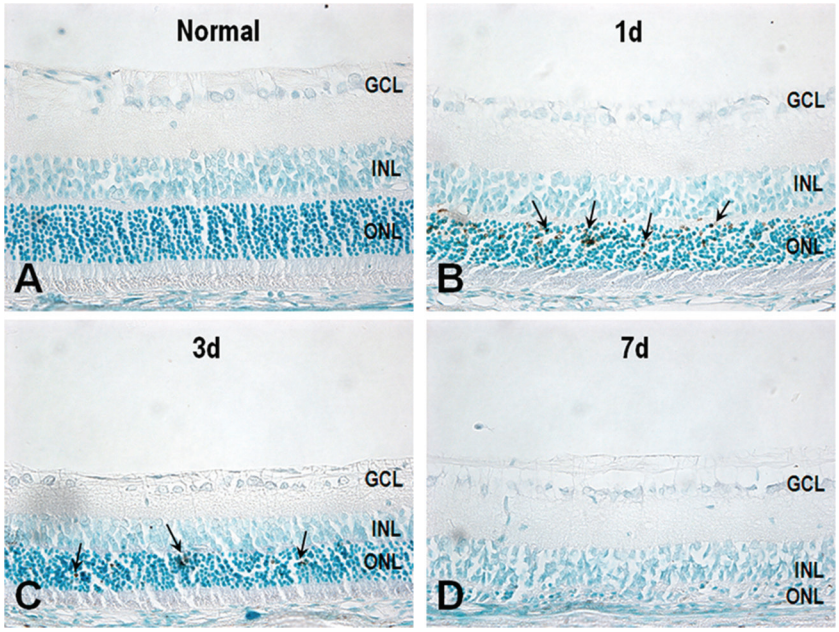

Figure 3. Representative examples of the photoreceptor cell death in the S2 region as analyzed by the TUNEL assay. Positive staining is absent in the normal rat retina (A). TUNEL-positive cells are seen in the ONL immediately after light exposure, peaked on day 1 (B), became less abundant on day 3 (C), and disappeared on day 7 (D). (black arrows: TUNEL-positive cells).

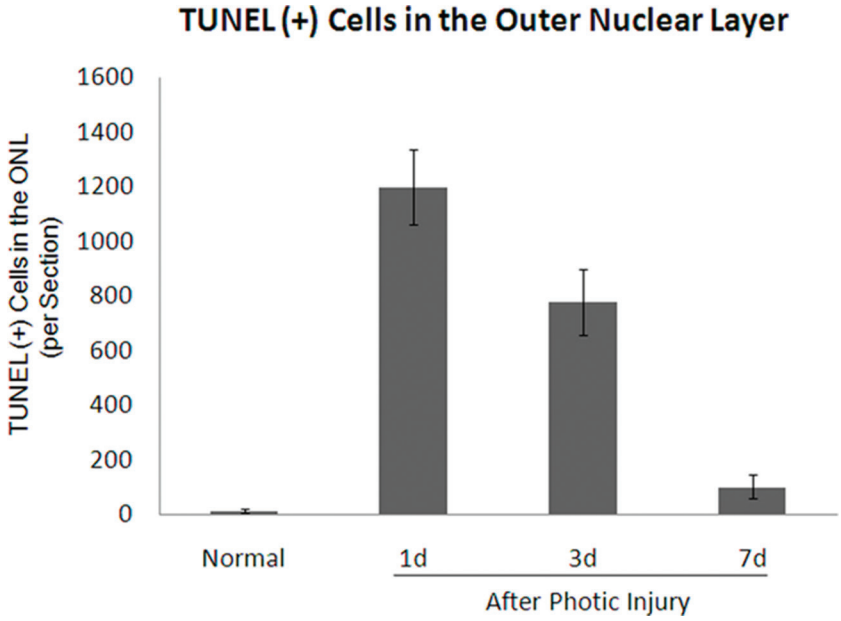

Figure 4. Quantitative analysis of the apoptotic cells in the outer nuclear layer (ONL) layer. Numbers of TUNEL positive cells in the ONL layer of the whole section before and 1, 3, and 7 days after light exposure were 14.9. ± 6.3., 1197.5. ± 137.8., 776.9. ± 119.6., and 102.2. ± 44.3., respectively. TUNEL stained cells peaked 1 day after photic injury. (n=10).

only one or two rows of the ONL remained, with overall disappearance of the IS/OS layer noted (Figure 1F). Quantitative analysis revealed that the ONL thickness began to reduce significantly on the 3rd day after photic injury (Figure 2).

The TUNEL assay was used to explore cell apoptosis within the retina. Positive staining was absent in the control rats (Figure 3A). TUNEL-positive cells were observed in the ONL immediately after light exposure and peaked on day 1 (Figure 3B). Cells apoptosis were

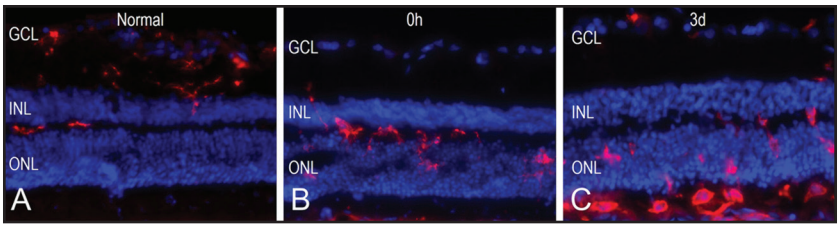

Figure 5. Immunolabeling of retinal microglia stained with OX42. (A) In normal retina, cell bodies of OX42-positive microglia were mainly seen in the GCL and IPL, with their processes extending to the INL. (B) Right after light exposure, microglia began to move to ONL. Their cell bodies were in the INL and OPL, but their processes has reached the ONL. (C) Three days after light exposure, the majority of OX-42 positive microglia reached the ONL and the subretinal space, with significant morphologic changes from ramified cells (A and B) to amoeboid cells (C). (GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer).

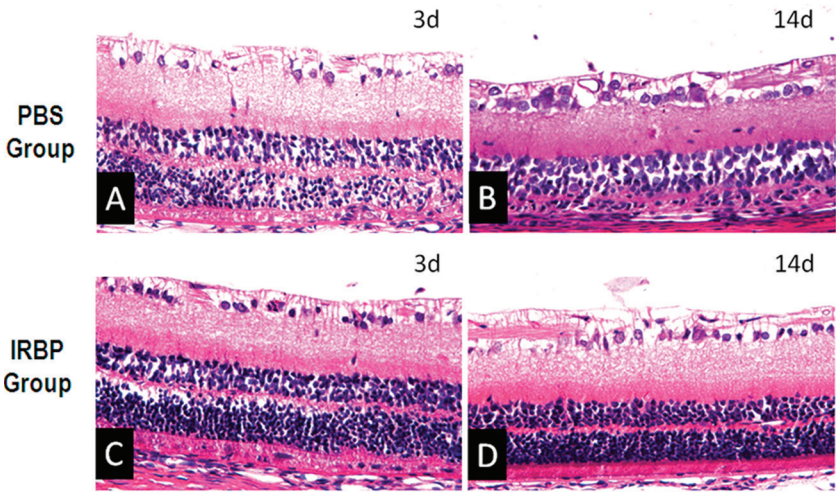

Figure 6. Photomicrographs of the retinas in both groups. Three days after photic injury, ONL thickness was not different between two groups (A and C). However, 14 days after light exposure, ONL in the IRBP group was significantly thicker than that in the PBS group (B and D), indicating the protective role of the IRBP treatment against the light injury. In the IRBP group, morphological changes of ONL on day 14 were similar to that on day 3. (ONL: outer nuclear layer).

less abundant in the ONL on day 3 (Figure 3C) and dramatically decreased on day 7 (Figure 3D). Quantitative analysis revealed that the number of TUNEL-positive cells in the whole section increased significantly at 1 day after exposure and then decreased thereafter (Figure 4).

4.2. Activation and migration of the retinal microglia in response to photic injury

The retinal microglia were specifically stained with the OX42 antibody. In the control retinas, a few OX42-positive microglia with thin cellular processes could be seen in the ganglion cell layer (GCL) and the inner plexiform layer (IPL). The outer retina and subretinal space showed no fluorescence (Figure 5A). Immediately after light exposure, OX42-positive microglia appeared in the outer retina (Figure 5B), increased rapidly, and reached their highest number at 3 days (Figure 5C). Activation and migration of the microglia did not show an apparent difference between the IRBP and PBS groups, suggesting that microglial activation was not inhibited by the IRBP treatment (data not shown).

4.3. Neuroprotective effect of the IRBP R16 peptide on the rat retina

The thickness of the ONL was markedly reduced in the PBS group at 14 days after photic injury (Figure 6A, 6B). By contrast, IRBP provided effective protection to the ONL (Figure 6C, 6D, and Figure 7). In the IRBP group, the morphology of the ONL on day 14 was similar to that observed on day 3.

Cell apoptosis at different time points after exposure to light was also analyzed with TUNEL assays. Treatment with the IRBP R16 peptides resulted in significantly fewer apoptotic cells on days 1, 3, and 7 (Figure 8), suggesting that IRBP protected photoreceptors through inhibiting cell apoptosis.

4.4. Activation of potential APCs after photic injury, as assessed by MHC-II and CIITA expression

Immunofluorescence showed that the expression of MHC-II strongly increased on day 1

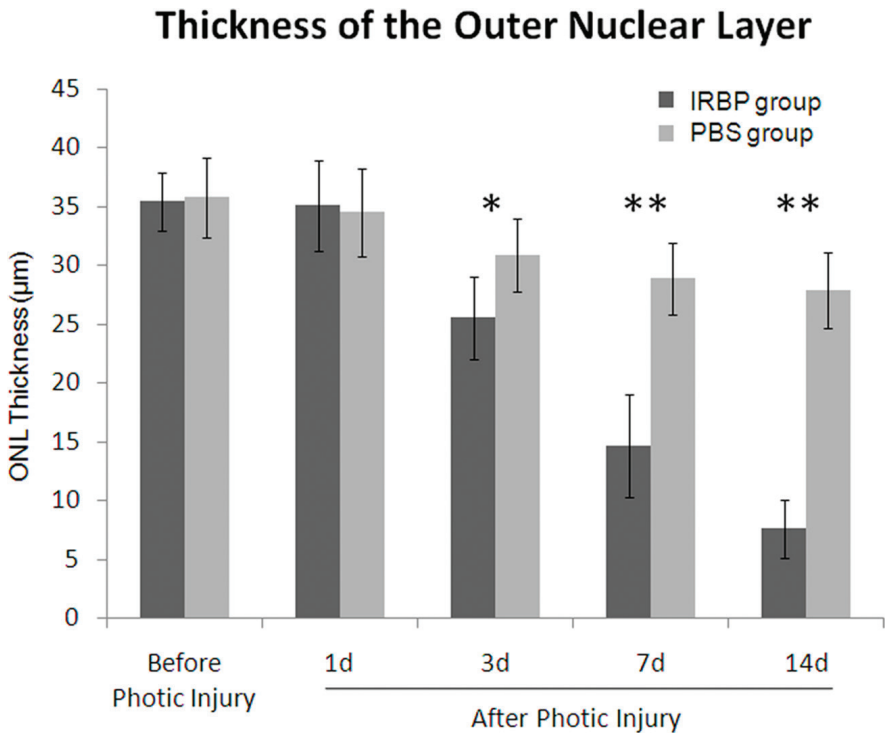

Figure 7. Comparative analysis of the outer nuclear layer (ONL) thickness between two groups. In the PBS group, ONL thickness on S2 region before and 1, 3, 7, and 14 days after light exposure were 35.4.2 ± 2.4.5μm, 35.1.1 ± 3.8.3μm, 25.5.1 ± 3.4.9μm, 14.6.4 ± 4.3.4μm, and 7.5.6 ± 2.5.0μm, respectively. In the IRBP group, ONL thickness were 35.7.5 ± 3.3.7μm, 34.4.9 ± 3.7.8μm, 30.9.0 ± 3.1.0μm, 28.8.6 ± 3.0.3μm, and 27.8.7 ± 3.2.5μm, respectively. From the day 3, ONL in the IRBP group became significantly thicker than the PBS group. Student's t-test for two groups (n = 10). *P < 0.0.1, **P < 0.0.01.

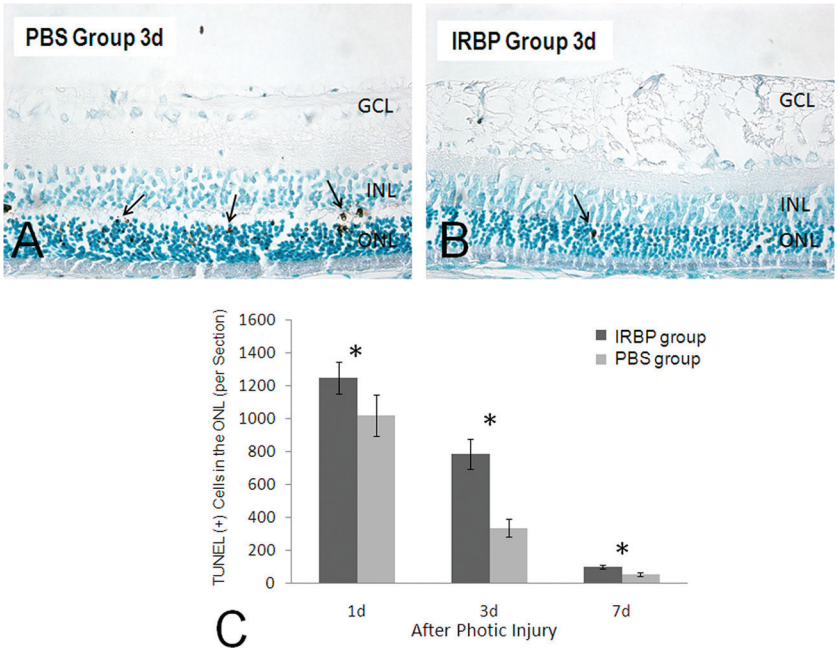

Figure 8. Protective effects of the IRBP treatment on light-induced photoreceptor apoptosis measured by TUNEL assay. In the S2 region, TUNEL positive cells in the ONL were more abundant in the PBS group (A) than in the IRBP group (B). (C) Statistical analysis showed that the number of TUNEL-positive cells was significantly lower in the IRBP group than in the PBS group on 1, 3 and 7 days after exposure to light. Student's t-test for two groups (n = 10). *P < 0.0.01. (IRBP: interphotoreceptor retinoid-binding protein; ONL: outer nuclear layer).

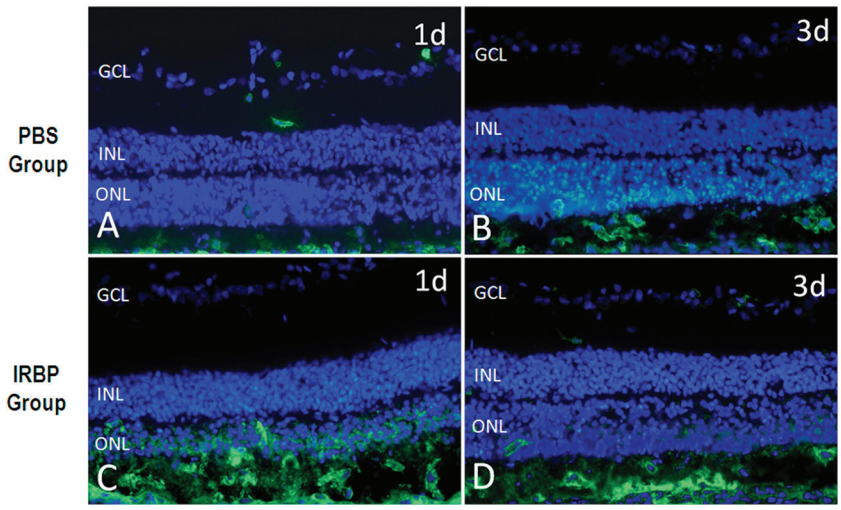

Figure 9. Immunolabeling of retinal MHC-II expression. Immunofluorescence of MHC-II was strongly increased on day 1 in the IRBP group (C and D), whereas in the PBS group it was not until day 3 that a slight increase of MHC-II could be seen (A and B). This suggested that MHC-II expression in retinas treated with the IRBP was not only advanced but also enhanced. (MHC-II: major histocompability complex-II; IRBP: interphotoreceptor retinoid-binding protein).

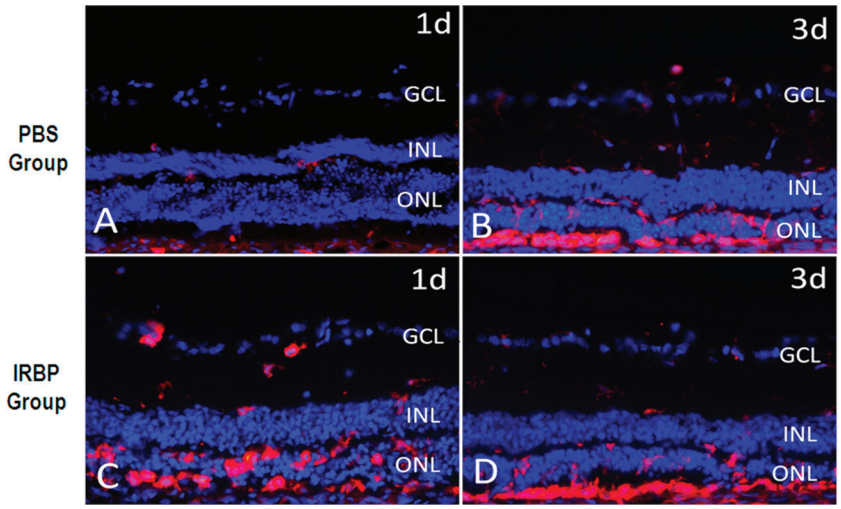

Figure 10. Immunolabeling of retinal CIITA expression. Immunofluorescence of CIITA was strongly increased on day 1 in the IRBP group (B and D), whereas in the PBS group it was not until day 3 that an increase of MHC-II could be seen (A and B). This suggested that CIITA expression in retinas treated with the R16 was both advanced and enhanced. (CIITA: class II major histocompatibility complex transactivator; IRBP: interphotoreceptor retinoid-binding protein).

after light exposure in the IRBP group, whereas in the PBS group, this increase was not detected until day 3 (Figure 9). Moreover, the expression of CIITA, the upstream molecule for MHC-II, was also delayed in the PBS group relative to the IRBP group (Figure 10). These findings were substantiated by the western blot results, which showed earlier and stronger expression of MHC-II and CIITA in the IRBP group (Figure 11).

4.5. TNF-α expression in the retina

Protein expression levels of TNF-α were quantitatively analyzed using western blotting. TNF-α expression in the IRBP group slightly increased after light injury. When compared to the PBS group, the IRBP group presented a significantly lower TNF-α level at all time points after light exposure (Figure 12).

5. DISCUSSION

In the present study, treatment with the IRBP R16 peptide effectively protected SPD rats against light-induced photoreceptor apoptosis and ONL loss. This protection was correlated with enhanced microglial antigen-presenting function and decreased retinal TNF-α protein levels.

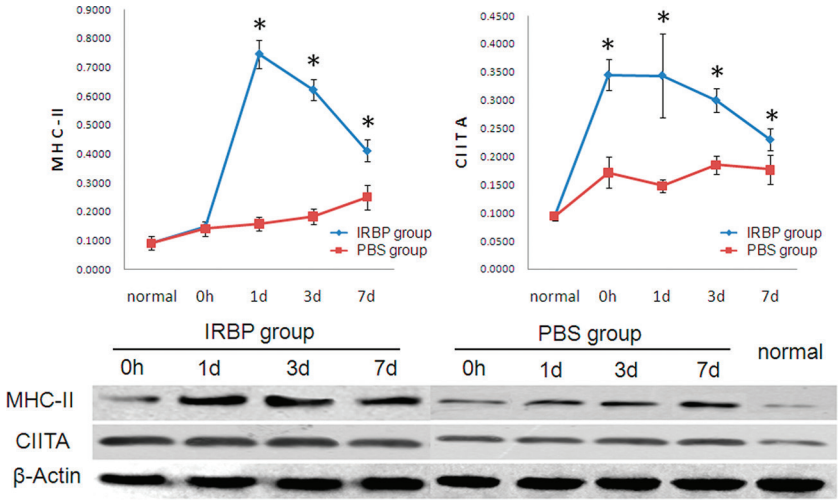

Figure 11. Levels of MHC-II and CIITA protein after photic injury as measured by Western blot. After photic injury, both MHC-II and CIITA expressions in the IRBP group increased more quickly and were significantly higher compared to those in the PBS group. β-actin was used as the loading control. Student's t-test for two groups (n = 8). *P < 0.0.01. (MHC-II: major histocompability complex-II; CIITA: class II major histocompatibility complex transactivator).

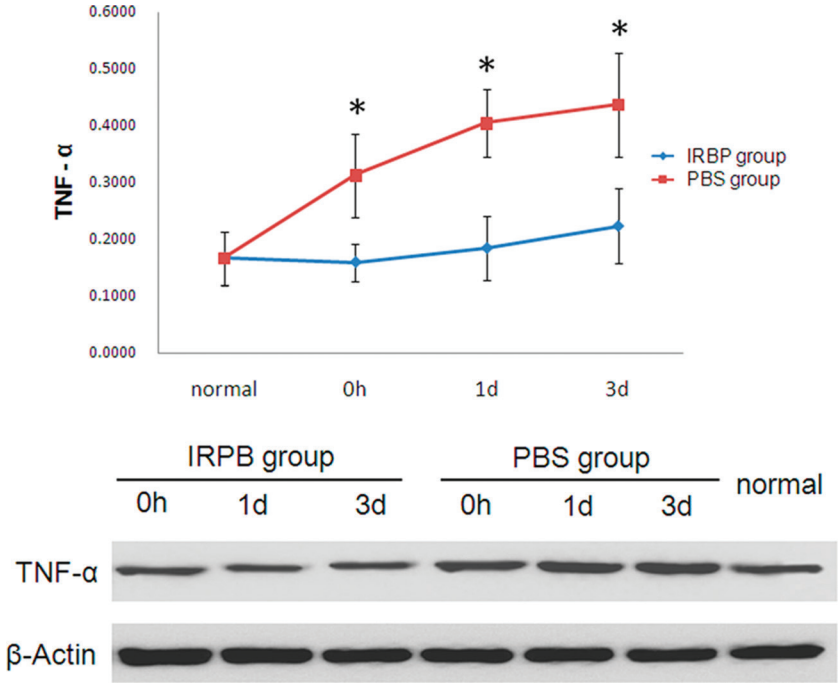

Figure 12. Level of TNF-α expression after photic injury as measured by Western blot. Before light exposure, TNF-α protein expression was not different between the IRBP groups and the PBS groups. After light exposure, TNF-α protein expression was significantly lower in the IRBP group at all the three time points. β-actin was used as the loading control. Student's t-test for two groups (n = 8). *P < 0.0.01. tumor necrosis factor-α (TNF-α).

The inflammatory reaction in nervous tissues is notorious since it causes irreversible neuronal damage (5,31-33). However, some experts believe that such inflammation could be beneficial to neurons if regulated under optimal control (7,34-35). Neurons that are directly injured will inevitably die (1, 36-37), but the neighboring neurons that managed to escape the primary injury or were not fatally injured will survive the secondary degeneration process mediated by the injury-evoked self-destructive compounds (2-6, 34, 38, 39). Thus, immunotherapies for controlling neural inflammation involve immunizing animals with the self-antigens (Ags) residing at the site of ongoing degeneration (3, 10, 40), or infusing them with autoimmune T cells specific to the self-Ags (2, 4, 7, 35, 39, 41-43).

Immunotherapies have also proven to be effective in treating retinal and optic nerve disorders (8-11, 21, 44, 45). One of the self-Ags commonly used for treating eye diseases is IRPB, a classic inducer for EAU models (19, 20, 25, 46). IRBP was found to be effective in protecting RGCs against various injuries such as high intraocular pressure (8), toxicity of intraocular glutamate injection (45), and optic nerve crush (21). Even though susceptible animals (e.g., the Lewis rat) will inevitably experience neuronal death caused by the transient EAU, the cost is clearly outweighed by the benefit (8,11, 21). Fortunately, there seems to be no such trade-off for the benefit obtained in SPD rats, which are resistant to EAU (8, 21). Importantly, the protective immunologic intervention is a highly antigen-specific process, as the protection is promoted exclusively by self-Ags residing in the injured tissues (21, 42, 45). For instance, IRBP could protect the RGCs against acute injuries (e.g., glutamate toxicity or optic nerve crush), which was not achieved with myelin antigens, which belong to the myelin sheath of the spinal cord and thus only show a protective effect against spinal cord diseases (21, 39, 43). Since IRBP is a major component of the interphotoreceptor matrix (47, 48), it is specifically located in the retinal photoreceptor layer and thus shows promise in protecting the retina from local injury.

The outcomes of neural injury most likely depend on the extent of control over the local immune response, and microglia are suggested to be the target for this control (45, 49). Activated microglia have dual but opposite functions. They express neuroprotective proteins such as neurotrophins and glutamate transporters that facilitate neuronal survival (50, 51). However, they also mediate neuronal death by releasing glutamate (52, 53), nitric oxide (54), IL-1β (55, 56), and TNF-α (57, 59). These conflicting functions indicate that the way in which the microglia are activated might determine their specific influence on the outcomes of the injury and that their properly timed and appropriate mode of activation might determine whether a protective immune response is induced (18).

Both *in vitro* (16, 17, 60) and *in vivo* (18) studies have concluded that one of the key characteristics of protective microglia is their role as APCs, which is generally represented by the expression of MHC-II molecules. The ability of microglia to function as APCs enables them to present antigens to, communicate with, as well as be controlled by circulatory helper T cells, thereby coordinating the post-injury immune events. In this study, we demonstrated that both MHC-II and its upstream signaling molecule CIITA were upregulated in the IRBP-treated group, indicating that one of the possible mechanisms for the neuroprotective effect of IRBP against light injury is enhancement of the microglial APC function.

Another explanation for the protective role of IRBP might be through inhibition of TNF-α, a proapoptotic factor secreted by activated microglia. TNF-α is cytotoxic to neurons capable of causing neuronal degeneration through mechanisms that have not yet been completely elucidated (61, 62). In the present study, upregulation of TNF-α began immediately after light exposure in the PBS group and continued to increase for 3 days, whereas TNF-α expression was much lower in the IRBP group (Figure 12). It is reasonable to assume that the lower TNF-α level would reduce photoreceptor death and that the most likely source of TNF-α is the activated microglia (57, 59). Other retinal cells, such as Müller cells, may release cytotoxic factors in response to injury, which can also aggravate the apoptotic progression of retinal photoreceptors.

Another prerequisite for an immune event to be neuroprotective is the time of its onset, since the beneficial response must occur early enough to fit itself into the therapeutic window for neuronal survival. Although different injuries or tissues have varying therapeutic windows, the onset of a protective immune response should be as early as possible (3, 18, 63, 64). In a susceptible animal, delay or decline of microglial activation and the consequent MHC-II expression were found to lead to greater neuronal loss (18). In the present study, IRBP treatment resulted in early and increased MHC-II expression in the retinal microglia, which might contribute to the better survival of the photoreceptors.

In contrast to our previous study in which the number of TUNEL-positive cells in naloxone-treated rats did not significantly decrease until 3 days after light exposure (24), the results of the present study demonstrated that apoptotic cells significantly decreased as early as 1 day after the light exposure (Figure 8). Unlike naloxone, which inhibits microglial activation, IRBP might induce a neuroprotective phenotype of the microglia, which allows them to readily react to and effectively fight against the primary photoreceptor injury even before the termination of light exposure. The possibility that IRBP has a direct neuroprotective effect on the photoreceptors without the influence of local microglia could be considered. However, two facts strongly exclude this possibility. First, comparison between the IRBP and PBS groups suggests that IRBP significantly reduced the TNF-α protein level immediately after light exposure (Figure 12), indicating that IRBP plays an antiapoptotic role through downregulating the TNF-α released by the microglia. Second, previous studies demonstrated that IRBP induced EAU and directly caused neuronal loss, despite its benefits (8, 11, 21), indicating that IRBP is an intrinsically harmful protein, especially in EAU-susceptible animals.

In the nervous tissues, the macroglia (astrocytes and Müller cells) and neurons are believed to originate

from the neuroectoderm, whereas the microglia share a common origin with circulatory monocytes (65, 67). The expression of ED1 is a distinguishing characteristic between resting microglia and circulatory macrophages (68, 69); however, it is still not easy to differentiate activated microglia from macrophages (70). Whether the microglia found in the ONL originate from the inner retina (22) or from the blood macrophages (71) remains equivocal. The present study demonstrated that there are dynamic migrations and morphological changes of the OX42-positive microglia before and after light exposure (Figure 5), suggesting that these microglia migrate from the inner retina. Our previous study showed a few ED1-positive cells that could not be found at either 1 day or 3 days after exposure to light were observed in the superior region at 7 day exposure (24). Therefore, it is speculated that the retinal microglia play a predominant role in light-induced photoreceptor degeneration while the blood-borne macrophages appear in the later stages when the blood–retina barrier is damaged.

In conclusion, the results of this study demonstrated that IRPB could protect retinal photoreceptors against light-induced injury. This protection coincided with advanced and enhanced microglial antigen-presenting function. One mechanism for this protection is the downregulation of TNF-α expression. Our findings shed light on a novel method of motivating self-protective immune reaction which effectively improved the prognosis of injuries to the neural tissues.

6. ACKNOWLEDGEMENTS

This work has been supported by National Basic Research Program of China (973 Program, No. 2013CB967503), by National Natural Science Foundation of China (No. 81170857), and by Shanghai Key Laboratory of Visual Impairment and Restoration, Fudan University, Shanghai 200031, China. The funding organization had no role in the design or conduct of this researchs.

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Key Words: Photoreceptor, IRBP, Light, Injury, Cell Death, Review

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