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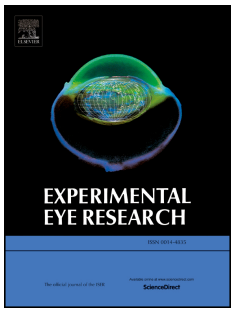

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Yen-Zhen Lu, Riccardo Natoli, Michele Madigan, Nilisha Fernando, Kartik Saxena, Riemke Aggio-Bruce, Haihan Jiao, Jan Provis, Krisztina Valter

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Photobiomodulation with 670nm light ameliorates Müller cell-mediated activation of microglia and macrophages in retinal degeneration

Authors and Affiliations

Yen-Zhen Lu[1], Riccardo Natoli[1,2], Michele Madigan[3,4], Nilisha Fernando[1], Kartik Saxena[1], Riemke Aggio-Bruce[1], Haihan Jiao[1], Jan Provis[1,2], Krisztina Valter[1,2]

[1]The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia.
[2]ANU Medical School, The Australian National University, Canberra, ACT, Australia.
[3]Save Sight Institute, Discipline of Clinical Ophthalmology, The University of Sydney, Sydney, NSW, Australia.
[4]School of Optometry and Vision Science, The University of New South Wales, Kensington, NSW, Australia.

Corresponding Author

Krisztina Valter
The John Curtin School of Medical Research,
Building 131, Garran Rd,
The Australian National University,
Canberra ACT 2601 Australia.
Email: krisztina.valter-kocsi@anu.edu.au

Abstract

Müller cells, the supporting cells of the retina, play a key role in responding to retinal stress by releasing chemokines, including CCL2, to recruit microglia and macrophages (MG/MΦ) into the damaged retina. Photobiomodulation (PBM) with 670nm light has been shown to reduce inflammation in models of retinal degeneration. In this study, we aimed to investigate whether 670nm light had an effect on Müller cell-initiated inflammation under retinal photo-oxidative damage (PD) *in vivo* and *in vitro*. Sprague-Dawley rats were pre-treated with 670nm light ($9J/cm^2$) once daily over 5 days prior to PD. The expression of inflammatory genes including CCL2 and IL-1β was analysed in retinas. *In vitro*, primary Müller cells dissociated from neonatal rat retinas were co-cultured with 661W photoreceptor cells. Co-cultures were exposed to PD, followed by 670nm light treatment to the Müller cells only, and Müller cell stress and inflammation were assessed. Primary MG/MΦ were incubated with supernatant from the co-cultures, and collected for analysis of inflammatory activation. To further understand the mechanism of 670nm light, the expression of COX5a and mitochondrial membrane potential (ΔΨm) were measured in Müller cells. Following PD, 670nm light-treated Müller cells had a reduced inflammatory activation, with lower levels of CCL2, IL-1β and IL-6. Supernatant from 670nm light-treated co-cultures reduced activation of primary MG/MΦ, and lowered the expression of pro-inflammatory cytokines, compared to untreated PD controls. Additionally, 670nm light-treated Müller cells had an increased expression of COX5a and an elevated ΔΨm following PD, suggesting that retrograde singaling plays a role in the effects of 670 nm light on Müller cell gene expression. Our data indicates that 670nm light reduces Müller cell-mediated retinal inflammation, and offers a potential cellular mechanism for 670 nm light therapy in regulating inflammation associated with retinal degenerations.

Keywords: Müller cells; 670nm light; photobiomodulation; retinal degeneration; oxidative stress; macrophages; inflammation; microglia

1. Introduction

Irradiation with low energy light wavelengths from far red to the near infrared spectrum (600nm-1000nm), termed as PBM, has been shown to display beneficial effects on various tissue injuries (Albarracin et al., 2011; Albarracin et al., 2013; Wong-Riley et al., 2005), such as accelerated wound healing in skin, decreased pain perception in joint disorders (Herranz-Aparicio et al., 2013) and reduced inflammation in autoimmune diseases (Brosseau et al., 2005). PBM has also been used to reduce neuroinflammation in rodent models of brain damage and spinal cord injury (Giacci et al., 2014; Hu et al., 2016).

PBM has been shown to be beneficial in human retinal diseases and animal models of age-related macular degeneration (AMD), diabetic retinopathy (DR) and retinitis pigmentosa (RP) (Abrahan et al., 2009; Albarracin et al., 2011; Geneva, 2016). It is proposed that cytochrome c oxidase (COX), the rate-limiting enzyme in terminal phosphorylation in the mitochondrial respiratory chain, is the most likely primary photoacceptor of 670nm light (Desmet et al., 2006; Karu, 1999). Exposure to 670nm light has shown to enhance COX activity in retinas (Begum et al., 2013; Kaynezhad et al., 2016) and primary neurons (Desmet et al., 2006; Wong-Riley et al., 2005), mediate the increase of redox states in mitochondria (Kaynezhad et al., 2016), increase ATP production (Calaza et al., 2015; Gkotsi et al., 2014; Wong-Riley et al., 2005) and up-regulate mitochondrial membrane potential ($\Delta\Psi m$) (Kokkinopoulos et al., 2013b). One of the beneficial effects of treatment with 670nm light is the apparent reduction of oxidative stress and the mitigation of the subsequent inflammatory response in the retina both of which are key features of several retinal diseases including AMD and DR (Tang et al., 2013; Whitcup et al., 2013).

However, due to the lack of understanding of the precise cellular signalling events during 670nm irradiation, there are still roadblocks in the translation of 670nm light therapy to the clinic (Hamblin, 2016). Previously we have demonstrated in the PD model that Müller cells, the principal macroglia of the retina, are the key source of the potent chemokine *Ccl2* in retinal (Rutar et al., 2012; Rutar et al., 2011a), which is responsible for the recruitment and activation of MG/MΦ in retinas (Grigsby et al., 2014; Natoli et al., 2017b; Rutar et al., 2015). We postulated that the anti-inflammatory effect of 670nm may be due to a reduction of Müller cell-mediated inflammation during retinal degeneration. Our previous *in vivo* study suggested that pre-treatment with 670nm light mitigated photo-oxidative damage-induced structural changes in Müller cells (Albarracin and Valter, 2012). However, this model did not allow us to investigate the direct effects of 670nm light on the activation of Müller cells. In this study, by using a co-culture system where primary Müller cells are exposed to the environment of damaged photoreceptors, we can mimic gliotic changes that occur during photoreceptor degeneration *in vivo*, while allowing us to investigate the effects of PBM directly on the Müller cells.

2. Materials and Methods

2.1 Animals and light exposure

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with ethics approval from the Australian National University Animal Experimentation Ethics Committee (Ethics ID: A2014/56). Albino Sprague-Dawley (SD) rats were born and raised in low light levels (5 lux) in a 12-hour light, 12-hour dark cycle. Food and water were available *ad libitum*. Twenty adult animals aged 100-120 postnatal (P) days were used for all experiments. Animals were separated into 4 experimental and control groups as follows: Control (n=5); Control + 670nm

(n=5); PD only (n=5); PD + 670nm (n=5). The animal received 670nm light prior to PD, according to previously described methods (Albarracin et al., 2011; Albarracin and Valter, 2012; Jager et al., 2008; Rutar et al., 2010). PD was induced by 2x36W COLDF2 fluorescent tubes, as described previously (Fernando et al., 2016) delivering 1000lux (147uW/cm^2) power density. Whole eyes and retinas were immediately collected after PD for histological analysis and RNA extraction. Five animals were used for each experimental group.

2.2 Maintenance of 661W photoreceptor-like cells

Murine photoreceptor-derived 661W cells were kindly gifted by Dr. Muayyad R. Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Centre, Oklahoma City, OK, USA). Cells for experimental purposes were used within five passages of authentication, and validation of authenticity was performed using gene expression of green cone pigments and cone arrestin. Cells were further validated for species authenticity (CellBank, Sydney, Australia). Cells were cultured in growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 6mM L-glutamine (Thermo Fisher Scientific, MA, USA) and antibiotic-antimycotic (100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Fungizone; Thermo Fisher Scientific), and incubated in dim conditions in a humidified atmosphere of 5% CO_2 at 37°C in the dark.

2.3 Preparation of rat primary Müller cells

Retinas were isolated from 36 SD rats aged P8-10 days following the modified methods described previously (Hicks and Courtois, 1990). Isolated retinas were immersed in DMEM supplemented with 6mM L-glutamine and antibiotic-antimycotic for 2 hours at 4°C. Retinas were dissociated into small fragments and then incubated with DMEM containing 0.1% trypsin, 70U/ml collagenase type IV, and antibiotic-antimycotic for 1 hour at 37°C. Cell

pellets were collected by spinning at 210g for 10 minutes at 37°C and then dissociated in fresh GM by triturating with plastic serological pipettes. Cell suspension was cultured in a T25 flask (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2 at 37°C. The GM was left unchanged for the initial 4 days; GM was replenished on day 5. After 7-8 days, cellular aggregations, which are attached to the base of the flask, were removed by agitating the media and the GM was replenished. Until cells reached 90% confluency, cells were detached with 0.25% trypsin/EDTA from a T25 flask and then expended in a T75 flask (Thermo Fisher Scientific). After reaching 90% confluency in a T75 flask, cells were subcultured in the appropriate plates for subsequent experiments. Primary Müller cell characteristics were confirmed using immunolabelling for vimentin, S100β, glutamate synthetase (GS) (data not shown).

2.4 Photo-oxidative damage in co-culture of 661W cell line with primary Müller cells

For mimicking *in vivo* interactions between photoreceptors and Müller cells, a transwell co-culture system was used. Primary Müller cells were seeded into 24-well plates at a density of $2.5x10^4$ cells per well or 6-well plates at density of $2x10^5$ cells per well in GM, and plates incubated in 5% CO_2 at 37°C. After 24 hours, in separate plates, 661W cells were seeded onto the membranes of transwell inserts (pore size 0.4µm; Corning, NY, USA) at a density of $4x10^3$ cells per insert (24-well transwell), or at a density of $5x10^4$ cells per insert (6-well transwell) in GM, and were incubated with 5% CO_2 at 37°C for 24 hours. Following incubation, inserts containing 661W cells were placed into wells seeded with Müller cells. The co-cultures were incubated in reduced-serum DMEM (supplemented with 1% FBS, L-glutamine and antibiotic-antimycotic) and exposed to 15,000 lux light ($2.2mW/cm^2$; irradiance measured with PM100D optical power meter, THORLABS, NJ, USA) from two white fluorescent lamps (2x10W T4 tri-phosphor 6500K daylight fluorescent tubes; Crompton, NSW, Australia), for 4.5 hours with 5% CO_2 at 37°C. Control plates were placed

in the same incubator, but shielded with aluminium foil to avoid light exposure. For air/gas exchange, small incisions were cut on the aluminium foil. Following 4.5 hours of PD, co-cultures were incubated under dim light conditions with 5% CO_2 at 37°C. After 24 hours of recovery, cells and supernatant were collected for analysis.

2.5 670nm red light treatment of co-cultures

The 670nm LED array (Quantum Devices) was applied to different co-culture groups (with or without PD stress) as follows. **(A)** PD + 670nm group - inserts containing 661W cells were removed from the co-culture during 670 nm light exposure. Only Müller cells were exposed to 670nm light ($9J/cm^2$) and were treated three times over the first 12 hours of recovery following PD. **(B)** Control + 670nm group - Müller cells received treatment with 670nm light using the same paradigm, but with no PD. For **(C)** PD only and **(D)** control groups, the inserts were removed from plates and only Müller cells were exposed to the 670nm LED array, but with the light source switched off.

2.6 Isolation and assessment of activation of rat MG/MΦ

Rat retinal microglia and macrophages (MG/MΦ) from SD rats (P50-60) were isolated using a fluorescence-activated cell sorter (FACS) (BD FACSAria II; BD Biosciences, NJ, USA), using previously described protocols with minor modifications (Fernando et al., 2016; Ma et al., 2013; Rutar et al., 2015). Isolated cells were subsequently cultured in GM containing mouse granulocyte-macrophage colony-stimulating factor (GM-CSF, 1ng/ml; Stem Cell Technologies, Vancouver, Canada) with 5% CO_2 at 37°C. Media was replaced every 3-4 days until cells reached 80% confluency.

Supernatant (SN) was collected from co-cultures of the control, 670nm only, PD only and PD+670nm groups. MG/MΦ cells were incubated with the SN of these groups for 24

hours with 5% CO_2 at 37°C. MG/MΦ were then collected for RNA extraction, or immunostained with CD11b/CD86 for flow cytometry. MG/MΦ were collected and fixed in 2% paraformaldehyde (PFA) for 10 minutes on ice. Cells were blocked with 1% BSA and then incubated with biotin CD86 (1:100; Biolegend), or anti-rat CD11b PE antibody in 1% BSA for 30 minutes on ice. Cells were washed once in PBS containing 0.2% Tween-20 and resuspended in the secondary antibody with streptavidin conjugated with Alexa-Fluor-488 (S32453; Thermo Fisher Scientific) for 30 minutes on ice. The expression of CD11b or CD86 was measured using FACSort (LSRII; BD, CA, USA) and data was analysed using FlowJo (FLOWJO, OR, USA).

2.7 Assessment of cell viability and cell toxicity

For measuring cell viability, an ATPlite 1 step assay (PerkinElmer, MA, USA) and an MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Sigma-Aldrich) were used according to the manufacturer's instructions and protocols previously described (Lu et al., 2013; Natoli et al., 2016). A CellTox assay (Promega, Madison, WI, USA) was performed to measure cell death according to previously described methods (Natoli et al., 2016).

2.8 Immunocytochemistry on primary Müller cells

Müller cells were seeded onto poly-L-lysine-coated (100µg/ml; Sigma-Aldrich) glass coverslips at a density of $30x10^4$ cells/mm^2 for 48 hours and then co-cultured with 661W cells with/without 670nm light as described above. Coverslips containing Müller cells were fixed with 2% PFA for 30 minutes and then washed twice with PBS. Immunocytochemistry was performed as described previously (Albarracin et al., 2011; Albarracin and Valter, 2012) using a primary antibody for COX5a (1:500, #ab110262, Abcam), an anti-mouse IgG (H+L) conjugated with biotin (SAB3701153; Sigma-Aldrich) and Streptavidin conjugated with

Alexa-Fluor-488 (S32453; Thermo Fisher Scientific). Fluorescence was visualized using a laser-scanning $A1^+$ confocal microscope (Nikon, Tokyo, Japan) and captured with NIS-Element AR software (Nikon).

2.9 Quantitative real-time PCR (qPCR)

Retinas were collected and stored in RNA stabilizer (RNAlater; Thermo Fisher Scientific) overnight at 4°C. RNA extraction was performed described previously (Rutar et al., 2011a), using a combination of TRIzol (Thermo Fisher Scientific) and an RNAqueous Total RNA Isolation kit (Thermo Fisher Scientific) following the manufacturer's instructions. cDNA was synthesised from extracted RNA using a Tetro cDNA Synthesis Kit (Bioline, London, UK), according to the manufacturer's protocol. Gene expression was determined by real-time quantitative PCR (qPCR), using Taqman hydrolysis probes (Table 1; Thermo Fisher Scientific) and Taqman Gene Expression Master Mix (Thermo Fisher Scientific), which were applied according to the manufacturer's instructions. qPCR reactions were run in duplicate using a QuantStudio Flex 12K instrument (Thermo Fisher Scientific). Data analysis was performed using the comparative cycle threshold method ($\Delta\Delta C_t$), which was normalised to the expression of the *Gapdh* reference gene.

2.10 In situ hybridization

To localise *Ccl2* mRNA expression in retinas, *Ccl2* was cloned from PCR products (550-bp amplicon) using cDNA synthesis from retinal RNA (as described above). A digoxigenin (DIG)-labelled riboprobe for *Ccl2* mRNA was synthesised according to our previous publication (Rutar et al., 2011b). *In situ* hybridisation was used on retinal cryosections as described previously (Cornish et al., 2005). Briefly, the *Ccl2* riboprobe was hybridized overnight at 55°C and then washed in a series of saline sodium citrate solutions (pH 7.4) at 60°C. The bound probe was visualised using NBT/BCIP.

2.11 Enzyme-Linked Immunosorbent Assay

Cell culture media were assayed for IL-6 (#R000B, R&D Systems, MN, USA) and CCL2 (#ab100777, Abcam, Cambridge, UK) using a sandwich enzyme-linked immunosorbent assay (Cornish et al.) as per the manufacturer's instructions.

2.12 Western blotting

Whole cell protein lysates were extracted using the Cellytic M buffer (Sigma-Aldrich) containing a Protease Inhibitor Cocktail (Sigma-Aldrich). Western blotting was performed according to previously described methods with minor modifications (Begum et al., 2013; Walker and Steinle, 2007). 20µg of denatured protein was loaded onto a 4-20% Mini-Protean TGX Precast Protein gel (Bio-Rad, CA, USA) followed by semi-dry transfer to a nitrocellulose membrane. To measure the protein expression of COX5a in cells, a COX5a primary antibody (1:500-1:1000, #ab110262, Abcam) was used, as well as a secondary antibody-peroxidase conjugate for visualisation (Bio-Rad). The protein was visualised with chemiluminescence using a Clarity Western ECL kit (Bio-Rad) and images captured and analysed using a Chemidoc MP with Image Lab software (Bio-Rad). The expression of COX5a was normalized to GAPDH.

2.13 Mitochondrial membrane potential (ΔΨm)

The JC-1 dye (Sigma-Aldrich) was used to assess changes in the mitochondrial membrane potential (ΔΨm) of living cells, following previously published methodology (Kokkinopoulos et al., 2013b; Smiley et al., 1991). Fluorescence of JC-1 (525nm/575nm) in cells was measured using flow cytometry (FACSort, LSRII) and analysed with FlowJo (FlowJo, OR, USA). Data is presented as a ratio of red to green fluorescence intensity.

2.14 Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA). Data were analysed using Tukey's multiple comparisons test, with $P<0.05$ considered to represent a statistically significant difference. All data is represented as the mean ± SEM.

3. Results

3.1 670nm light suppressed cytokine expression following PD *in vivo*

We examined the expression of *Il-1β* and *Ccl2* in rat retinas following photo-oxidative damage (PD) and assessed the effect of 670nm light treatment. The expression of *Il-1β* and *Ccl2* increased significantly in PD retinas compared to dim-reared controls ($P<0.05$, Figure 1A, B). 670nm light-treated animals had significantly reduced levels of *Il-1β* and *Ccl2* expression compared to untreated PD retinas ($P<0.05$). *Il-1β* and *Ccl2* expression was comparable between 670nm-treated dim-reared animals and dim-reared controls (without 670nm treatment or PD, Figure 1A, B).

For localisation of *Ccl2* in the PD retinas, we examined *Ccl2* expression using *in situ* hybridisation (Figure 1C-F). In PD retinas (Figure 1E) a large number of *Ccl2*-positive cells were apparent in the inner nuclear layer, where the nuclei of Müller cells reside. Conversely, PD retinas treated with 670 nm light had fewer *Ccl2*-positive cells in the INL compared to untreated PD retinas (Figure 1F). Quantification of the number of *Ccl2*-positive cells in the INL demonstrated that 670nm light significantly suppressed cells in the INL to express *Ccl2* post-PD compared to untreated PD retinas ($P<0.05$, Figure 1G).

3.2 670nm light reduced stress in primary Müller cells co-cultured with damaged 661W cells

We assessed Müller cell changes under photoreceptor damage-initiated stress, and investigated whether treatment with 670nm light had an effect on these changes. Co-culturing

primary Müller cells with photo-oxidative damaged-661W cells did not have a significant effect on Müller cell death (Figure 2D), survival (MTT, Figure 2C), and their ability to produce ATP (Figure 2B). Treatment with 670nm light had no significant effect on these factors (Figure 2B-D). When assessing activation of Müller cells, we found that expression of *Gfap*, a marker of gliosis, was significantly down-regulated in 670nm-treated Müller cells compared to non-treated stressed cells ($P<0.05$, Figure 2E). *Rlbp1* has been linked with the maintenance of normal metabolic homeostasis in the retina. There was a significant reduction in *Rlbp1* expression in stressed Müller cells (Figure 2F). Treatment with 670nm prevented this loss of expression in stressed Müller cells ($P<0.05$, Figure 2F).

To further confirm whether 670nm light influences the function of Müller cells in PD, the neuroprotective effect of Müller cells on 661W photoreceptor cells was measured. In the absence of Müller cells, PD increased cell death (Figure 2I), reduced viability (MTT, Figure 2H) and reduced ATP production (Figure 2G) in 661W cells, compared to non-damaged 661W cells. When co-cultured with Müller cells, 661W cells had elevated levels of ATP and MTT and a reduced level of cell death following PD, compared to control 661W cells ($P<0.05$). Co-culture with Müller cells treated with 670nm light resulted in increased cell survival and ATP production of 661W cells, as well as reduced cell death. However, these changes were not significantly different from those co-cultured with non-treated Müller cells (Figure 2G-I). Co-culture with Müller cells, treated or un-treated with 670nm, did not alter viability or death of control (non-PD) 661W cells (Figure 2G-I).

3.3 670nm light suppressed oxidative stress and inflammation in Müller cells following PD

A key feature of retinal degenerations is the increased production of free radicals and oxidative stress (Nita and Grzybowski, 2016). We examined the gene expression levels of

two NADPH oxidases, *Nox1* and *Nox4*. *Nox1* gene expression significantly increased in activated Müller cells compared to controls, while the expression of *Nox4* did not change significantly (Figure 3B, C). Treatment with 670nm light significantly reduced the expression of both *Nox1* and *Nox4* in activated Müller cells ($P<0.05$, Figure 3B, C).

Another hallmark of neurodegenerative diseases is the activation of the immune response (Chen and Xu, 2012). We examined the expression of CCL2, a potent chemokine produced by Müller cells shortly after retinal injury, as well as IL-6, a pro-inflammatory cytokine. Müller cells activated by the supernatant of PD-damaged 661W cells showed an increased gene and protein expression of CCL2 (Figure 3D, F). Treatment with 670nm light reduced CCL2 expression significantly (Figure 3D, F), confirming our *in vivo* findings (Figure 1B, G). *Il-6* gene expression did not change in activated Müller cells, but was significantly reduced by 670nm irradiation ($P<0.05$, Figure 3E). IL-6 protein levels were significantly lower in activated Müller cells than controls, and 670nm treatment reduced IL-6 protein levels further ($P<0.05$, Figure 3G).

3.4 670nm light treatment has no effect on NLRP3 inflammasome activation in Müller cells

NLRP3 inflammasome activation has been found in retinal degenerations including AMD (Doyle et al., 2012a; Tarallo et al., 2012). Using the *in vivo* PD model, we have previously shown an increase in the expression of IL-1β in recruited microglia and macrophages and subsequent NLRP3 inflammasome activation (Natoli et al., 2017b). Using our *in vitro* model, we investigated whether this inflammasome activation is Müller cell related, and if 670nm light treatment can influence inflammasome activation by its direct effect on Müller cells. The gene expression levels of *Casp8*, *Nlrp3*, *Pycard*, *Casp1* and *Il-18* were not significantly different in the activated Müller cells compared to controls. Treatment

with 670nm light did not have any altering effect on their expression (Figure 4B-F). The expression of *Il-1β* increased significantly in activated Müller cells, and 670nm treatment significantly reduced the expression level of this pro-inflammatory cytokine ($P<0.05$, Figure 4G), confirming our finding in *in vivo* (Figure 1A).

3.5 Mitochondrial function is improved by 670nm light following PD

Cytochrome c oxidase (COX) is the terminal component of the electron transport chain in mitochondria and is the most likely photoacceptor of 670nm light (Desmet et al., 2006; Diaz, 2010; Karu, 1999). COX5a is one of 13 subunits of the COX enzyme in mammals. To understand whether COX participates in the effects of 670nm light on Müller cells, the expression of COX5a was measured in Müller cells. Low expression of COX5a was detected in the cytoplasm of Müller cells co-cultured with normal 661W cells (Figure 5B). Treatment with 670nm light of control Müller cells resulted in a significantly increased COX5a protein expression. In activated Müller cells, COX5a expression was significantly higher than control cells, and this expression further increased when activated cells were treated with 670nm ($P<0.05$, Figure 5B). Western blot analysis was used to quantify these changes (Figure 5C). Treatment with 670nm light increased the protein levels of COX5a in both control and activated Müller cells ($P<0.05$, Figure 5C, D). The increased expression of COX5a in control Müller cells following 670nm treatment confirmed the mechanistic effects of PBM. However, exposure to PD led to a higher expression of COX5a in Müller cells ($P<0.05$).

The mitochondrial membrane potential ($\Delta\Psi m$) is an important driver of ATP production and thus is a measure of mitochondrial function. The $\Delta\Psi m$ significantly dropped in activated Müller cells compared to controls ($P<0.05$, Figure 5E). Treatment with 670nm

did not alter ΔΨm in control cells, but increased in activated Müller cells, compared to untreated activated cells ($P<0.05$, Figure 5E).

3.6 670nm light regulated Müller cell-mediated activation of microglia and macrophages (MG/MΦ) following PD

After incubation with control supernatant (SN) (Figure 6B), or 670nm SN (Figure 6C), primary MG/MΦ displayed a ramified morphology with defined processes, indicating a resting state. However, the shape of MG/MΦ became more amoeboid with smaller processes following incubation with SN from activated Müller cells (PD SN, Figure 6D), indicating activation of these MG/MΦ. After incubation with SN from 670-treated activated Müller cells (PD+670nm SN, Figure 6E), MG/MΦ were more ramified and processes extending from cell soma were apparent, indicating that 670nm reduced activation of MG/MΦ.

We used flow cytometry to detect the fluorescence intensity of CD11b (Figure 6F-G) and CD86 expression (Figure 6H-I) after stimulation with differing SN to assess their activation state. MG/MΦ had a higher expression of CD11b and CD86 after incubation with PD SN compared to cells incubated with PD+670nm SN ($P<0.05$, Figure 6F-I), suggesting that PBM reduced macrophage activation. To further confirm MG/MΦ activation, we assessed the gene expression of *Ccl2*, *Il-1β, Tnfα*, *Il-6, Il-10* and *Sod2,* which are all markers of macrophage activation (Figure 6J-O). The expression of these genes was increased in MG/MΦ incubated in PD SN, indicating that supernatant from PD-stressed Müller cells have an activating effect on these cells. Treatment with 670nm significantly lowered MG/MΦ activation as suggested by the reduced expression of these genes ($P<0.05$, Figure 6J-O).

4. Discussion

Treatment with 670nm red light has been shown to reduce inflammation in retinal diseases (Geneva, 2016), however, there is still a lack of understanding of the precise cellular mechanisms underpinning its anti-inflammatory effects. The current study offers insight into cellular signalling pathways influenced by photobiomodulation during retinal degeneration, and demonstrates that treatment with 670nm light reduces Müller cell gliosis and subsequent MG/MΦ activation. Firstly, 670nm light suppressed glial activation by reducing the expression of pro-inflammatory cytokines, chemokines and oxidative stress components in Müller cells exposed to damaged photoreceptors. Secondly, 670nm light reduced activation of MG/MΦ by influencing the expression of inflammatory activators by Müller cells. Thirdly, we demonstrated that a potential mechanism of action of 670nm light is through the support of mitochondrial membrane potential ($\Delta\Psi m$) to stabilise mitochondrial function, and the improvement of metabolic function in stressed Müller cells.

4.1 670nm light modulates Müller cell reactive gliosis

In our earlier *in vivo* studies, we found that 670nm treatment reduced photoreceptor death following PD, and mitigated Müller cell stress (Albarracin et al., 2011; Albarracin and Valter, 2012). However, what remained unclear was whether 670nm light directly regulates Müller cell stress, or indirectly influences their activation through the damaged photoreceptors (Abrahan et al., 2009). In the current study, as only Müller cells were exposed to 670nm light, we were able to isolate the direct effects of 670nm on these macroglia.

We show that treatment with 670nm light reduces Müller cell stress in isolated cells, as evidenced by their reduced expression of *Gfap* following PD, which is consistent with previous *in vivo* studies using PD (Albarracin et al., 2011; Marco et al., 2013) and ageing (Begum et al., 2013). GFAP is a well-known marker of Müller cell stress and gliosis in retinal diseases (Albarracin et al., 2011; Bringmann et al., 2009; Bringmann et al., 2006;

Bringmann and Wiedemann, 2012). Mice deficient in GFAP exhibit less glial scars after retinal detachment (Nakazawa et al., 2007; Verardo et al., 2008), indicating a reduced activation of Müller cells. This may lead to the reduced expression of CCL2 and subsequent macrophage infiltration into retinas following damage (Nakazawa et al., 2007).

4.2 670nm light reduces Müller cell-mediated activation of MG/MΦ

In the present study, we found the rapid upregulation of *Ccl2* and *Il-1β* in the PD model *in vivo*, and by using a co-culture system of 661W photoreceptors and primary Müller cells *in vitro*, we have confirmed that the source of these factors are the Müller cells. Furthermore, using supernatant from activated Müller cells, we were able to initiate activation of MG/MΦ, confirming the direct link between Müller cell and macrophage activation. We have previously demonstrated that inhibition of *Ccl2* and *Il-1β* reduces the infiltration of microglia and macrophages into the damaged outer retina in PD models (Natoli et al., 2017b; Rutar et al., 2012). The accumulation of MG/MΦ in the subretinal space is a well-established feature of retinal degenerations (Fernando et al., 2016; Knickelbein et al., 2015), which is associated with photoreceptor death (Natoli et al., 2017a; Zhao et al., 2015).

The present study has demonstrated that treatment with 670nm light mitigates *Ccl2* and *Il-1β* expression in the retina *in vivo*, and in primary Müller cells *in vitro*. Further, we found that 670nm light-treated Müller cells were able to mitigate MG/MΦ activation. This indicates that 670nm light targets the expression of pro-inflammatory cytokines and chemokines by Müller cells. Previous studies have found that 670nm light resulted in reduced activation and recruitment of MG/MΦ in retinal damage *in vivo* (Albarracin et al., 2011; Begum et al., 2013; Kokkinopoulos, 2013a; Kokkinopoulos et al., 2013b), but to our knowledge, this is the first report that demonstrated the direct effect of 670nm light on Müller cells and its downstream effect of reduced MG/MΦ activation.

The inflammasome has been linked to the progression of retinal degenerations such as AMD (Doyle et al., 2012a; Tarallo et al., 2012), and generation of IL-1β is one of the direct consequences of NLRP3 inflammasome activation (Ambati et al., 2013; Schroder and Tschopp, 2010). In the current study, we demonstrated that the irradiation of Müller cells with 670nm light leads to down-regulation of IL-1β in response to PD. However, it does not influence the expression of the NLRP3 inflammasome in these cells, indicating that the production of IL-1β may be inflammasome-independent in some retinal cell types (Netea et al., 2015). Our results showed that Müller cells are not major contributors to inflammasome activation, further supporting the view that the RPE and microglia/macrophages are the primary sites of inflammasomes (Doyle et al., 2012b; Kataoka et al., 2015; Kauppinen et al., 2016; Tseng et al., 2013).

4.3 670nm light increased COX5a expression and elevated ΔΨm

We have shown that 670nm light treatment leads to an increase in *Rlbp1* in Müller cells. RLBP1 is required for the maintenance of normal metabolic function (Taylor et al., 2015), so the increase towards control levels suggests that treatment with 670nm light supports Müller cells in maintaining normal cellular functions, to withstand tissue stress caused by PD and to protect photoreceptors. In addition, we also found that the treatment with 670nm light increased the ΔΨm and expression of COX5a in Müller cells under PD. COX is complex IV of the mitochondrial respiratory chain, known to absorb energy from far red to near infrared light (Schroeder et al., 2007). This can trigger a higher ΔΨm, which generates ATP in cells (Verardo et al., 2008). Kaynezhad and colleagues reported that 670nm light increased the level of oxidized COX, for 1-2 hours after a single 5-minute exposure (Kaynezhad et al., 2016). Elevated production of ATP and higher protein expression of COX were observed in models of retinal degeneration. The ΔΨm was increased in RPE cells in response to 670nm light in aged mice (Begum et al., 2013; Kokkinopoulos et al., 2013b). The

current study is consistent with these results, and although we did not detect a higher level of ATP production in 670nm light-treated Müller cells, higher levels of ΔΨm and COX5a were observed in this study. Therefore, we speculate that ATP production is a consequence of up-regulated ΔΨm and COX5a.

PBM is known to potentially influence cellular DNA and RNA synthesis (Karu, 1999). As photoacceptors are located in the mitochondria, Karu et al. proposed that mitochondrial retrograde signalling can be triggered with PBM (Karu, 2008). PBM-induced changes in ΔΨm, ROS, and calcium mobilization may be associated with mitochondrial retrograde signalling that leads to modifications in DNA and RNA expression in the nucleus. A growing body of evidence suggests that increased ΔΨm is related to changes in gene expression. Gavish and colleagues reported an increase in ΔΨm in human keratinocytes immediately after exposure to 780nm light with a subsequent reduction in IL-6 gene expression 2 hours later (Gavish et al., 2004). Treatment with an inhibitor of the electron transport chain abolished PBM-induced gene expression changes in fibroblasts following irradiation with 760nm or 1140nm light (Schroeder et al., 2007). Therefore, our data strongly support the hypothesis that PBM stimulates mitochondrial retrograde signaling providing further insight into the cellular mechanisms underlying PBM. The understanding of these mechanisms are crucial in the efforts to transition light-based therapy (PBM) from experimental models to clinical acceptance and clinical applications.

5. Conclusions

Our findings suggest that 670nm light can directly affect Müller cells and mitigate stress-induced inflammation, which subsequently can reduce microglia/macrophage activation and recruitment. Further, the maintenance of mitochondrial function in Müller cells may enhance their tissue support functions, and thereby contribute to the neuro-protective effects of 670nm light. These data suggest that 670nm light plays a key role in controlling

inflammation during retinal stress. As a non-invasive and relatively non-expensive treatment, 670nm light has adjuvant therapeutic potential for retinal degenerations where inflammation, macrophage recruitment and photoreceptor loss play a key role.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Authors' contributions

YZL designed the experiments, conducted the experiments as well as analysis, and wrote the paper; RN conceived the study, designed the experiments, and revised paper; MM conceived the study, designed the experiments, and revised paper; NF edited and revised the paper; KS conducted the experiments and acquired data; RAB supported techniques. HJ supported techniques; JP conceived the study and interpreted data; KV obtained funding, conceived the study, designed the experiments, and revised paper; all contributing authors have read and approved the final version of the paper.

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Table 1. Taqman hydrolysis probes used for qPCR

Gene Symbol	**Gene Name**	**Catalog Number**	**Entrez Gene ID**
Casp1	Caspase 1	Rn00562724_m1	25166
Casp8	Caspase 8	Rn00574069_m1	64044
Ccl2	Chemokine (C-C motif) ligand 2	Rn01456716_g1	24770
Cox5a	Cytochrome c oxidase, subunit Va	Rn00821806_m1	252934
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1	24383
Gfap	Glial fibrillary acidic protein	Rn00566603_m1	24387
Il-1β	Interleukin 1 beta	Rn00580432_m1	24494
Il-6	Interleukin 6	Rn01410330_m1	24498
Il-10	Interleukin 10	Rn00563409_m1	25325
Il-18	Interleukin 18	Rn01422083_m1	29197
Nlrp3	NLR family, pyrin domain containing 3	Rn04244620_m1	287362
Nox1	NADPH oxidase 1	Rn00586652_m1	114243
Nox4	NADPH oxidase 4	Rn00585380_m1	85431
Pycard	PYD and CARD domain containing	Rn00597229_g1	282817
Rlbp1	Retinaldehyde binding protein 1	Rn01477965_m1	293049
Sod2	Superoxide dismutase 2, mitochondrial	Rn00690588_g1	24787
Tnf	Tumor necrosis factor	Rn00562055_m1	24835

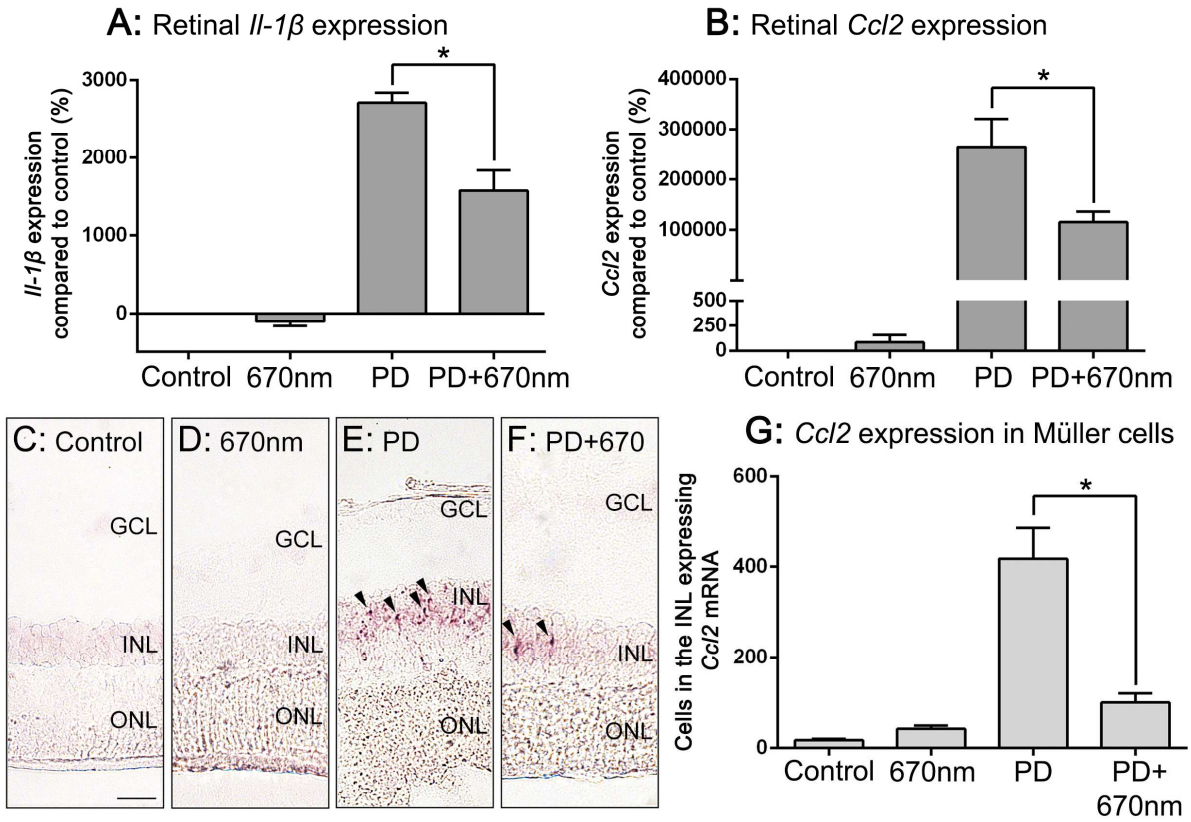


Figure 1. *In vivo* expression of cytokines was mitigated by 670nm light treatment following photo-oxidative damage (PD) in rat retinas. **A-B:** PD significantly increased levels of *Il-1β* (A) and *Ccl2* (B) in retinas compared to dim-reared controls (P<0.05). However, *Il-1β* and *Ccl2* were significantly reduced after 670nm light treatment compared to PD retinas (P<0.05). **C-F:** *Ccl2 in situ* hybridization revealed that numerous *Ccl2*-positive cells (arrows) were present in the INL of PD retinas (E) compared to dim-reared controls (C). After 670nm light treatment, a lower number of *Ccl2*-positive cells were detected in PD retinas (F). **G:** 670nm light significantly reduced the number of *Ccl2*-positive Müller cells in the INL following PD (P<0.05). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. N=5 was used for all experimental comparisons, except for (B) (N=10), and was performed in biological duplicate. The data is presented as the mean ± SEM. * denotes a significant change (P<0.05).

Colour should be used

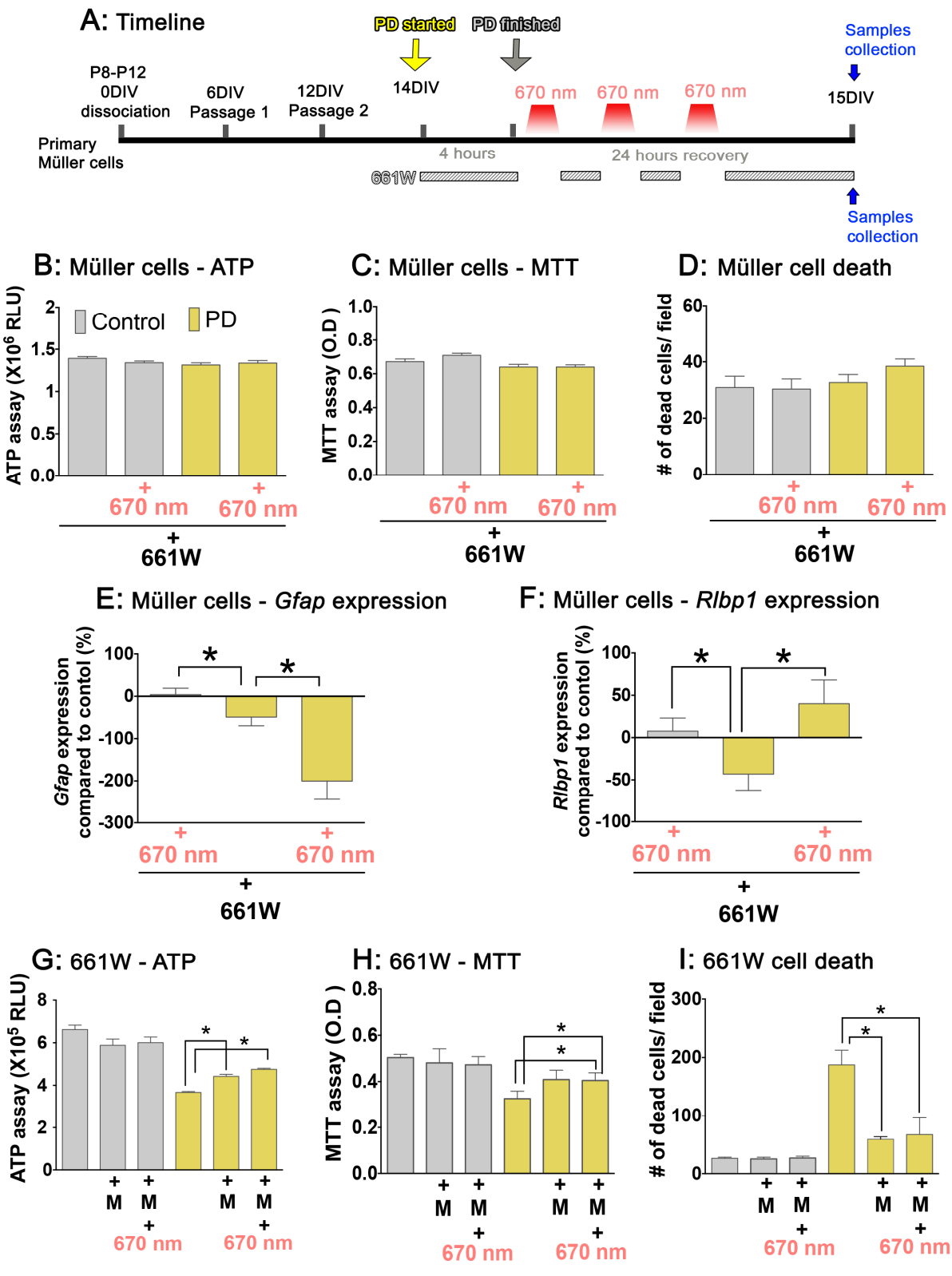
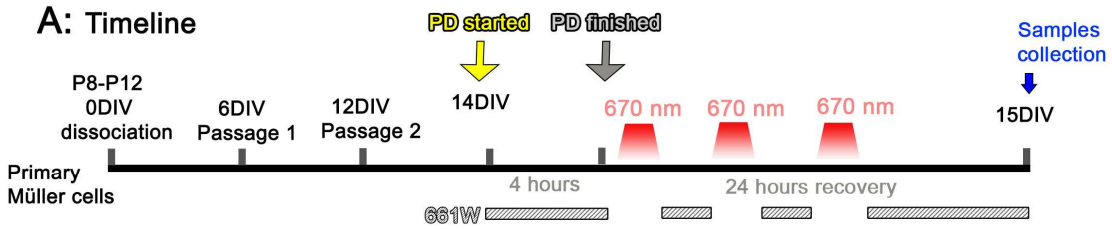

A: Timeline
PD started
PD finished
Samples collection
P8-P12 0DIV dissociation
6DIV Passage 1
12DIV Passage 2
14DIV
670 nm
670 nm
670 nm
15DIV
Primary Müller cells
4 hours
24 hours recovery
661W
Samples collection
B: Müller cells - ATP
ATP assay (X10^6 RLU)
Control
PD
670 nm
670 nm
661W
C: Müller cells - MTT
MTT assay (O.D)
670 nm
670 nm
661W
D: Müller cell death
# of dead cells/ field
670 nm
670 nm
661W
E: Müller cells - Gfap expression
Gfap expression compared to contol (%)
670 nm
670 nm
661W
F: Müller cells - Rlbp1 expression
Rlbp1 expression compared to contol (%)
670 nm
670 nm
661W
G: 661W - ATP
ATP assay (X10^5 RLU)
M
670 nm
H: 661W - MTT
MTT assay (O.D)
I: 661W cell death
# of dead cells/ field

Figure 2. 670nm light treatment reduced primary Müller cell stress when co-cultured with 661W cells exposed to photo-oxidative damage (PD). **A:** Timeline of experimental paradigm. **B-D:** Neither PD nor 670nm light influenced ATP (B), MTT (C) and cell death (D) in Müller cells. **E:** 670nm light reduced *Gfap* expression in Müller cells following PD ($P<0.05$). **F:** Expression of *Rlbp1* was elevated in 670nm-treated Müller cells compared to untreated Müller cells ($P<0.05$). **G-I:** Following PD, co-culturing Müller cells (M) with 661W cells increased ATP (G) and MTT (H) and diminished cell death (I) in 661W cells compared to 661W cells cultured alone ($P<0.05$). ATP, MTT and cell death were not affected by 670nm light treatment. DIV; days *in vitro*. N=6 was used for all experimental comparisons and was performed in biological triplicate. The data is presented as the mean ± SEM. * denotes a significant change ($P<0.05$).

Colour should be used


A: Timeline
PD started
PD finished
Samples collection
P8-P12
0DIV
dissociation
6DIV
Passage 1
12DIV
Passage 2
14DIV
670 nm
670 nm
670 nm
15DIV
Primary
Müller cells
4 hours
24 hours recovery
661W

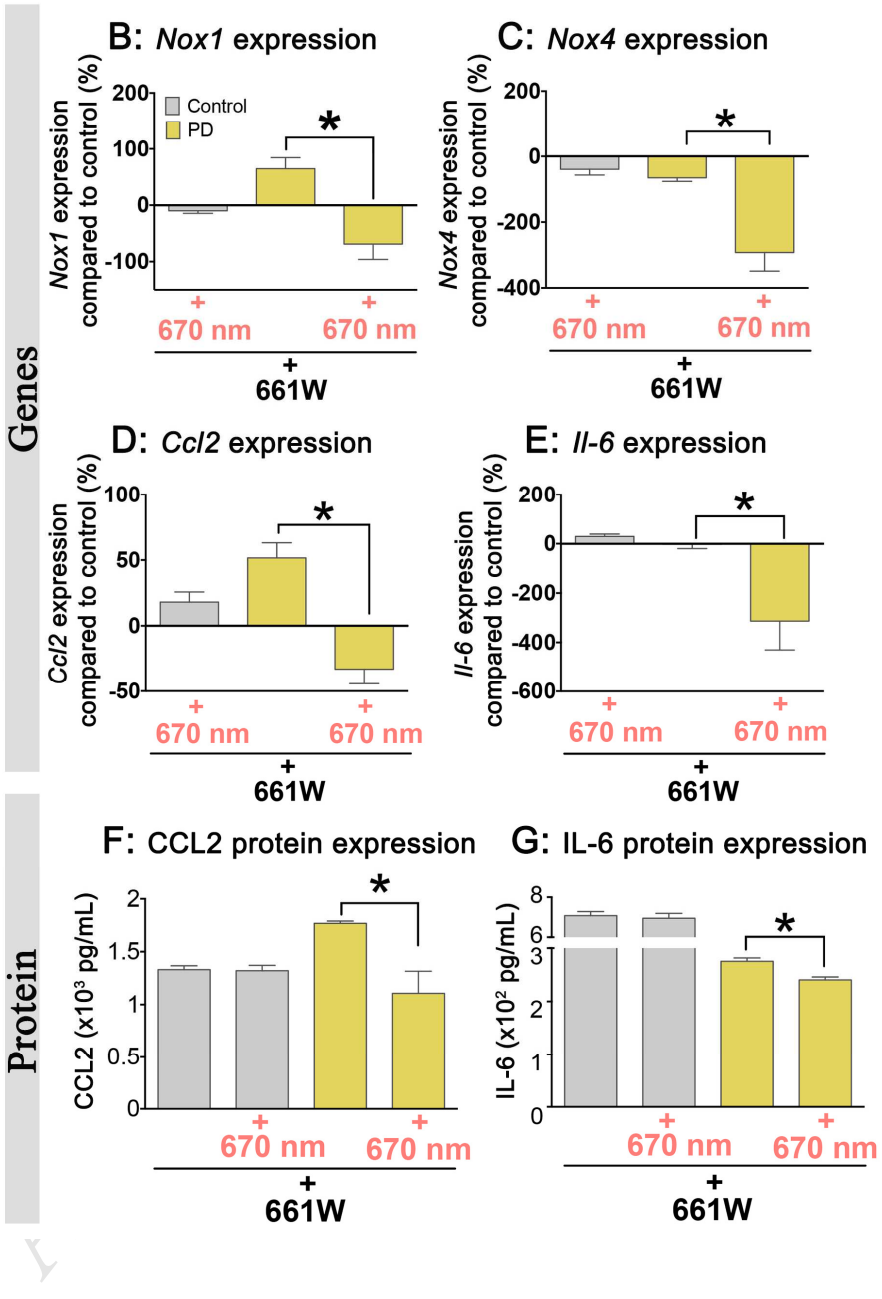

Genes
B: Nox1 expression
Nox1 expression compared to control (%)
Control
PD
C: Nox4 expression
Nox4 expression compared to control (%)
D: Ccl2 expression
Ccl2 expression compared to control (%)
E: Il-6 expression
Il-6 expression compared to control (%)
670 nm
661W
Protein
F: CCL2 protein expression
CCL2 (x10³ pg/mL)
G: IL-6 protein expression
IL-6 (x10² pg/mL)

Figure 3. Müller cell-derived inflammation was mitigated by exposure to 670nm light following photo-oxidative damage (PD). **A:** Timeline of experimental paradigm. **B-E:** Müller cells exposed to 670nm light had significantly reduced *Nox1* (B), *Nox4* (C), *Ccl2* (D) and *Il-6* (E) expression compared to untreated Müller cells ($P<0.05$). **F-G:** CCL2 and IL-6 in co-culture supernatant was significantly decreased by 670nm light post-PD ($P<0.05$). DIV; days *in vitro*. N=6 was used for all experimental comparisons and was performed in biological triplicate. The data is presented as the mean ± SEM. * denotes a significant change ($P<0.05$).

Colour should be used

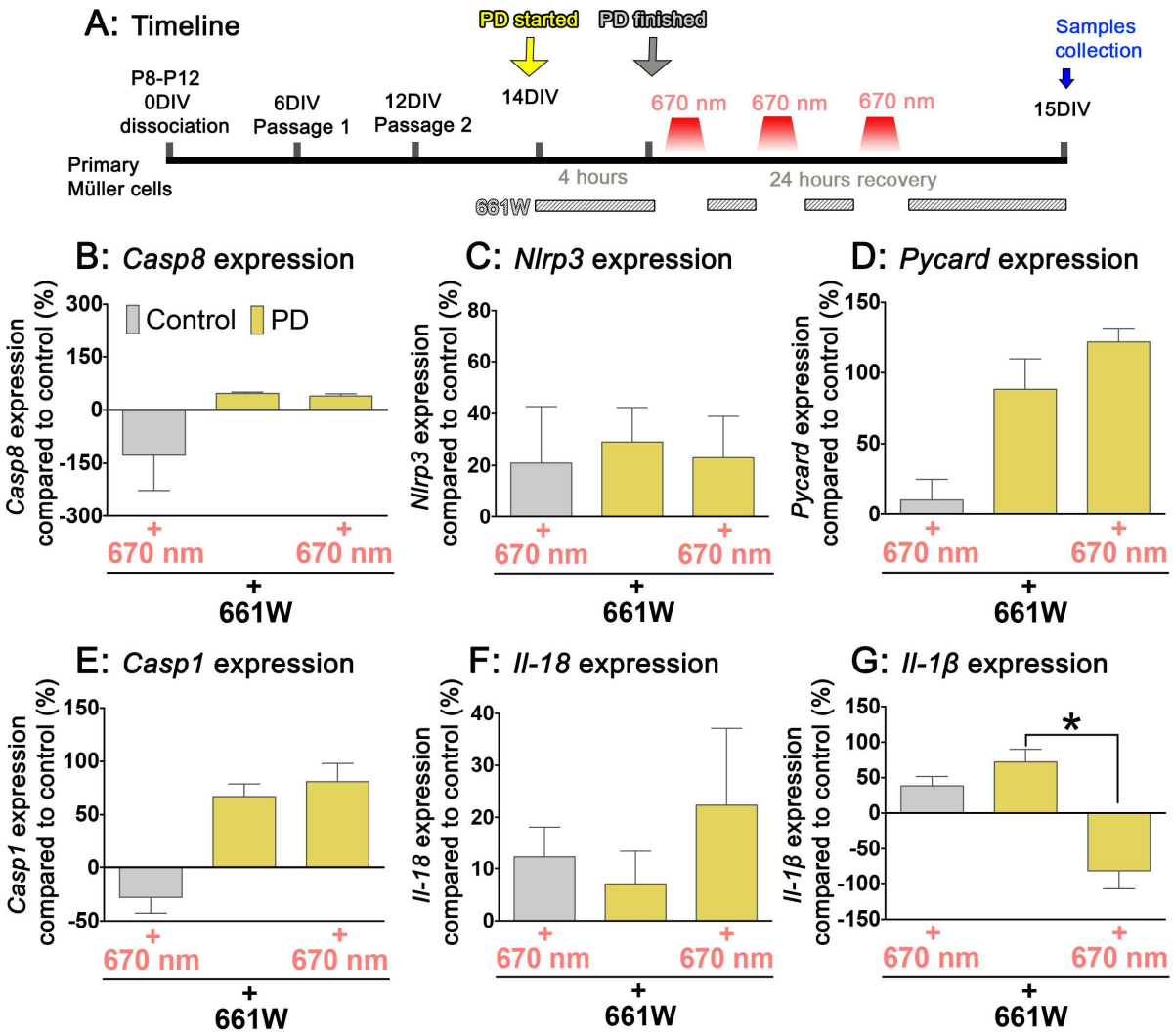


Figure 4. The effects of 670nm light on activation of the NLRP3 inflammasome in Müller cells following photo-oxidative damage (PD). **A:** Timeline of experimental paradigm. **B-F:** Expression of inflammasome genes including *Casp8* (B), *Nlrp3* (C), *Pycard* (D), *Casp1* (E), and *Il-18* (F) were comparable between 670nm-treated and untreated Müller cells following PD (P>0.05). **G:** *Il-1β* expression was significantly lowered in 670nm-treated Müller cells compared to untreated Müller cells (P<0.05). DIV; days *in vitro*. N=6 was used for all experimental comparisons and was performed in biological triplicate. The data is presented as the mean ± SEM. * denotes a significant change (P<0.05).

Colour should be used

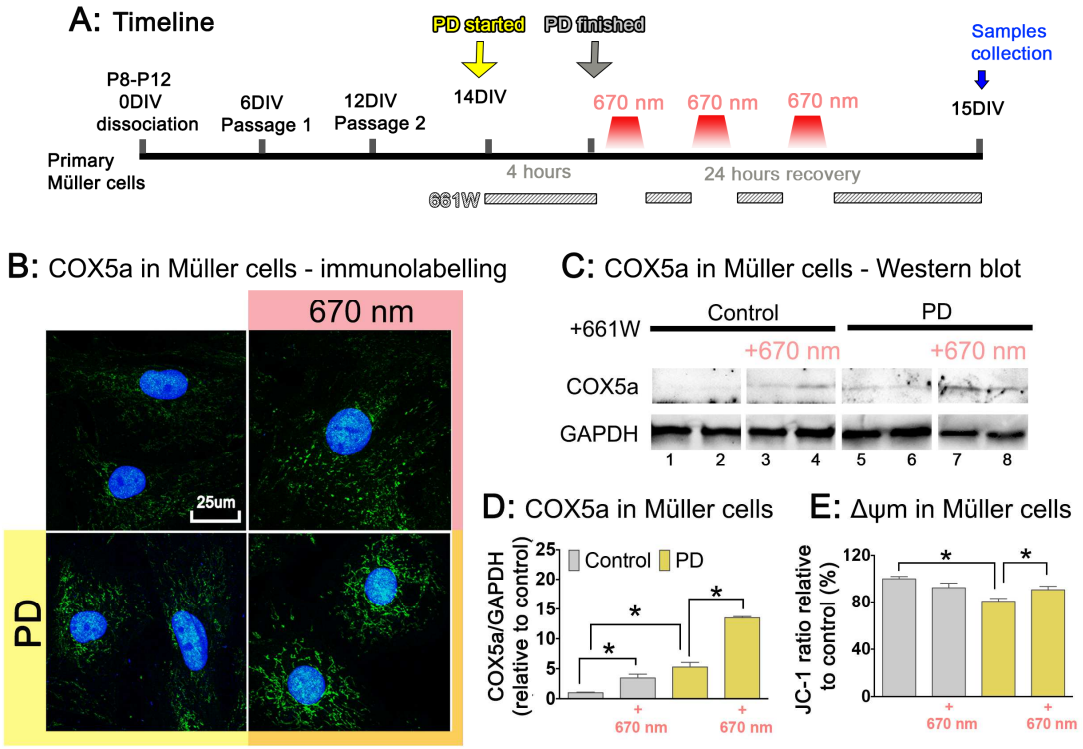


Figure 5. The activation of cytochrome oxidase (COX5a) was enhanced in 670nm-treated Müller cells after photo-oxidative damage (PD). **A:** Timeline of experimental paradigm. **B:** Müller cells were immunolabelled with COX5a antibody (green). Low levels of COX5a were detected in Müller cells co-cultured with normal 661W cells. Following PD, a higher intensity of COX5a was detected in Müller cells compared to controls. The intensity of COX5a was further elevated by 670nm light in Müller cells compared to untreated Müller cells after PD. **C-D:** Western blotting quantification relative to GAPDH expression confirmed the higher expression of COX5a by Müller cells treated with 670 nm light ($P<0.05$). **E:** The JC-1 ratio in mitochondria to cytoplasm was used to measure mitochondrial membrane potential (ΔΨm). Following PD, 670nm light triggered a higher level of ΔΨm in 670nm-treated Müller cells compared to untreated Müller cells ($P<0.05$). DIV; days *in vitro*. N=6 was used for all experimental comparisons and was performed in biological triplicate. The data is presented as the mean ± SEM. * denotes a significant change ($P<0.05$).

Colour should be used

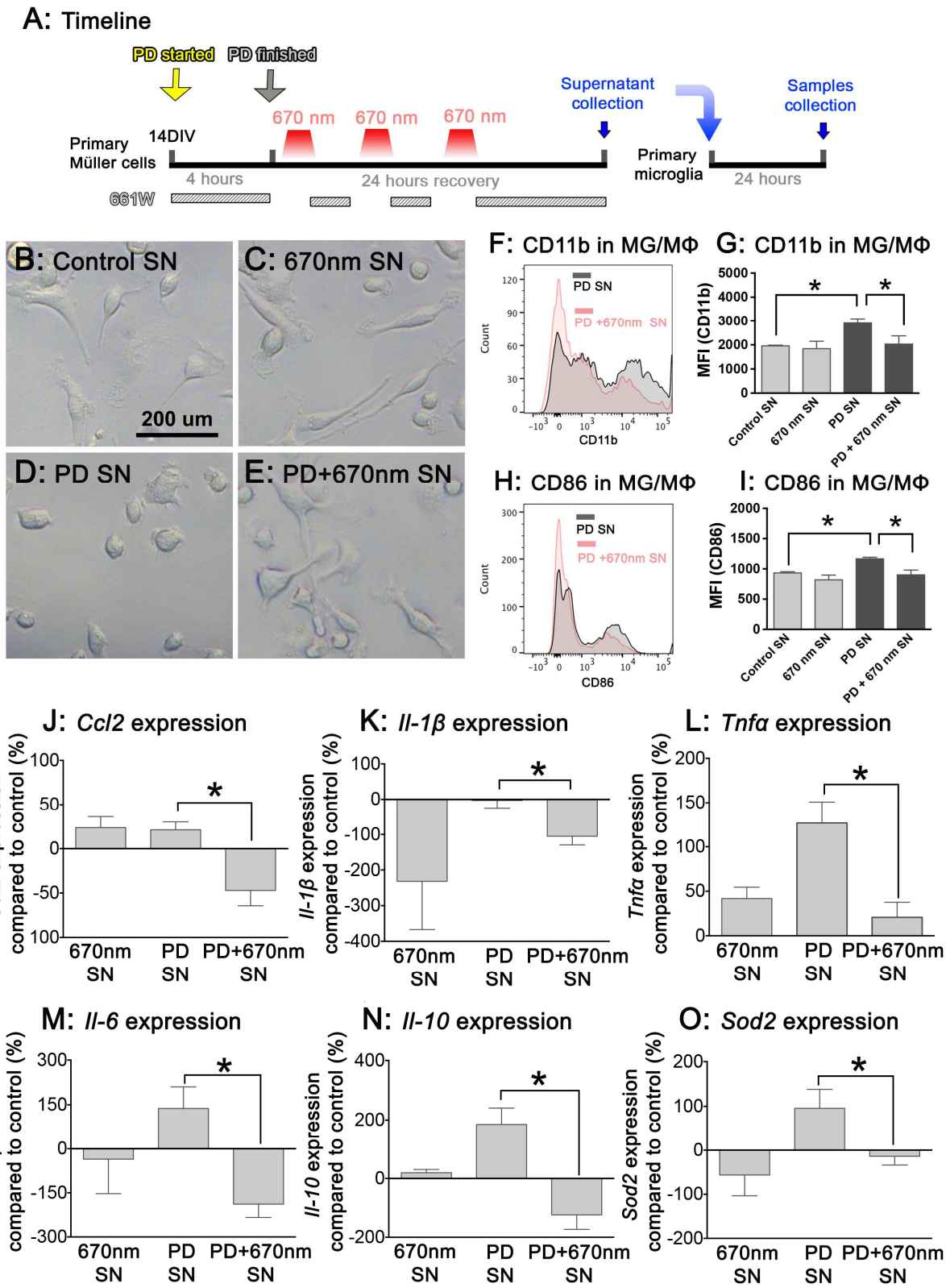

A: Timeline
PD started
PD finished
670 nm
670 nm
670 nm
Supernatant collection
Samples collection
Primary Müller cells
14DIV
4 hours
24 hours recovery
661W
Primary microglia
24 hours
B: Control SN
C: 670nm SN
200 um
D: PD SN
E: PD+670nm SN
F: CD11b in MG/MΦ
PD SN
PD +670nm SN
Count
CD11b
G: CD11b in MG/MΦ
MFI (CD11b)
Control SN
670 nm SN
PD SN
PD + 670 nm SN
H: CD86 in MG/MΦ
CD86
I: CD86 in MG/MΦ
MFI (CD86)
J: Ccl2 expression
Ccl2 expression compared to control (%)
K: Il-1β expression
Il-1β expression compared to control (%)
L: Tnfa expression
Tnfa expression compared to control (%)
M: Il-6 expression
Il-6 expression compared to control (%)
N: Il-10 expression
Il-10 expression compared to control (%)
O: Sod2 expression
Sod2 expression compared to control (%)
670nm SN
PD SN
PD+670nm SN

Figure 6. 670nm light ameliorated Müller cell-mediated activation of primary microglia/macrophages (MG/MΦ) following photo-oxidative damage (PD). **A:** Timeline of experimental paradigm. **B-E:** After incubation with supernatant (SN) of the PD group (D), MG/MΦ cells displayed an amoeboid morphology. However, a ramified shape was observed in MG/MΦ incubated with SN of the PD+670nm group (E). **F-I:** A higher mean of fluorescence intensity (MFI) of CD11b (F, G) and CD86 (H, I) was found in MG/MΦ incubated with SN of the PD+670 group compared to SN of the control group ($P<0.05$). However, MG/MΦ incubated with SN of the PD+670 group had a lower MFI of CD11b and CD86 compared to the PD group ($P<0.05$). **J-O:** Reduced gene expression of *Ccl2* (J), *Il-1β* (K), *Tnfα* (L), *Il-6* (M), *Il-10* (N), and *Sod2* (O) was found in MG/MΦ incubated with SN of the PD+670 group compared to the PD group ($P<0.05$). DIV; days *in vitro*. N=6 was used for all experimental comparisons and was performed in biological triplicate. The data is presented as the mean ± SEM. * denotes a significant change ($P<0.05$).

Colour should be used

Photobiomodulation with 670nm light ameliorates Müller cell-mediated activation of microglia and macrophages in retinal degeneration

Highlights

- Photobiomodulation using 670 nm light directly affects Müller cells and mitigates their pro-inflammation reactions on retinal microglia/macrophages following photo-oxidative stress
- Photobiomodulation using 670 nm light maintains mitochondrial function in Müller cells, thereby supporting the retinal homeostasis and providing the protection to photoreceptors during the photo-oxidative stress.
- We suggest that photobiomodulation using 670 nm light is a non-invasive and non-expensive treatment and can be used as an adjuvant therapeutic approach to reduce inflammation in retinal degeneration.