Glutathione-S-transferase omega 1-1 (GSTO1-1) is a member of a family of glutathione S-transferase enzymes, with many reported roles including protein protection via glutathionylation and modulating oxidative stress and inflammation.\(^1\)\(^-\)\(^5\) Protein-S-glutathionylation is a posttranslational modification whereby mixed disulfides are formed between protein thiols and glutathione (GSH), to protect these groups from being oxidized to sulfonic acids, and the protein becoming irreversibly inactivated.\(^4\) In contrast to this role, GSTO1-1 has also been shown to be localized to the inner segment of cone photoreceptors in the retina.\(^1\)\(^-\)\(^2\)\(^,\)\(^3\) GSTO1-1 has also been shown to be involved in glutathionylation, toll-like receptor signaling, and calcium channel regulation.\(^1\)\(^-\)\(^3\) GSTO1-1 dysregulation has been implicated in oxidative stress and inflammation, and contributes to the pathogenesis of several diseases and neurological disorders; however, its role in retinal degenerations is unknown. The aim of this study was to investigate the role of GSTO1-1 in modulating oxidative stress and consequent inflammation in the normal and degenerating retina.

**RESULTS.** GSTO1-1 was localized to the inner segment of cone photoreceptors in the retina. GSTO1-1’s role in modulating oxidative stress and inflammation in the normal and degenerating retina was explored by using Gsto1-1 knockout mice. The expression and localization of GSTO1-1 were investigated with immunohistochemistry and Western blot. Changes in the expression of inflammatory (Ccl2, Il-1β, and C3) and oxidative stress (Nrf2, Nqo1, Gpx3, and Gpx4) genes were investigated via quantitative real-time polymerase chain reaction. Retinal function in Gsto1-1 knockout mice was investigated by using electroretinography.

**CONCLUSIONS.** These results indicate that GSTO1-1 is required for inflammatory-mediated photoreceptor death in retinal degenerations. Targeting GSTO1-1 may be a useful strategy to reduce oxidative stress and inflammation and ameliorate photoreceptor loss, slowing the progression of retinal degenerations.

**Keywords:** oxidative damage, inflammation, retinal degeneration, knockout animals, GSTO1-1
light exposure, a history of smoking, and obesity. In addition, photoreceptors are rich in polyunsaturated fatty acids, also making them vulnerable to lipid peroxidation. As the central retina has a high concentration of photoreceptors, oxidative damage tends to accumulate in this area, contributing to the pathogenesis of retinal degeneration. Given the naturally high oxidative state of the retina, it is logical that there are tightly regulated cellular defense mechanisms in place, to protect the retina from oxidative damage and the ensuing inflammatory response. Oxidative stress and inflammation are implicated in the pathogenesis of many retinal degenerations, including AMD, diabetic retinopathy, and retinitis pigmentosa. Here, we aimed to characterize the role of GSTO1-1 in the retina by using Gsto1−/− mice, and investigate its effect on oxidative stress and inflammation in the degenerating retina, using a rodent model of photo-oxidative damage (PD) in which oxidative stress and inflammation have been shown to contribute to progressive focal retinal degeneration. The results indicate that GSTO1-1 has a damaging effect in retinal degenerations and demonstrate that knocking out GSTO1-1 reduces oxidative stress and inflammation, and related photoreceptor cell death in the retina in a model of retinal degenerations.

**METHODS**

**Animal Handling and Photo-Oxidative Damage**

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the ANU Animal Experimentation Ethics Committee (Ethics ID: A2014/56). Adult male and female C57BL6 wild-type (WT) and Gsto1−/− mice (aged between 60-90 postnatal days) were bred and reared under 12-hour light/dark-cycle conditions (5 lux) with free access to food and water. Gsto1−/− mice were supplied by Taconic Biosciences (Derwood, MD, USA), model TF1210 Gsto1/C0, and were subsequently backcrossed to the C57BL6 strain. The origin and characterization of the Gsto1−/− mice has been previously described in detail. Age-matched WT and Gsto1−/− mice were randomly assigned to PD and dim-reared (DR) control groups (n = 6 per group). Animals in the PD group were continuously exposed to 100 K lux white LED light for a period of 5 days, as described previously, while DR mice were maintained in 12-hour light (5 lux)/dark-cycle conditions.

**Measurement of Retinal Function Using Electroretinography**

Full-field scotopic electrotoretinography (ERG) was performed to assess the retinal function of DR controls and animals after 5 days PD, as previously described. Mice were dark-adapted overnight before being anaesthetized with an intraperitoneal injection of Ketamine (100 mg/kg; Troy Laboratories, Gleneden, NSW, Australia) and Xylazil (10 mg/kg, Troy Laboratories). Both pupils were dilated with one drop each of 2.5% wt/vol phenylephrine hydrochloride and 1% wt/vol tropicamide (Bausch and Lomb, Rochester, NY, USA). A single- or twin-flash paradigm was used to elicit a mixed response from rods and cones, and an isolated cone response, respectively. Flash stimuli for mixed responses were provided by an LED-based system (FS-250A Enhanced Ganzfeld; Photometric Solutions International, Huntingdale, VIC, Australia), over a stimulus intensity range of 6.3 log cd s m−2 (range, −4.4 to 1.9 log cd s m−2). Amplitudes of the a-wave and b-wave were analyzed by using LabChart 8 software (AD Instruments, Dunedin, New Zealand) and data were expressed as the mean wave amplitude ± SEM (μV).

**Tissue Collection and Preparation**

Animals were euthanized with CO2 following functional ERG analysis. The superior surface of the left eye from each animal was marked and enucleated, then immersed in 4% paraformaldehyde (PFA) for 3 hours. Eyes were then cryopreserved in 15% sucrose solution overnight, embedded in OCT medium (Tissue Tek, Sakura, Japan), and cryosectioned at 12 µm in a parasagittal plane (superior to inferior) with a CM 1850 Cryostat (Leica, Mt. Waverley, Victoria, Australia). To ensure accurate comparisons were made for histologic analysis, only sections containing the optic nerve head were used for analysis (n = 6 per experimental group). The retina from the right eye of each mouse used was excised through a corneal incision and placed into RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C overnight and then stored at −80 °C until further use (n = 6 per experimental group). Some retinas were collected into Cellytic M buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) containing a Protease Inhibitor Cocktail (Sigma-Aldrich) to extract whole cell protein lysates and then stored at −80 °C until further use (n = 6 per experimental group).

**Immunolabelling**

Immunohistochemical analysis of retinal cryosections was performed as previously described. Details of primary antibodies used are displayed in Table 1. Fluorescence was visualized and images obtained by using a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan). Images panels were analyzed by using ImageJ (http://imagej.nih.gov/ij/ provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and assembled with Photoshop CS6 software (Adobe Systems, San Jose, CA, USA).

<table>
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<th>Antibody</th>
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For IBA-1 immunohistochemistry, the number of IBA-1+ cells (a marker of retinal microglia and macrophages) was counted across the superior and inferior retina in retinal cryosections. This quantification was performed on two retinal sections per mouse and averaged. Retinal cryosections were stained with the DNA-specific dye bisbenzimide (1:10,000; Sigma-Aldrich) to visualize the cellular layers. The thickness of the outer nuclear layer (ONL) was determined by counting the number of rows of nuclei (photoreceptor cell bodies) in the area of retinal lesion development (1 mm superior to the optic nerve head), to quantify photoreceptor survival. In addition, ONL thickness was calculated as the ratio of the thickness of the ONL to the whole retinal thickness (outer limiting membrane to the inner limiting.
membrane), to take into account any obliquely cut sections. The process of ONL photoreceptor cell row quantification was performed five times per retina, on two retinal sections at comparable locations per mouse, while the ONL thickness measurement from each retina was the average of two retinal sections at comparable locations (1 mm superior to the optic nerve head).

**TUNEL Staining and Quantification**

Terminal deoxynucleotidyl transferase (TdT)-deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) was used as a measure of photoreceptor cell apoptosis. TUNEL in situ labelling was performed on retinal cryosections by using a TdT enzyme (Cat No. 5335366001; Sigma-Aldrich) and biotinylated dUTP (Cat No. 11093070910; Sigma-Aldrich) as previously described. In each retinal section, the total number of TUNEL+ cells was counted along both the superior and inferior retina. This process of quantification was performed on two retinal sections per animal and was calculated as the average number of TUNEL+ cells per retinal section. Images of TUNEL staining were captured with the A1 confocal microscope at ×10 magnification.

**661W Cell Culture**

Murine photoreceptor-derived 661W cells (kindly gifted by Muayyad R. Al-Ubaidi, Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA) within five passages of authentication were used for these experiments. Validation of authenticity was performed by using gene expression of green cone pigments and cone arrestin. Cells were further validated for species authenticity (CellBank, Sydney, Australia). These cells were cultured as previously published, in growth medium containing Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 6 mM L-glutamine (Thermo Fisher Scientific), and antibiotic-antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin; Thermo Fisher Scientific). Cells were maintained in dim conditions in a humidified atmosphere of 5% CO2 at 37°C and passed by trypsinization every 3 to 4 days.

At passage two, 661W cells were split and seeded into two 24-well plates (at a density of 2.5 × 10⁴ cells per well) and into two 8-well chamber slides (5000 cells per well) in growth medium, and were incubated overnight in dim conditions in a humidified atmosphere of 5% CO2 at 37°C. The following day, cells in the 24-well plate and 8-well chamber slides were incubated in reduced-serum DMEM (supplemented with 1% FBS, L-glutamine, and antibiotic-antimycotic) and after 24 hours were exposed to 15,000 lux light (2.2 mW/cm²; irradiance measured with PM100D optical power meter; THORLABS, Newton, NJ, USA) from two white fluorescent lamps (2 × 10W T4 tri-phosphor 6500K daylight fluorescent tubes; Crompton, Minto, NSW, Australia) for 5 hours with 5% CO2 at 37°C. For dim control cells, one plate and one chamber slide in the incubator were completely wrapped in aluminum foil to avoid light exposure. For air/gas exchange, six small incisions were cut on the aluminum foil. Following incubation, cells in both the dim and PD chamber slides were washed in PBS before being fixed in 4% PFA for 2 hours at 4°C and then were maintained in PBS at 4°C until further use. Cells in each of the 24 wells were washed with PBS and were then triturated either in TRIzol (Thermo Fisher Scientific) and stored at −80°C until further use (n = 6 per experimental group), or placed into 1 ml lysis M buffer (Sigma-Aldrich) containing a Protease Inhibitor Cocktail (Sigma-Aldrich) and then stored at −80°C until further use (n = 6 per experimental group).

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from the retinal and 661W cell samples as described previously, using a combination of TRIzol (Thermo Fisher Scientific) and an RNAqueous Micro Total RNA Isolation kit (Thermo Fisher Scientific). The concentration and purity of each RNA sample was assessed by using the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Following purification of RNA, cDNA was synthesized from 1 μg of each RNA sample by using a Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer’s protocol. Gene expression was measured by using mouse-specific TaqMan hydrolysis probes (Thermo Fisher Scientific), as shown in Table 2. The TaqMan probes, cDNA, and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) were plated in a 384-well transparent plate. Each reaction was performed in technical duplicate and was carried out by using a QuantStudio 12 K Flex RT-PCR machine (Thermo Fisher Scientific). Analysis was performed by using the comparative C_t method (ΔΔC_t). Results were analyzed as a percentage change relative to DR control samples, and normalized to two reference genes, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and β-actin (Actb).

**Western Blot**

Western blot was used to measure the protein expression of GSTO1-1 in retinas from WT and Gsto1+/− mice. The blot was performed by using whole retinal and 661W cell protein lysates according to previously described methods. Twenty micrograms of denatured protein was loaded into a 4% to 20% Mini-Protein TGX Precast Protein gel (Bio-Rad, Hercules, CA, USA) followed by semidry transfer to a nitrocellulose membrane. To detect protein expression of GSTO1-1 in the retina, a GSTO1-1 primary antibody (1:200; kindly gifted by PGB) was used along with a standard GST-ubiquitin protein (1:100; also gifted by PGB), as well as a secondary rabbit anti-horseradish peroxidase conjugate for visualization (Bio-Rad). The protein was visualized with chemiluminescence using a Clarity Western ECL kit (Bio-Rad) and images were captured and analyzed by using a Chemidoc MP with Image Lab software (Bio-Rad).

### TABLE 2. TaqMan Hydrolysis Probes Used for qRT-PCR

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<td>GAPDH</td>
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Glutathione Assay

A glutathione assay was conducted to compare the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in order to determine the oxidative state of both DR and PD WT and Gsto1−/− retinas. The assay was performed with whole retinal protein lysates and a De-proteinising Sample Preparation Kit (TCA, Ab204708, Abcam, Cambridge, UK) and a GSH/GSSG Ratio Detection Assay Kit (Fluorometric - Green, ab138881; Abcam), according to the manufacturer’s protocols.

Statistical Analysis

All graphing and statistical analyses were performed with Prism 6 (GraphPad Software, San Diego, CA, USA). An unpaired Student’s t-test, 1-way ANOVA, or 2-way ANOVA with Tukey’s multiple comparison post test was used to determine the statistical outcome; a P value of <0.05 was considered statistically significant.

RESULTS

In Vivo and In Vitro Expression and Localization of Gsto1-1 in the Retina

Gsto1-1 protein and Gsto1 gene expression were absent from Gsto1−/− mouse retinas (Fig. 1). In WT animals, Gsto1-1 was detected at 28 kDa by Western blot, but was not found in Gsto1−/− retinas (Fig. 1A). Quantification of gene expression levels by quantitative real-time polymerase chain reaction (qRT-PCR) confirmed an absence of Gsto1 gene expression in Gsto1−/− DR retinas and in Gsto1−/− retinas subjected to 5 days PD (P < 0.05; Fig. 1B). These data indicate a complete functional knockout of Gsto1-1 in the Gsto1−/− mouse retina. There was no change in Gsto1-1 protein or gene expression between WT PD mice and WT DR controls (P > 0.05; Figs. 1A, 1B).

Localization of Gsto1-1 in the WT retina by immunohistochemistry indicated that Gsto1-1 was strongly expressed in retinal cone photoreceptors (Gsto1-1 and peanut agglutinin [PNA] colabelling; Fig. 1D, inlet window), but not rod photoreceptors (no colabelling with rhodopsin; Fig. 1K, inlet window) and was seen specifically within the inner segment of cones, highly expressed along the superior retina, and weakly in these cells along the inferior retina (Figs. 1C-J). In Gsto1−/− mice, cone inner segments were weakly labelled with Gsto1-1 antibodies in DR and PD animals (Figs. 1E, 1J). Gsto1-1 immunoreactivity was also detected in inner nuclear layer (INL) cells in DR and PD WT retinas, which we tentatively identify as amacrine cells (Figs. 1C, 1D, 1G, 1H). This labelling pattern was not observed in either DR or PD Gsto1−/− retinas (Figs. 1E, 1F, 1I, 1J). In vitro studies of 661W mouse photoreceptor-like cell line also showed increased Gsto1 levels following 5 hours PD by qRT-PCR (Fig. 1M) and immunocytochemistry (Fig. 1N). Gene expression of oxidative stress markers heme oxygenase-1 (Hmox1); nuclear factor, erythroid derived 2, like 2 (Nrf2); and NAD(P)H quinone dehydrogenase 1 (Nqo1) also increased in 661W cells after 5 hours PD (Fig. 1M).

Changes in Oxidative Stress in Gsto1−/− Retinas Following Photo-Oxidative Damage

To investigate the effect of Gsto1-1 deficiency on oxidative stress production in the retina, the gene expression changes of oxidative stress markers Hmox1, Gpx3, Nox1, Sod2, Nrf2, and Nqo1 (Figs. 2A–F) and glutathione-specific gamma-glutamylcysteine synthetase (Gcbs1), a glutathione degradation enzyme (Fig. 2G), as well as the total ROS/reactive nitrogen species (ROS/RNS) levels, were measured in WT and Gsto1−/− mice, before (DR) and after PD (Fig. 2H). A significant increase in the gene expression of Nrf2 (P < 0.05; Fig. 2E) and Nqo1 (P < 0.05; Fig. 2F), as well as in the total ROS/RNS levels, was detected in WT PD retinas compared to WT DR retinas (P < 0.05; Fig. 2H). Nrf2 expression was also found to decrease between both WT DR and Gsto1−/− DR mice and between WT DR and WT PD mice (P < 0.05; Fig. 2G). While there was a significant increase in the gene expression of oxidative stress markers Hmox1 (P < 0.05; Fig. 2A), Gpx3 (P < 0.05; Fig. 2B), Nrf2 (P < 0.05; Fig. 2E), and Nqo1 (P < 0.05; Fig. 2F), following PD in Gsto1−/− mice compared to Gsto1−/− DR controls, a significant decrease was seen in the gene expression of Nqo1 (P < 0.05; Fig. 2F) as well as in the total ROS/RNS levels in Gsto1−/− PD retinas compared to WT PD retinas (P < 0.05; Fig. 2H). This result overall indicates a reduced oxidative state in Gsto1-1-deficient mice following PD.

Gsto1-1 Deficiency Reduces Inflammation and Activated Microglia/Macrophage Accumulation in the Retina Following Photo-Oxidative Damage

The number of microglia/macrophages accumulating in the outer retina (photoreceptor layer and subretinal space) of WT and Gsto1−/− mice was determined by using IBA-1 immunoreactivity following PD. There was a significant increase in the total number of outer retinal IBA-1+ cells following PD in both WT and Gsto1−/− retinas compared to respective DR controls (P < 0.05; Fig. 3A). No significant difference in outer retinal IBA-1+ cell counts was observed between WT and Gsto1−/− PD groups (P > 0.05; Fig. 3A). However, analysis of IBA-1+ cell morphology (ramified versus amoeboid) indicated a significantly reduced number of amoeboid cells in the outer retina of Gsto1−/− PD retinas compared to WT PD retinas (P < 0.05; Fig. 3B).

To investigate the effect of Gsto1-1 deficiency on key inflammatory markers known to be upregulated in the PD model, inflammatory gene expression was investigated by using qRT-PCR. Results showed a significant downregulation in the expression of Ccl2 (P < 0.05; Fig. 3G), Ifn-β (P < 0.05; Fig. 3H), and C3 (P < 0.05; Fig. 3I) in Gsto1−/− PD retinas compared to WT PD retinas. On the other hand, there was a statistically significant increase in the expression of all three inflammatory genes between WT DR and WT PD groups (P < 0.05; Figs. 3G–I), there was only a statistically significant upregulation of C3 between Gsto1−/− DR and Gsto1−/− PD mice (P < 0.05; Fig. 3I). No statistical difference in Ccl2 or Ifn-β expression between Gsto1−/− DR and Gsto1−/− PD mice (P > 0.05; Figs. 3G, 3H) was observed. Overall, these data indicate that Gsto1−/− deficiency reduces inflammation and activated microglia/macrophage accumulation in the retina following PD.

Retinal Function Is Increased and Cell Death Decreased in Gsto1−/− Retinas Following Photo-Oxidative Damage

The level of photoreceptor cell death was measured and compared in the retinas of WT and Gsto1−/− mice by using a TUNEL assay. Following PD, analyses showed that there was a significantly decreased number of TUNEL+ photoreceptors (P < 0.05; Fig. 4A) and a significantly increased number of photoreceptor cell rows (P < 0.05; Fig. 4B) in Gsto1−/− mice compared to WT mice. This change in photoreceptor cell survival was also reflected by using ERG, which demon-
Figure 1. Localization and expression of GSTO1-1 in the retina and in 661W cells before (dim-reared, DR) and after photo-oxidative damage (PD).

(A) GSTO1-1 expression was detected via Western blot, producing a 28-kDa band in WT but not Gsto1−/− retinas. No change was seen in GSTO1-1 protein expression in WT mice after PD. (B) Gsto1 gene expression was significantly reduced in both the DR and PD Gsto1−/− mice compared to their respective controls (n = 6, P < 0.05), while no change was seen in Gsto1 gene expression in WT PD mice. (C–J) Representative images of GSTO1-1 immunolabelling showed GSTO1-1 (green) to be highly expressed on the inner segments of cone (PNA, red) photoreceptors (outlined arrows), on the superior retina (SR), and weakly in the inferior retina (IR) in WT DR (D) and WT PD (H) mice. GSTO1-1 was also found to be weakly expressed in the same locations in Gsto1−/− DR (F) and Gsto1−/− PD (J) mice. GSTO1-1 was also expressed in the INL (filled arrows) on the SR as well as IR in WT DR (C, D) and PD (G, H) mice. (K) GSTO1-1 did not colocalize with rod photoreceptors (no colabelling with rhodopsin, red). (L) Negative control. (M) Gsto1 expression was significantly increased after 5 hours PD as compared to dim conditions (P < 0.05) in 661W cells. An increase in Nrf2, Nqo1, and Hmox1, markers of oxidative stress, was also seen to significantly increase in 661W cells after 5 hours PD as compared to dim controls (n = 6, P < 0.05) (K). (N) GSTO1-1 (green) was found in all 661W cells in both dim and PD conditions. n = 6, results were analyzed with 1-way ANOVA followed by Tukey’s multiple comparison post test. Asterisks denote a significant change where P < 0.05. Scale bar: 25 μm (C–L) and 10 μm (N).
Changes in oxidative stress gene (A–G) and protein (H) expression in Gsto1−/− retinas compared to WT in both DR and following 5 days PD damage. (A–G) A significant increase in gene expression was seen in the expression of Hmox-1 (A) and Gpx3 (B) between Gsto1−/− DR and Gsto1−/− PD mice (P < 0.05), and a significant decrease was found in the expression of Gpx3 between WT DR and Gsto1−/− DR mice (B) (P < 0.05). No statistical differences were shown between any other groups for these genes, or between any group for the expression of genes Nox1 (C) or Sod2 (D) (P > 0.05). A significant increase was found in the expression of Nrf2 (E) between WT DR and WT PD mice (P < 0.05), and between Gsto1−/− DR and Gsto1−/− PD mice (P < 0.05); however, there was no difference seen in expression of Nrf2 between WT PD and Gsto1−/− PD mice (P > 0.05). (F) Nqo1 gene expression was significantly downregulated in both DR and PD Gsto1−/− mice compared to respective controls (P < 0.05). (G) Gene expression changes of glutathione-specific gamma-glutamylcyclotransferase 1 (Chac1), which degrades glutathione, showed a significant decrease in expression between WT DR and Gsto1−/− DR mice (P < 0.05), and between WT DR and WT PD mice (P < 0.05). No significant change, however, was seen in Chac1 expression between WT PD and Gsto1−/− PD mice (P > 0.05). (H) The total levels of ROS/RNS were measured in the retinas with results showing a significant increase in the total ROS/RNS levels between WT DR and WT PD retinas (P < 0.05), as well as a significant decrease between WT PD and Gsto1−/− PD retinas (P < 0.05). n = 5; results were analyzed with 1-way ANOVA followed by Tukey’s multiple comparison post test. Asterisks denote a significant change where P < 0.05.
Figure 3. The effect of Gsto1−/− on inflammation and the accumulation of microglia/macrophages in the retina following 5 days PD. (A) Following PD, a significant increase in the total number of IBA-1+ microglia/macrophages was seen in the outer retina (ONL and subretinal space) in both WT PD and Gsto1−/− PD mice compared to their respective controls (P > 0.05). No difference in the total number of IBA-1+ cells was seen between WT DR and Gsto1−/− DR mice or between WT PD and Gsto1−/− PD mice. (B) Significant increase in the ratio of amoeboid (activated) to ramified (resting) macrophages was seen in WT PD compared to Gsto1−/− PD retinas (P < 0.05). (C-F) Representative images showing IBA-1+ labelling (green) in the outer retina. Thin ramified processes of IBA-1+ microglia/macrophages (filled arrows) can be seen projecting into the ONL in WT DR (C) and Gsto1−/− DR (D) retinas, with larger and more numerous ramified processes seen in Gsto1−/− PD retinas (F). In comparison, in WT PD retinas (E), numerous rounded (amoeboid) IBA-1+ cells can be seen in the ONL and subretinal space (outlined arrows). (G-I) Inflammatory markers were quantified by qRT-PCR in whole retinas. Ccl2 (G), Il-1β (H), and C3 (I) expression significantly increased in WT PD mice compared to WT DR mice (P < 0.05) and was significantly reduced in Gsto1−/− PD mice compared to WT PD mice (P < 0.05). In addition, C3 expression increased significantly in Gsto1−/− PD mice compared to Gsto1−/− DR mice (P < 0.05); however, there was no difference in the expression of Ccl2 or Il-1β between these groups (P > 0.05). n = 6; results were analyzed with either an unpaired Student's t-test, or 1-way ANOVA followed by Tukey's multiple comparison post test. Asterisks denote a significant change where P < 0.05. Scale bar: 20 μm.
FIGURE 4. The effect of Gsto1<sup>−/−</sup> on retinal function and cell death in the retina following 5 days PD. (A) The total number of TUNEL<sup>+</sup> cells in the ONL was significantly increased in both the WT PD and Gsto1<sup>−/−</sup> PD retinas compared to their respective WT DR and Gsto1<sup>−/−</sup> controls (P < 0.05). In addition, the total number of TUNEL<sup>+</sup> cells was significantly reduced in the retinas of Gsto1<sup>−/−</sup> PD mice compared to WT PD retinas (P < 0.05). (B) A significantly reduced number of photoreceptor cell rows was seen in WT DR and Gsto1<sup>−/−</sup> DR retinas and their respective WT PD and Gsto1<sup>−/−</sup> PD controls. There was a significant increase in photoreceptor cell nuclei survivability in Gsto1<sup>−/−</sup> PD compared to WT PD retinas (P < 0.05). (C–F) Representative images show the immunolabelling of TUNEL<sup>+</sup> cells (red) in the ONL as well as the ONL thickness (white line). TUNEL<sup>+</sup> cells (filled arrows) can be seen in both the WT DR (C) and Gsto1<sup>−/−</sup> DR (D) retinas, while there are significantly more TUNEL<sup>+</sup> cells in the WT PD retina (E), and only a slight increase in TUNEL<sup>+</sup> cells in the Gsto1<sup>−/−</sup> PD retina (F) compared to DR controls. (G–J) Retinal function was measured before (DR) and after 5 days PD by using ERG. There was no statistical difference observed in either the a-wave (G) or b-wave (H) between WT DR and Gsto1<sup>−/−</sup> DR mice (P < 0.05). Following PD, there was a significant improvement in both the a-wave (I) and b-wave (J) function between Gsto1<sup>−/−</sup> PD and WT PD mice (P < 0.05). n = 5–10; results were analyzed with 1-way ANOVA followed by Tukey’s multiple comparison post test or a 2-way ANOVA for ERG analysis. Asterisks denote a significant change where P < 0.05. Scale bar: 20 µm.
strated that WT PD mice had significantly reduced retinal function for both a-wave ($P < 0.05$; Fig. 4I) and b-wave ($P < 0.05$; Fig. 4J), compared to Gsto1–/– PD mice. No statistical difference ($P > 0.05$) in the number of TUNEL photoreceptor cells (Fig. 4A), photoreceptor cell rows (Fig. 4B), or ERG function (Figs. 4G, 4H) was observed between WT DR and Gsto1–/– DR mice.

GSTO1-1 Increases Total Glutathione Levels in the Retina Following Photo-Oxidative Damage

As glutathione exists in both an antioxidant reduced (GSH) state and an oxidized (GSSG) state, the ratio of GSH to GSSG in the retina can be used as a measure of cell health, as high levels of GSSG accumulate during increased oxidative stress. A glutathione assay was used to determine what effect GSTO1-1 had on the GSH to GSSG ratio in the retina before and after PD. There was a statistically significant increase in GSH ($P < 0.05$; Fig. 5A), GSSG ($P < 0.05$; Fig. 5B), and total glutathione (GSH+GSSG) levels ($P < 0.05$; Fig. 5C) in WT PD retinas compared to WT DR retinas. Levels of GSH were also found to be significantly reduced in Gsto1–/– PD mice compared to WT PD mice ($P < 0.05$; Fig. 3A); however, while there was a reduction in the levels of GSSG and total glutathione in Gsto1–/– PD mice, it was not statistically significant ($P > 0.05$; Figs. 5A–D) from either control levels or WT PD at 5 days. In addition, there was no statistical difference in the ratio of GSH to GSSG between any of the groups ($P > 0.05$; Fig. 5D). There was also no statistical difference in the levels of GSH, GSSG, total glutathione, or GSH to GSSG ratio between WT DR and Gsto1–/– DR mice ($P > 0.05$; Figs. 5A–D).

DISCUSSION

This is the first study to determine a possible role of GSTO1-1 in mediating retinal inflammation, which can contribute to the pathogenesis of retinal degenerations. Firstly, our data show that GSTO1-1 is present in the mouse retina, localized to the inner segment of cone photoreceptors, and is maximally expressed in the region of the retina most susceptible to oxidative stress, the central superior retina. Secondly, Gsto1–/– mice had lower levels of markers of oxidative stress, a reduction in photoreceptor cell death, and improved retinal function as compared to WT controls. Thirdly, following PD-induced oxidative stress, Gsto1–/– mice had a reduced inflammatory response, accompanied by a decrease in activated microglia/macrophage accumulation in the outer retina as compared to WT controls. Lastly, our results indicate that while there were changes in the levels of reduced and oxidized forms of glutathione (GSH and GSSG, respectively) in PD WT compared to DR WT and Gsto1–/– retinas, the overall ratio of GSH to GSSG (an indicator of redox status) remained unchanged between all experimental groups. Overall, these data indicate a role for GSTO1-1 in the progression of retinal degenerations and indicate that GSTO1-1 may be a potential target for the treatment of retinal degenerations where oxidative stress and inflammation are key features of disease progression.

The Role of GSTO1-1 in the Normal Retina

Mice deficient in GSTO1-1 have been previously shown to be phenotypically normal, however display reduced monocyte and eosinophils counts, compared to controls.1 We observed no difference in retinal structure, number of photoreceptors, macrophage recruitment, retinal function, or oxidative stress gene expression in Gsto1–/– mice, with the exception of reduced levels of antioxidant enzymes Gpx3 and Nqo1. Reduced levels of Gpx3 and Nqo1 suggest that GSTO1-1 deficiency decreases oxidative stress under normal conditions in the retina, consistent with previous findings from models of obesity.1 Glutathionylation and deglutathionylation of proteins by GSTO1-1 has been well documented in many other tissues.2,34–36 Despite this we saw no change in GSH, GSSG, or total glutathione levels between Gsto1–/– and WT control retinas, indicating a novel role of GSTO1-1 in the normal and degenerating retina. Given the cone photoreceptor-specific labelling demonstrated in this article, we suggest that GSTO1-1 may act at the interface of oxidative stress and inflammatory pathways and may therefore contribute to photoreceptor cell death following light-induced PD. This potential role will be the focus of the remainder of the discussion.

GSTO1-1 Is Primarily Localized to the Inner Segment of Cone Photoreceptors

In rodents, the central superior retina (1–3 mm from the optic nerve) is known to be highly susceptible to PD-induced focal photoreceptor cell death,30–37–38 in a region functionally comparable to the human macula, known as the “area centralis.”39–39 GSTO1-1 appeared to be most highly expressed in this area, specifically localized to the inner segments of the cones, with photoreceptor inner segments known to be rich in mitochondria to accommodate their high rates of metabolism and energy usage,40 and in endoplasmic reticulum (ER), where calcium homeostasis is maintained41 and posttranslational modification of proteins occurs, including glutathionylation. Although weak GSTO1-1 labelling was also seen in the retina of Gsto1–/– mice, likely antibody binding to another compensatory GST, no expression was detected in Western blot analysis or qRT-PCR of Gsto1–/– retinas.

In drosophila, GSTO1-1 is involved in regulating mitochondrial ATP synthase activity and modulating oxidative stress and has been suggested as a contributor to the neurodegeneration in Parkinson’s disease.1 Photoreceptors have the highest levels of ROS production in the retina,15,18 a large source of which comes from mitochondria in the photoreceptor inner segments.42,43 In a healthy state, ROS, including free radicals, superoxide anion, singlet oxygen, and hydrogen peroxide, are quenched by cellular defense mechanisms, for example, by antioxidants such as glutathione (GSH).17 However, an accumulation of ROS can overburden the antioxidant capabilities of the cell, leading to cell death.44 It has also been reported that ER can respond to oxidative stress in the retina, with previous studies showing that in both PD retinas and 661W cells, intracellular Ca2+ levels increase in the ER, producing ROS and ultimately leading to ER stress and photoreceptor cell death.45,46 GSTO1-1 has been shown to play a role in modulating the activity of the ER calcium release channel, ryanodine receptor (RyR),47 and could potentially play a similar role in retinal cone cells during oxidative stress.

Although not investigated further in this study, the underlying mechanism of interactions with these cellular organs is likely to explain the reduced oxidative stress response seen in the GSTO1-1-deficient mice. As it is known that cones are very susceptible to oxidative damage over time,48 the suggested cellular location of GSTO1-1, combined with the known role of GSTO1-1 as a modulator of oxidative stress, indicates a potential role of GSTO1-1 in modulating oxidative stress in the retina.

In addition to cone labelling, GSTO1-1 expression was also seen in a large, rounded cell type in the INL in both the superior and inferior sides of the retina of WT mice, possibly amacrine cells. Further experiments are necessary to verify this labelling pattern and to determine the potential role of GSTO1-1.
differences were noted in the expression of oxidative stress. 49 Although there was no significant change where the ratio of GSH to GSSG between any of the groups (P > 0.05). In addition there was no statistical difference in the levels of GSH between WT PD and Gsto1−/− PD mice (A) (P < 0.05). While there was a large reduction in the levels of GSSG (B) and total glutathione (C) between WT PD and Gsto1−/− PD mice; it was not statistically significant (P > 0.05). In addition there was no statistical difference in the ratio of GSH to GSSG between any of the groups (D) (P > 0.05). n = 5; results were analyzed by unpaired Student's t-test. Asterisks denote a significant change where P < 0.05.

In these cells, however, it is not the focus of this study. Nevertheless, given the specificity of Gsto1-1 labelling in the cone photoreceptors, Gsto1-1 could be playing a role in modulating the effects of oxidative stress in these cells of the INL as well.

Oxidative Stress and Photoreceptor Cell Death Is Promoted by Gsto1-1 in Retinal Degenerations

We report that the gene expression of Gsto1-1 and oxidative stress markers Hmox1, Nrf2, and Nqo1 concurrently increased in 661W photoreceptor-like cells, following PD. HMOX1 is an integral membrane protein in the ER and is a key regulator of inflammatory processes, which is known to be induced by high levels of oxidative stress. 49 Although there was no significant difference seen in the expression of Hmox1 in WT retinas compared to Gsto1−/− retinas following PD, photoreceptor-specific changes may be masked by whole retinal changes. 50,51 Recent results from Menon et al. show that expression levels of Nqo1, an antioxidant enzyme target of the NRF2 pathway, are significantly lower in liver lysates from Gsto1−/− mice, indicating a reduced oxidative state in these Gsto1−/− mice compared to controls. The present study confirms these findings, with significantly lower levels of Nqo1 found in DR and PD Gsto1−/− mice compared to their respective WT controls. As GSTO1-1 is a downstream target of NRF2, the expression of Nrf2 was not expected to change in the Gsto1−/− mice compared to WT and may be expressed at higher levels in an attempt to compensate for the lack of downstream GSTO1-1 in the deficient mice. 52 While no differences were noted in the expression of oxidative stress genes Nox1, Sod2, and Gpx3 between WT PD and Gsto1−/− PD retinas, a large reduction in total ROS/RNS levels was detected in the Gsto1−/− PD mice compared to PD controls, indicating that Gsto1-1 may instead act at the protein level inducing the production of ROS and RNS, potentially through the activation of TLR signaling and NFκB pathways. 7

Glutathione is an intracellular antioxidant and is considered to be one of the most important ROS scavengers. In normal healthy cells, approximately 90% of glutathione is in its reduced form, GSH, however, following oxidative stress, GSSG (oxidized form) accumulates and the ratio of GSH to GSSG becomes an important indicator of oxidative stress in tissues. However in this study, the individual levels of GSH and GSSG were both equally higher in WT PD than Gsto1−/− PD mice, which resulted in no significant difference in the GSH to GSSG ratio between these groups, indicating no relative change in redox status.

The increase seen in GSH and GSSG levels in WT PD mice is supported by the gene expression analysis of Chac1, an enzyme involved in the cleavage and degradation of glutathione which, when overactivated, causes glutathione depletion and apoptosis. 53,54 Results demonstrated that there was a significant reduction in Chac1 expression in Gsto1−/− DR mice compared to WT DR mice, with expression remaining equally low in Gsto1−/− DR controls following PD, suggesting that the levels of glutathione degradation in the group was a consequence of Gsto1-1 deficiency and not PD. In contrast however, Chac1 was found to be significantly reduced in WT PD mice compared to WT DR controls, indicating that there is a reduction in the degradation of glutathione in these mice, potentially to combat increased levels of ROS/RNS seen...
following PD. Despite no changes in the redox state of each of the experimental groups, we postulate that the increased levels of GSH and GSSG seen in the WT PD mice are due to an increased oxidative state as supported by data in this study. It remains unclear if the detrimental effects of GSTO1-1 on the retina following PD are occurring through the glutathionylation cycle, or perhaps if GSTO1-1 is modulating the glutathionylation of a specific protein rather than the overall redox potential as previously described.7,11

**GSTO1-1 Mediates an Inflammatory Response in Retinal Degenerations**

It has been well characterized in photo-oxidative retinal degeneration that an increase in oxidative stress and photoreceptor cell death leads to the release of proinflammatory cytokines, macrophage accumulation, and complement deposition.25,26,29,35,55 Release of proinflammatory cytokines (including IL-1β and CCL2) and complement production (including C3) are characteristic features of many retinal degenerations.24–26,29,58–62 Modulation of GSTO1-1 leads to the posttranslational modification and subsequent activation of IL-1β,11,65 a fundamental component in the early inflammatory response of many neurodegenerative diseases. Our results demonstrate that following PD, Gsto1−/− retinas showed reduced levels of Il-1β, Ccl2, and C3, compared to WT controls. CCL2 is a potent chemokine known to recruit macrophages to areas of retinal damage.26,52 We observed that Gsto1−/− microglia/macrophages were less amoeboid with processes, but not cell bodies, extending into the ONL, indicating a reduced activation state as compared to WT microglia/macrophages. Upon recruitment to the outer retina, activated microglia and macrophages are known to produce IL-1β and C3, which ultimately exacerbates photoreceptor cell death.29 Therefore, the reduction in expression of these inflammatory markers in the Gsto1−/− microglia/macrophages also indicates a reduced inflammatory state. In addition, as high levels of Chac1 are known to initiate apoptosis via GSH depletion, a significant reduction in this gene suggests a lower apoptotic state in GSTO1-1-deficient mice. This hypothesis is supported by results from this study demonstrating that Gsto1−/− mice had reduced photoreceptor cell death following PD. While a significantly higher level of TUNEL+ cells were shown in WT PD mice compared to both controls and Gsto1−/− PD mice, a correlative increase in Chac1 expression was not found. It is possible that as gene expression was only investigated at one time point (5 days PD), levels of Chac1 expression may have been significantly increased as compared to DR controls before this time point, possibly during initial photoreceptor cell death, and requires further investigation.

One of the most characterized functions of GSTO1-1 is its activity as a thioredoxin reductase enzyme, with reported roles in both glutathionylation, to protect proteins against oxidative damage and inactivation,4 as well as deglutathionylation of proteins in different tissues and diseases.2,3,5,10,12,35,36 Although the precise signaling mechanisms that GSTO1-1 is acting through in our model to promote photoreceptor cell death are unknown, it has been recently reported that GSTO1-1 may act through the TLR4 pathway in LPS-stimulated macrophages via the glutathionylation of the MyD88 adaptor-like (MAL) protein56 to induce ROS production and proinflammatory cytokine release.7,10,11,36 The study by Menon et al.10 has shown a decrease in the production of ROS, IL-1β, and HIF-1α (responsible for maintaining activated macrophages in an inflammatory state) in GSTO1-1−/− deficient macrophages and highlights the essential role of GSTO1-1 in LPS/TLR4-mediated inflammatory cascades.10 Although we did not detect GSTO1-1 protein expression in microglia/macrophages in the current study, it has been reported that TLR4 is present on both retinal photoreceptors and 661W cells.59,65 It is therefore possible that GSTO1-1 is mediating inflammatory cascades via this pathway, promoting the recruitment of macrophages to the damaged photoreceptors and consequently inducing photoreceptor cell death. This concept is supported by the reduced levels of ROS, inflammation, and photoreceptor cell death seen in Gsto1−/− mice following PD and indicates that targeting GSTO1-1 may be beneficial in slowing the progression of retinal degenerations and warrants further investigations via the use of a GSTO1-1 inhibitor delivered to the retina.

**Conclusions**

This study describes a damaging role for GSTO1-1 in retinal degenerations, through the promotion of oxidative stress and inflammation, leading to photoreceptor cell death, with mice deficient in GSTO1-1 showing a reduction in the damaging levels of oxidative stress and inflammation that is central to the progression of retinal degenerations. While further research into the possible mechanisms by which GSTO1-1 acts in the retina is necessary, this study highlights that GSTO1-1 may be a possible therapeutic avenue for slowing the progression of retinal degenerations.

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**References**

GSTO1-1 Regulates Photoreceptor Survival


