Eye-Directed Overpressure Airwave-Induced Trauma Causes Lasting Damage to the Anterior and Posterior Globe: A Model for Testing Cell-Based Therapies

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Abstract

Purpose: Characterization of the response of the Balb/c mouse to an eye-directed overpressure airwave, with the hypothesis that this mouse strain and model is useful for testing potential therapeutics for the treatment of traumatic eye injury.

Methods: The left eyes of adult Balb/c mice were exposed to an eye-directed overpressure airwave. Intraocular pressure (IOP) was measured and eyes were inspected for gross pathology changes. Optical coherence tomography and histology were used to examine the structural integrity of the retina and optic nerve. Immunohistochemistry, *in vivo* molecular fluorophores, and a multiplex enzyme-linked immunosorbent assay were utilized to identify changes in cell death, neuroinflammation, and oxidative stress.

Results: This model induced a transient increase in IOP, corneal injuries, infrequent large retinal detachments, retinal pigment epithelium (RPE) vacuolization, glial reactivity, and retinal cell death. Both the corneal damage and RPE vacuolization persisted with time. Optic nerve degeneration occurred as early as 7 days postinjury and persisted out to 60 days. Retinal cell death, increased levels of reactive oxygen species, and neuroinflammation were detected at 7 days postinjury.

Conclusions: The injury profile of the Balb/c mouse is consistent with commonly observed pathologies in blast-exposed patients. The damage is throughout the eye and persistent, making this mouse model useful for testing cell-based therapies.

Introduction

OCULAR INJURIES FROM blast exposure are a significant problem. A recent study identified eye trauma in 66% of veterans with mild blast-induced traumatic brain injury (TBI),¹ and since 2000, over 300,000 veterans have been diagnosed with TBI.² Ocular blast injuries also affect civilian populations caught up in warfare, terrorist attacks, and explosions.^{3,4} Blast injuries fall into 4 major categories: damage from the overpressure airwave (primary), fragments and debris (secondary), structural collapse on the body or the impact of the body on a surface (tertiary), or chemical and thermal burns (quaternary).⁵ The secondary and tertiary aspects of blast can cause both closed-(contusions and lacerations) and open-globe injuries (penetrating and perforating insults).^{3,6,7} Closed-globe injuries are more insidious than open-globe injuries as they can remain undetected and cause subtle loss of vision over time.⁷ Vision problems and ocular damage have been detected months after the initial injury, suggesting that the damage is long-lasting.¹

Our laboratory developed and characterized a murine model of closed-globe eye trauma that recapitulates common injuries and vision loss observed in blast-exposed veterans.^{8–10} The model produces an overpressure airwave (primary aspect of blast) directed at the mouse eye while the head and body are shielded. Thus, the model does not address additional injury that could be incurred due to free head movement or the tertiary aspect of blast trauma. We have previously described the effects of this injury in the C57Bl/6J and DBA/2J mouse strains.^{9,10} Upon visual examination, the eye of the C57Bl/6 mouse appears normal after this trauma, yet we detected small rare retinal detachments and increased oxidative stress beginning at 3 days posttrauma. Retinal cell death was first detected at 1-month postblast and correlated with vision loss.^{8,9} In contrast, in the posttrauma DBA/2J mouse eye, we found frequent severe anterior pathologies, larger retinal detachments with epiretinal membranes, immune infiltrate, increased oxidative stress, retinal cell death by 3 days postblast and more rapid and severe vision loss.¹⁰ The DBA/2J lacks a fully functional

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anterior chamber-associated immune deviation, possibly explaining the greater injury response.¹¹ The immune response in the DBA/2J may be similar to an open-globe trauma without the additional complication of bacterial pathogens since the eye was intact.

In this study, we describe the effects of an eye-directed overpressure airwave in the Balb/c mouse. Notably, it responds differently from the other 2 strains, suggesting possible genetic factors that influence injury severity. We present evidence that this injury in the Balb/c mouse recapitulates key pathologies observed in blast-exposed veterans and leads to a robust, lasting injury profile in both the anterior and posterior globe. Thus, the Balb/c may be an ideal mouse strain for testing potential cell-based therapeutic interventions for eye trauma.

Methods

Animals

Adult Balb/c mice (The Jackson Laboratory) between 8 and 12 months of age were used in this study. Mice were maintained on a 12-h light–12-h dark cycle and provided food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision and Ophthalmic Research.

Ocular trauma

Eye injury was induced as previously described.⁸ Briefly, Balb/c mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (105/8 mg/kg) and secured and padded within a housing chamber. The housing chamber was placed inside a pipe. The left eye of the mouse was positioned against the hole in the pipe, which was aligned with the barrel of the paintball marker. All experiments were performed at an air pressure of 26 psi in the morning. Mice were provided gel recovery food (ClearH₂O) for the first 3 days postinjury.

Gross pathology

The eyes of awake Balb/c mice were assessed before injury and at 0, 3, 7, 14, 28, and 60 days later using an SZX16 stereomicroscope (Olympus). Representative images were taken using a DP71 camera (Olympus). Eyes were examined for the presence of corneal abrasions, corneal edema, hyphemas, cataracts, and corneal neovascularization (CNV).

Intraocular pressure

Intraocular pressure (IOP) was measured in awake mice before and after trauma using a TonoLab rebound tonometer (Colonial Medical Supply Co.).

Ultra-high resolution optical coherence tomography

Balb/c mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (25/10 mg/kg). A 1% tropicamide solution was used to dilate the eyes. Genteal lubricating eye gel (Alcon) was used to keep the eyes moist. The

mice were wrapped in gauze, placed in a holding chamber, and head position was stabilized with a bite bar. A BiopTigen ultra-high resolution spectral domain optical coherence tomography system with a mouse retinal bore (BiopTigen LLC) was used to image the retinas.

In vivo oxidative stress probes

All mice were given Refresh eye drops (Allergan) immediately postblast to preserve cornea clarity.¹⁰ Shortly before injections, CM-H₂DCFDA (Thermo Fisher Scientific) and dihydroethidium (DHE; Thermo Fisher Scientific) were diluted in sterile phosphate buffered saline (PBS) at 1:10 dilution and covered with foil. Mice were anesthetized with 3% inhalable isoflurane (Vet Equip) and placed on the stage of an Olympus SZ51 stereomicroscope (Olympus). Anesthesia was maintained during injections using a nose cone (Vet Equip). The mouse eyes were dilated using 1% Tropicamide eye drops (Akorn, Inc.). A Hamilton No. 802 microliter syringe (Hamilton Company) with a small-gauge needle (Hamilton Company) was used to puncture the mouse eye and inject 1 μ L of either probe into the vitreous chamber.

During optimization of our protocol, we determined that peak fluorescence occurred at 1 h postinjection for CM-H₂DCFDA and 30 min postinjection for DHE. Thus, all animals injected with CM-H₂DCFDA were imaged 1 h postinjection and all animals injected with DHE were imaged 30 min postinjection. Before imaging, the mice were anesthetized with 3% isoflurane (Vet Equip) and their eyes were dilated using 1% Tropicamide eye drops (Akorn, Inc.). Refresh lubricating eye drops (Allergan) was applied to the mouse eyes before imaging to prevent corneal dryness. The Micron IV retinal imaging microscope (Phoenix Research Labs) was used to capture brightfield images of the retina, as well as fluorescent images of the probes. A fluorescein filter was used to excite CM-H₂DCFDA and a near-infrared filter was used to excite DHE.

Tissue collection

Mice were euthanized by an overdose of Avertin delivered through intraperitoneal injection and transcardially perfused with 4% paraformaldehyde (PFA; Electron Microscopy Sciences) and PBS. The tissues were collected and stored in either 4% PFA (for immunohistochemistry) or 4% PFA with 0.5% glutaraldehyde (Electron Microscopy Sciences, for resin).

Retina histology

For histological analysis, eyecups were bisected to allow infiltration of Spurr's resin (Electron Microscopy Sciences), sectioned at 1 μ m-thickness on a Reichardt-Jung Ultracut E microtome (Leica Microsystems) and stained with Toluidine Blue. Representative images were collected on an Olympus PROVIS AX70 with a 60 $\times$ oil objective lens. To quantify retinal pigment epithelium (RPE) damage, a grading scale was developed to classify the vacuoles: 1 (normal, very infrequent, and small); 2 (small and infrequent); 3 (small and frequent); 4 (large and infrequent); and 5 (large and frequent). The number of pyknotic nuclei in the outer nuclear layer (ONL) or inner nuclear layer (INL) was quantified within a single section of retina through the middle of each eye. A masked experimenter performed the imaging.

Optic nerve histology

Optic nerves were placed in 1% osmium tetroxide in 0.1M cacodylate buffer, dehydrated in a graded ethanol series and embedded in Spurr's resin (Electron Microscopy Sciences). Starting from the proximal (near the eye) end of the optic nerve, 1 μ m-thick sections were collected from a Reichert-Jung Ultracut E microtome and stained with 1% *p*-phenylenediamine in 50% methanol (Sigma-Aldrich). Sections were imaged on an Olympus PROVIS AX70 microscope using a 100 $\times$ oil immersion objective lens. Imaging was performed in a masked fashion.

Retina immunohistochemistry

For immunohistochemistry, eyes were cryoprotected in 30% sucrose overnight at 4°C, embedded in Tissue Freezing Medium (Triangle Biomedical) and then sectioned on a cryostat (Fisher). Ten micrometer-thick sections were collected on 12 slides, with each slide containing representative sections from the entire eye. The sections were rinsed with PBS and incubated at room temperature in normal donkey serum at 1:20 in 0.1M phosphate buffer with 0.5% bovine serum albumin and 0.1% Triton X-100 (PBT) for 2 h, followed by an overnight incubation at 4°C in either anti-glial fibrillary acidic protein (GFAP) (1:400; Dako) or anti-nitrotyrosine (1:500; Millipore) in PBT. The sections were rinsed with PBS and incubated with a donkey anti-rabbit secondary antibody at a 1:200 dilution (Life Technologies) for 2 h at room temperature, rinsed with PBS and mounted in VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Imaging was performed on a Nikon Eclipse epifluorescence microscope (Nikon). The tissue was assessed in a masked fashion.

Quantification of fluorescence intensity

Nonoverlapping, but adjacent images of all areas of all retinal sections were collected using the same gain and exposure settings. Fluorescence was quantified within a rectangle of a set width and a height equivalent to the height of the retinal layer of interest using ImageJ according to previously published methods.¹²

TdT dUTP Nick End Labeling quantification

Retina sections were labeled with the TdT dUTP Nick End Labeling (TUNEL) Apoptosis Detection Kit adhering to the manufacturer's protocol (Merck Millipore) and mounted with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole. TUNEL-positive cells within the ONL, INL,

and ganglion cell layer (GCL) were counted and the lengths of the regions with TUNEL-positive cells (affected regions) were measured using NIS Elements Advanced Research software (Nikon). The total length of each retinal section with TUNEL-positive cells (affected section) was also measured. To determine the percentage of the retina with cell death, we measured and summed the lengths of all sections on the slide. Then, we divided the sum of affected region lengths by the total length of all sections and multiplied this value by 100, which yielded the percentage of retina with TUNEL-positive cells. Quantification was performed in a masked fashion.

Multiplex enzyme-linked immunosorbent assay

Retinas were sonicated and run in singlet on the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel I 25 plex assay as per the manufacturer's protocol (EMD Millipore). Plates were read on a Luminex MAGPIX with xPONENT software (Thermo Fisher Scientific) using settings stated in the manufacturer's protocol. Multiplex enzyme-linked immunosorbent assay plates were read and analyzed through the use of the Vanderbilt Hormone Assay and Analytical Services Core.

Statistical analysis

The median and range were calculated and presented for each data set. A one-way analysis of variance with a Bonferroni's *post-hoc* test was performed on the body weight, IOP, and TUNEL data using GraphPad Prism software (GraphPad).

Results

Overpressure airwave trauma does not alter body weight, but increases IOP

No significant changes in body weight were observed within the first 2 weeks postinjury (Fig. 1A). At the time of blast, the mice weighed 21 ± 0.2 g [Avg $\pm$ standard error of the mean (SEM)]. The weight remained consistent at 3, 7, and 14 days posttrauma: 20 ± 0.3 g, 21 ± 0.3 g, and 21 ± 0.5 g. At 28 days posttrauma, the mice weighed significantly more than at baseline (23 ± 0.3 g). There was no further increase at 60 days posttrauma (23 ± 0.5 g).

Within the first week postinjury, IOP was not significantly different than baseline (Fig. 1B). Before trauma mice had an average IOP of 12 ± 0.3 mmHg (Avg $\pm$ SEM). IOP remained consistent at 12 ± 0.4 mmHg and 12 ± 0.6 mmHg at 3 and 7 days posttrauma, respectively. IOP significantly increased over baseline values at both 14 and 28 days posttrauma to

FIG. 1. Eye-directed overpressure airwave-induced trauma had no effect on weight and caused a transient rise in intraocular pressure. (A) Box and whisker plot of mouse weight at blast (0 days) and over time out to 60 days (60 d) postblast. (B) Box and whisker plot of intraocular pressure measured at blast (0 d) and over time out to 60 d postblast. *** $P < 0.001$, **** $P < 0.0001$.

TABLE 1. QUANTIFICATION OF GROSS PATHOLOGY OVER TIME AFTER EYE-DIRECTED BLAST

Type of injury	0 days (43) ^a	3 days (38)	7 days (29)	14 days (5)	28 days (15)	60 days (5)
Corneal abrasions	0	6 (16)	2 (6.9)	5 (100)	0	0
Corneal edema	8 (19) ^b	12 (32)	22 (76)	0	5 (33)	5 (100)
CNV	0	2 (5.3)	5 (17)	2 (40)	4 (27)	3 (60)
Hyphema	0	0	1 (3.4)	0	0	0
Corneal scarring	0	0	4 (14)	0	8 (53)	0
Traumatic Cataract	3 (7)	3 (7.9)	4 (14)	0	0	0

^aTotal number of eyes examined.

^bNumber of eyes with pathology (percentage).

CNV, corneal neovascularization.

20±3 mmHg and 16±0.7 mmHg, respectively. IOP remained at 16±1.2 mmHg, but was not significantly different from baseline at 60 days posttrauma.

Eye-directed blast induces anterior pathologies

Immediately posttrauma, we observed corneal edema, CNV, and traumatic cataract in 15% of the mice (Table 1; Fig. 2A, B). The incidence of pathology increased over time posttrauma. At 28 days posttrauma, the hyphemas and corneal abrasions resolved, but corneal edema, CNV, corneal scarring, and traumatic cataract remained in 42%, 37%, 42%, and 10% of eyes, respectively. At 60 days posttrauma, only corneal edema and CNV remained, which were present in 100% and 60% of eyes, respectively. Due to the prevalence of anterior pathologies, we did not assess visual function.

Eye-directed blast exposure causes retinal detachments

Most retinas appeared similar to nontrauma controls (Fig. 3A). Large retinal detachments were detected in approximately one-third of blast-exposed eyes (Fig. 3B, C). The appearance of the retinal detachments with high and low areas is suggestive of a fusion of many small retinal detachments (Fig. 3B, C). The retinal detachments were present in the mid-peripheral retina, which appeared as mottled and/or darkened areas in the fundus images (Fig. 3D, E, arrowheads).

Eye-directed blast exposure damages cornea, retina, and RPE

Naive control eyes had healthy corneas (Fig. 4A), retinas, and RPE (Fig. 4E). In contrast, at 7 days posttrauma, the

corneal epithelium was thinned and CNV was evident in the stroma (Fig. 4B). Also at 7 days posttrauma, small, infrequent vacuoles (grade 2±0.4) were present in the RPE and the retina contained an average of 11±2 pyknotic nuclei in the ONL and 1±0.6 pyknotic nuclei in the INL (Fig. 4F). At 28 days posttrauma, the average grade for RPE vacuoles was 3±0.7, indicative of small, frequent vacuolization. The ONL and INL at this time point contained 14±9 and 14±13 pyknotic nuclei, respectively. At 60 days posttrauma, the corneas remained abnormal, with signs of edema and separation of the epithelium from the stroma (Fig. 4C, D). RPE vacuolization also remained high (grade 3±0.6) at this time point (Fig. 4G). Finally, pyknotic nuclei could still be detected in the ONL (3±0.4) and INL (2±0.4) (Fig. 4H).

Optic nerve degeneration occurs after eye-directed blast exposure

In the optic nerves from nonblast mice, the axons were tightly ensheathed by myelin and the axoplasm was clear (Fig. 5A). In contrast, at 7, 28, and 60 days posttrauma, the optic nerves contained degenerating axon profiles, marked by collapsed and thickened myelin (Fig. 5B–D, arrows).

Eye-directed blast exposure causes corneal and retinal cell death

In the corneas from nonblast mice there was a small number of TUNEL-positive cells as a result of natural turnover of the corneal epithelial cells, but no cell death in the stroma or endothelium (Fig. 6A). There was also no cell death in retinas from nonblast mice (Fig. 6D). In contrast, 3 days posttrauma, the cornea contained TUNEL-positive

FIG. 2. Eye-directed overpressure airwave-induced trauma caused anterior pathologies. Representative images of common pathologies detected after blast: (A) corneal edema (arrow), (B) cataract (arrowhead), and corneal neovascularization (arrows), (C) cataract, corneal edema, and hyphema (arrow).

FIG. 3. Eye-directed overpressure airwave-induced trauma induced large retinal detachments. (A, B) OCT B-line scans of retinas from (A) uninjured eye and (B) 3 days postblast eye. (C) Three-dimensional reconstruction of a stack of OCT B-line scans from a postblast eye showing multiple retinal detachments. (D, E) En face OCT images of postblast retinas showing the peripheral localization and grouping of the retinal detachments (arrowheads). OCT, optical coherence tomography; ON, optic nerve.

nuclei beyond natural turnover (data not shown) and $16 \pm 4\%$ of the retina was TUNEL positive (Fig. 6C). At 7 days posttrauma, the cornea contained TUNEL-positive nuclei in every layer (Fig. 6B) and $51 \pm 12\%$ of the retina contained TUNEL-positive cells (Fig. 6E), which was significantly higher than the amount of cell death observed at 3 and 28 days posttrauma (Fig. 6C). Cell death persisted at 28 days posttrauma, with $14 \pm 4\%$ of the retina containing TUNEL-positive nuclei (Fig. 6F). At all time points, the majority of the TUNEL-positive nuclei were located in the ONL.

Neuroinflammation occurs in the first week posttrauma

In the retinas from nonblast mice, GFAP immunolabeling was present in the Müller glia endfeet and astrocytes (Fig. 7A). In contrast, GFAP immunolabeling extended into the Müller glial process at 3 days (data not shown), 7 days (Fig. 7B), and 28 days posttrauma (Fig. 7C). These changes were uniform throughout the retina at all time points post-injury. We then assessed if the glial cell hypertrophy and infiltrating macrophages (Fig. 4F, H) also correlated with

FIG. 4. Eye-directed overpressure airwave-induced trauma injures the cornea, retina, and RPE. Representative brightfield micrographs of cornea (A–D) and retina/RPE (E–H) from uninjured (A, E), 7 days postblast (B, F), and 60 days postblast (C, D, G, H) mice. Arrowheads in (F) and (H) indicate condensed nuclei. Arrows in (F) and (G) indicate an infiltrating cell. Arrowheads in (G) indicate RPE vacuoles. Scale bars in (A) and (E) represent 25 μm and apply to (A, B, E–H). RPE, retinal pigment epithelium.

FIG. 5. Eye-directed overpressure airwave-induced trauma causes optic nerve degeneration. Representative brightfield micrographs of optic nerves from uninjured (**A**), 7 days postblast (**B**), 28 days postblast (**C**), and 60 days postblast (**D**) mice. Arrows indicate degenerating axons. Scale bar represents 50 μ m.

increased levels of proinflammatory cytokines and chemokines (Fig. 7D, E). A statistically significant increase was detected for keratinocyte chemoattractant (KC) (ie, CXCL1) at 3 days posttrauma as compared to the 3 days sham control ($P \leq 0.05$; Fig. 7D). The levels of IL-1 α , IP-10 (ie,

CXCL10), and IL-10 appeared equally increased at both 3 and 7 days after trauma, but statistical significance was not reached as compared to the matched sham controls. Variability was very high, potentially due to the focal nature of the injury, the early time point, and the small number of

FIG. 6. Eye-directed overpressure airwave-induced trauma induces cell death in the cornea and retina. Representative fluorescence micrographs of TUNEL in cornea (**A**, **B**) and retina (**D**–**F**) from uninjured (**A**, **D**), 3 days postblast (**E**), 7 days postblast (**B**), and 28 days postblast (**F**) mice. TUNEL (red), DAPI (blue). Scale bar represents 50 μ m. (**C**) Box and whisker plot of retinal TUNEL quantification at 3, 7, and 28 days postblast, $**P < 0.01$ and $***P < 0.001$. DAPI, 4',6-diamidino-2-phenylindole; Epi, corneal epithelium; S, stroma; Endo, corneal endothelium; DAPI, 4',6-diamidino-2-phenylindole.

FIG. 7. Eye-directed overpressure airwave-induced trauma mild neuroinflammation. (A–C) Representative fluorescence micrographs of GFAP immunolabeling in retinas from (A) uninjured, (B) 7 days postblast, and (C) 28 days postblast retinas. Anti-GFAP (green), DAPI (blue). Scale bar represents 50 μm. (D, E) Bar graphs of cytokines altered or trending toward an increase after injury. Since changes at 3 and 7 days posttrauma were comparable for most cytokines we also show all sham and all trauma groups combined. There is a trend for an increase in IL-1α that does not reach statistical significance. Both IP-10 and IL-10 levels were increased when comparing the all-sham and all-blast groups. KC levels were increased in the 3 days posttrauma group as compared to the 3 days sham group. There was a trend for an increase in MCP-1 at 3 days, but not 7 days posttrauma. * $P \leq 0.05$, # $P \leq 0.0001$; S, sham, T, trauma.

samples ($n = 4-7$). Therefore, we combined the 3- and 7-day groups together to create the all-trauma and all-sham groups. A statistically significant increase was detected in both IP-10 ($P \leq 0.0001$) and IL-10 ($P \leq 0.05$) in the all-trauma group as compared to all sham. MCP-1 (CCL2) trended higher at 3 days, but not 7 days after trauma. Because of the different response at 3 and 7 days posttrauma, there was no statistically significant difference between the all-sham and all-blast group. However, there was a significant difference between the all-sham and the 3 days postblast group ($P \leq 0.05$; Fig. 7D).

Reactive oxygen species are increased after trauma

Using the *in vivo* fluorescent markers CM-H₂DCFDA and DHE, an increase in reactive oxygen species (ROS) was detected at 7 days posttrauma (Fig. 8A–D). No fluorescence was detected in sham eyes after injection of either probe (Fig. 8A, C). In contrast, fluorescence for both markers was present after trauma. The eyes with the greatest amount of fluorescence are shown. In particular, levels of superoxide appear to be elevated at 7 days posttrauma (Fig. 8D). Imaging and quantification of immunofluorescence for nitrotyrosine, as an indicator of peroxynitrite, was also performed (Fig. 8E–H). Nitrotyrosine immunolabeling was

significantly increased in the inner, but not outer retina at 7 days posttrauma.

Discussion

The safety and efficacy of cell-based therapies must be tested in animal models before moving to the clinic. Trauma-induced damage is advantageous over inherited diseases for testing cell-based therapeutic approaches because there is no underlying genetic abnormality to overcome. Furthermore, the lasting damage to the RPE and cornea in the model presented in this study makes it ideal for testing cell-based therapies directed at replacing or regenerating the RPE, cornea, or optic nerve or promoting proper tissue healing of the corneal epithelium.

Optic nerve degeneration occurred rapidly in the Balb/c after blast in our model. We did not detect any degenerating axon profiles in the C57Bl/6J and the DBA/2J optic nerves until 28 days postinjury.^{9,10} In contrast, the Balb/c optic nerves contained degenerating axons as early as 7 days postinjury. Traumatic optic neuropathy, which can occur after blast or blunt trauma,^{6,13,14} is induced by 2 different injury mechanisms: primary (shearing of the axons and ischemia) and secondary (initial damage and death of the retinal ganglion cells).¹⁵ Given that the majority of

FIG. 8. Eye-directed overpressure airwave-induced trauma induces oxidative stress. (A–D) Maximal fluorescence detected by *in vivo* imaging with markers of reactive oxygen species. (A, B) *In vivo* images of CM-H₂DCFDA fluorescence, a general indicator of reactive oxygen species. Showing no fluorescence in sham eyes (A) and green fluorescence at 7 days postinjury (B). (C, D) *In vivo* images of dihydroethidium fluorescence, an indicator of superoxide showing no fluorescence in sham eyes (C) and red fluorescence at 7 days postinjury (D). (E, F) Representative epifluorescence micrographs of nitrotyrosine immunolabeling (green) of sham (E) and 7 days posttrauma (F) retinas double labeled with DAPI (blue). Scale bar represents 50 μ m. (G, H) Bar graph quantification of nitrotyrosine immunolabeling showing an increase in the inner (G), but not outer (H) retina. $^{\#}P \leq 0.0001$.

the TUNEL-positive nuclei were located in the ONL, it is unlikely that the retinal ganglion cells die before axon degeneration, supporting a role for primary optic nerve injury. The Balb/c is more susceptible to optic nerve crush than the C57Bl/6J and the DBA/2J,¹⁶ so it is possible that genetic differences are driving the early onset of axonal degeneration in the Balb/c postblast. Furthermore, this model may be more representative of clinical traumatic optic neuropathy than the optic nerve crush or axotomy models.

Vacuolization of the RPE, a symptom of oxidative stress,¹⁷ occurred in all 3 mouse strains postinjury. RPE damage was also reported in 36% of blast-exposed soldiers.¹³ In contrast to the C57Bl/6J and DBA2J, RPE vacuolization did not resolve in the Balb/c.^{9,10} The RPE is an important component of the blood–retina barrier¹⁸ and is essential for photoreceptor health and function.¹⁹ Enduring oxidative damage to the RPE could potentially cause loss of ocular immune privilege and ensuing inflammation. Oxidative stress and blood–brain-barrier permeability also occur in models of blast-

induced TBI, suggesting a similar mechanism in the eye and brain.^{20,21}

Consistent with the RPE damage, levels of ROS were increased early after trauma from an eye-directed overpressure airwave. We detected increases in superoxide and peroxynitrite, in particular. The increases in these ROS support our other findings that early and short-term treatment with systemic erythropoietin results in increased oxidative stress and cell death.²² This was likely induced by the increase in hematocrit and, therefore, iron into the retina, which pushed the Fenton reaction resulting in increased production of the damaging hydroxyl radical. These results suggest that treatment with antioxidants within the first week after trauma may reduce the downstream neuronal damage.

Delayed neuronal cell death after blast exposure is a common finding in models of blast-induced TBI.^{23,24} The accelerated cell death in the DBA/2J and Balb/c as compared to the C57Bl/6 in our model might be due to the greater inflammatory response exhibited by the former 2 strains, including Müller cell reactivity and peripheral immune cell infiltration.^{9,10} Reactive Müller cells can cause retinal edema, secrete inflammatory cytokines, and promote macrophage infiltration, which we detected histologically.^{25,26} We also detected increases in some proinflammatory cytokines and chemokines as early as a few days post-trauma, including IP-10 and KC. IL-1 α can induce neuronal expression of IP-10, KC, and MCP-1.²⁷ While we did not detect a statistically significant increase in IL-1 α , levels at both time points trended up and might explain the increases in these chemokines. These 3 chemokines are also increased in other models of central nervous system (CNS) injury and can act on both glia and neurons to mediate the recruitment and infiltration of peripheral immune cells into the CNS.^{28,29} This suggests that there may be an increase in microglia in the retina after eye-blast-induced trauma. While acute neuroinflammation is protective, chronic neuroinflammation can contribute to neurodegeneration, thus this pathway is also a potential therapeutic target.

The increase in IL-10 in the combined 3 and 7 days posttrauma retinas is suggestive of an endogenous protective response. IL-10 is an anti-inflammatory cytokine that inhibits activation of macrophages, microglia, and astrocytes and blocks production of proinflammatory cytokines and inducible nitric oxide synthase, resulting in lower levels of peroxynitrite.^{30–36} Gene delivery of IL-10 decreases nitrotyrosine immunolabeling and preserves ganglion cells in an optic nerve axotomy model.³⁷ This strategy may also be effective in the treatment of eye trauma due to an overpressure airwave.

Significant changes in IOP occurred in both the Balb/c and C57Bl/6J after blast-induced injury using our model.⁸ In addition, a recent study reported increased IOP in 13% of blast-exposed patients.³ Increased IOP and corneal injuries, especially hyphema, are risk factors for the development of posttraumatic glaucoma.^{38,39} Interestingly, IOP was increased postinjury in the Balb/c and decreased postinjury in the C57Bl/6J. These strain differences may be due to the large number of corneal injuries in the Balb/c (a risk factor for increased IOP) and the paucity of corneal injuries in the C57Bl/6J posttrauma. The change in IOP is suggestive of posttraumatic glaucoma, however, this will need to be confirmed in future long-term studies with a larger cohort of

mice and using additional measures of glaucoma such as angle recession.

In conclusion, the Balb/c mouse model of ocular trauma due to an eye-directed overpressure airwave provides a good platform for testing cell-based therapies to replace/restore the cornea, optic nerve, or RPE. These therapies are much needed, as there are currently no treatment options for eye trauma patients.

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