CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Glaucoma after Ocular Surgery or Trauma

The Role of Infiltrating Monocytes and Their Response to Cytokine Inhibitors

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Accepted for publication
July 7, 2020.

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Glaucoma is a frequent and devastating long-term complication following ocular trauma, including corneal surgery, open globe injury, chemical burn, and infection. Postevent inflammation and neuroglial remodeling play a key role in subsequent ganglion cell apoptosis and glaucoma. To this end, this study was designed to investigate the amplifying role of monocyte infiltration into the retina. By using three different ocular injury mouse models (corneal suture, penetrating keratoplasty, and globe injury) and monocyte fate mapping techniques, we show that ocular trauma or surgery can cause robust infiltration of bone marrow–derived monocytes into the retina and subsequent neuroinflammation by up-regulation of Tnf, Il1b, and Il6 mRNA within 24 hours. This is accompanied by ganglion cell apoptosis and neurodegeneration. Prompt inhibition of tumor necrosis factor- α or IL-1 β markedly suppresses monocyte infiltration and ganglion cell loss. Thus, acute ocular injury (surgical or trauma) can lead to rapid neuroretinal inflammation and subsequent ganglion cell loss, the hallmark of glaucoma. Infiltrating monocytes play a central role in this process, likely amplifying the inflammatory cascade, aiding in the activation of retinal microglia. Prompt administration of cytokine inhibitors after ocular injury prevents this infiltration and ameliorates the damage to the retina—suggesting that it may be used prophylactically for neuroprotection against post-traumatic glaucoma. (*Am J Pathol* 2020, 190: 2056–2066; <https://doi.org/10.1016/j.ajpath.2020.07.006>)

Glaucoma is a frequent and devastating long-term complication after acute ocular trauma, including corneal surgery, open globe injury, chemical burn, and infection.^{1–17} Intraocular pressure (IOP) elevation in the healing stage has usually been considered the primary reason for post-traumatic glaucoma; however, recent animal studies have demonstrated that inflammation might also be an important, IOP-independent pathway, justifying further investigation.^{18–22} At the molecular level, tumor necrosis factor- α (TNF- α) has been shown to be a key mediator of such neuroinflammation, and its prompt inhibition using a monoclonal antibody has been markedly protective against ganglion cell damage—the hallmark of glaucoma.^{18,20,23–28} TNF- α up-regulation has also been found in the blood of patients implanted with an artificial cornea (Boston

Keratoprosthesis), even years after the surgery, which may be a factor in the progression of glaucoma despite apparently normal IOP in keratoprosthesis recipients.²⁹ In summary, these data suggest that targeted immunomodulation should be able to reduce the severity of late glaucoma following acute injury to the eye.

Supported by the Boston Keratoprosthesis Research Fund, Massachusetts Eye and Ear (X.C., F.L., C.Z., J.C., C.H.D., E.I.P.), the Eleanor and Miles Shore Fund (E.I.P.), the Massachusetts Lions Eye Research Fund (E.I.P.), NIH National Eye Institute core grant P30EY003790, R01 grant 5R01EY029437-03 (E.I.P.), the National Key R&D Program of China Project 2017YFA0103204 (L.W. and X.C.).

Disclosures: None declared.

The present study was designed to further explore the development of post-traumatic glaucoma and particularly to investigate a possible amplifying pathway, effectuated by infiltration of bone marrow–derived monocytes into the retina. This is important because it was recently shown that monocytes can contribute to sustained neurodegeneration of the retina, long after chemical injury.^{30–34}

Materials and Methods

Animals

All animal-based procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH's *Guide for the Care and Use of Laboratory Animals*.³⁵ Animal Care Committee protocol approval was obtained at the Massachusetts Eye and Ear (Boston, MA). The 6- to 8-week-old (body weight, approximately 25 g) C57BL/6J, *Cx3cr1*^{EGFP/EGFP}, and B6. *Thy1*^{YFP} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Cx3cr1*^{+/EGFP} mice were generated by mating C57BL/6J with *Cx3cr1*^{EGFP/EGFP}. Bone marrow chimeras were generated, as previously described.³¹ Briefly, C57BL/6J mice were myelodepleted with three i.p. injections of busulfan (Sigma-Aldrich, St Louis, MO) (35 mg/kg) administered 7, 5, and 3 days before bone marrow transfer. Bone marrow cells (5×10^6 total) from *Cx3cr1*^{+/EGFP} mice were transferred to the myelodepleted C57BL/6J mice through tail vein injection 1 month before corneal injury. Bactrim was given ad lib for 15 days after myelodepletion to avoid infection.

The mouse strain C57BL/6J is a well-characterized wild-type black mouse, used in most of studies in immune studies. The *Cx3cr1*^{+/EGFP} reporter mouse is based on a BL/6 wild-type background and expresses green fluorescent protein under the fractalkine receptor *Cx3cr1* gene promoter. This labels mature monocytes/macrophages and microglia, and is commonly used in neuroglia biology. The *Thy1*^{YFP} mouse is also a BL/6 background mouse strain, genetically modified to express yellow fluorescent protein under the *Thy1* gene promoter. *Thy1* is expressing in retinal ganglion cells and nerve axons. Bone marrow *Cx3cr1*^{+/EGFP} mice were produced to trace and distinguish peripheral monocytes/macrophages from yolk sac–derived microglia.

Corneal Suture Model

A single 11-0 nylon suture (2881 G; 11-0 Ethilon black 5" BV50-3 taper; Johnson & Johnson, Neenah, WI) was placed full thickness in the cornea. Topical Polytrim antibiotic (Bausch & Lomb Inc., Bridgewater, NJ) was applied after the procedure, followed by tarsorrhaphy using 8-0 nylon sutures (Sharp; Surgical Specialties Co., Wyomissing, PA). Immediately after the procedure, mice received s.c. buprenorphine injection every 12 hours (0.1 mg/kg; Indivior, North Chesterfield, VA) for 3 days after surgery. Eyes were analyzed at 24 hours and 7 days after the injury.

Autologous Penetrating Keratoplasty Model

All surgeries were performed in anesthetized mice using a surgical microscope (S7; Zeiss, Oberkochen, Germany). A drop of 0.5% phenylephrine hydrochloride (Altaire Pharmaceuticals, New York, NY) was applied for pupil dilation 15 minutes before the procedure. Corneas were marked with a 2-mm–diameter biopsy punch (Miltex, Plainsboro, NJ). Viscoelastic (Viscoat; Alcon, Irvine, CA) was injected through a groove with a 30-gauge cannula (Rumex, Clearwater, FL) to maintain a deep anterior chamber. Corneas were trephined and then excised using Vannas scissors (Storz Instruments Company, San Dimas, CA), and immediately resutured back to the same eye using 11-0 nylon sutures (Sharp; Angiotech Pharmaceuticals, Vancouver, BC, Canada) in interrupted manner. Topical Polytrim (Allergan, Madison, NJ) antibiotic was applied after the procedure, followed by tarsorrhaphy using 8-0 nylon sutures. Immediately after the procedure, mice received s.c. buprenorphine injection every 12 hours for 3 days after surgery. Eyes were analyzed at 24 hours and 7 days after the injury.

Penetrating Keratoplasty with Globe Trauma Model

Controlled globe trauma was performed surgically by penetrating the cornea using a 2-mm trephine and extending the injury to the iris with iridotomy and to the lens with mechanical lens removal (forceps) (Katena, Denville, NJ). The cornea was then immediately resutured using 11-0 nylon sutures, and topical Polytrim antibiotic was administered to the eye, followed by tarsorrhaphy with 8-0 nylon sutures. Eyes were analyzed at 24 hours and 7 days. Immediately after the procedure, mice received s.c. buprenorphine injection every 12 hours for 3 days after surgery.

Recombinant IL-1 β Injection into the Anterior Chamber

Anterior chamber tap and inject of 1 μ L of recombinant IL-1 β (1 μ g; catalog number 211-11B; PeproTech, Cranbury, NJ) was performed using a 33-gauge needle in BMT *Cx3cr1*^{+/EGFP} reporter mice. Eyes were treated with topical Polytrim antibiotic and enucleated 24 hours later for retinal analysis using real-time quantitative RT-PCR and confocal microscopy.

Systemic Administration of Pharmaceutical Agents

Dexamethasone, 20 mg/kg (Baxter, Deerfield, IL), infliximab, 10 mg/kg (Remicade; Janssen Biotech, Inc., Horsham, PA), anakinra, 10 mg/kg (Kineret; Sobi, Inc., Waltham, MA), and a combination of infliximab (10 mg/kg) + anakinra (10 mg/kg) were injected intraperitoneally immediately after the procedure. For control, IgG (10 or 20 mg/kg; catalog number 500-M00; PeproTech, Rocky Hill, NJ) was injected intraperitoneally.

Figure 1 Ocular surgery or trauma causes rapid infiltration of bone marrow–derived C-X3-C motif chemokine receptor 1–positive (CX3CR1⁺) monocytes into the retina. **A–D:** Clinical presentation of mouse eyes with no injury (**A**), penetrating corneal suture injury (**B**), penetrating keratoplasty (PK; **C**), and PK with globe trauma (Globe; **D**) at 24 hours. **E–H:** Flat mount retinal confocal images of BL6;Cx3cr1⁺/EGFP chimeras infiltrated by bone marrow–derived CX3CR1⁺ monocytes within 24 hours of the injury. **I:** Quantification of the relative number of infiltrated CX3CR1⁺/EGFP monocytes showing significant increase following injury. **J:** Schematic representation of flat mount retinas, showing the optic nerve (central white circle), the retinal vessels (red lines), and the infiltrating CX3CR1⁺ monocytes (green oval). **K:** Flow cytometry of retinas from Cx3cr1⁺/EGFP reporter mice. Retinal monocytes/macrophages were selected using the CX3CR1⁺ and CD45⁺ markers. Two monocyte/macrophage subpopulations were identified, the CD45^{hi} (peripheral monocytes) and CD45^{lo} (microglia). Further analysis confirms the infiltration of CX3CR1^{lo} (peripheral monocytes) after surgical injury, whereas the CX3CR1^{hi} microglia number remains unchanged. **I:** Analysis of variance analysis with Tukey correction for multiple comparison. **K:** Unpaired *t*-test. *n* ≥ 8 mice per group (**I**); *n* = 4 mice per group (**K**). ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Scale bars = 100 μm (**E–H**). Ctrl, naive control; EGFP, enhanced green fluorescent protein; NoTx, no treatment control.

Figure 2 Tumor necrosis factor- α (TNF- α) or IL-1 β inhibition prevents CD45⁺ myeloid cell infiltration into the retina after ocular surgical injury. **A–G:** Flow cytometry (**A–F**) and quantification (**G**) of the relative number of C-X3-C motif chemokine receptor 1-positive (CX3CR1^{+/EGFP}) infiltrating monocytes into the retina at 24 hours after penetrating keratoplasty (PK) injury. **A** and **G:** PK injury causes marked infiltration of CX3CR1⁺ monocytes into the retina within 24 hours. **B** and **G:** Systemic administration of corticosteroids [dexamethasone (Dex)] has minimal effect in suppressing monocyte infiltration after ocular trauma (at clinical acceptable doses). **D–G:** In contrast, TNF- α or IL-1 β inhibitor (at clinically acceptable doses) prevents peripheral CX3CR1⁺ monocyte infiltration into the retina after surgery (**D**, **E**, and **G**), whereas concomitant inhibition of TNF- α and IL-1 β has an additive effect, causing further reduction in peripheral monocyte infiltration (**F** and **G**). **G:** Analysis of variance analysis with Tukey correction for multiple comparison. $n = 4$ mice per group (**A–G**). * $P < 0.05$, **** $P < 0.0001$. Anak, anakinra (anti-IL-1 β); Ctrl, naive control; EGFP, enhanced green fluorescent protein; Inf, infliximab (anti-TNF- α); NoTx, no treatment control.

Quantitative Real-Time PCR

Eyes were harvested for quantitative real-time PCR at 24 hours after the injury. Corneal and retinal tissues were surgically dissected, placed in cryovials, and frozen in liquid nitrogen. Retinal RNA isolation was performed using RNeasy Mini Kit (74,106; Qiagen, Valencia, CA) and quantified using nanodrop spectrophotometer (Nanodrop 2000; Thermo Scientific, Waltham, MA). Each sample was normalized, and cDNA synthesis was performed using SuperScript IV VILO (11756050; ThermoFisher, Carlsbad, CA). cDNA (1 μ L) was used for RT-PCR. The following TaqMan probes were used: *TNFA* (Mm99999068_m1; Life Technologies, Woburn, MA), *IL1B* (MmMm00434228_m; Thermo Scientific), and *IL6* (Mm00446190_m1; Thermo Scientific). The average C_T value was used in the analysis. C_T values were normalized using *18S* (Hs99999901_s1; Thermo Scientific), and ranges were normalized based on naive control tissue. $\Delta\Delta C_T$ algorithm was used to compare mRNA levels.

TUNEL Labeling and Quantification

Eyes were enucleated 24 hours after the injury, fixed in 4% paraformaldehyde, and embedded in optimal compound temperature OCT (Tissue Tek; Sakura, Leiden, the Netherlands). Multiple sagittal cryosections of approximately 10 μ m in thickness were obtained from the center and periphery of the globe. Tissue sections were transferred

to positive charged glass slides (Superfrost Plus; 75 $\times$ 25 mm; 1-mm thickness; VWR, Radnor, PA) and stored at -80°C for further processing. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) labeling was performed on tissue sections using the Roche TUNEL kit (12156792910; Roche, Indianapolis, IN), as previously published.²⁰ Slides were mounted with DAPI (UltraCruz; sc-24941; Santa Cruz Biotechnology, Dallas, TX) and coverslip, and imaged using epifluorescent microscopy (Leica DM5500 B; Leica, Wetzlar, Germany). The percentage of TUNEL expression in the peripheral retina was calculated as the area ratio of TUNEL/DAPI (%) using Particle Analysis of ImageJ software version 1.52p (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Quantitative data were presented as means $\pm$ SD.

Cellular Assessment by Flow Cytometry

Eyes were harvested 7 days after the injury for flow cytometry, and retinas were digested to generate single cells for flow cytometry using collagenase Type I and Papain Dissociation System (Worthington Biochemical Corp., Lakewood, NJ). Allophycocyanin anti-mouse CD45 antibody (clone 1040) and PerCP anti-mouse/human CD11b antibody (clone M1/70) were used. All antibodies were purchased from BioLegend (San Diego, CA). Cells were analyzed on a BD LSR II cytometer (BD Biosciences, San Jose, CA) using FlowJo software version 10.6.2 (Tree Star, Ashland, OR).

Figure 3 Concomitant inhibition of tumor necrosis factor- α (TNF- α) and IL-1 β suppresses C-X3-C motif chemokine receptor 1-positive (CX3CR1⁺) monocyte entry into the retina following surgical or globe trauma. **A–D:** Confocal images of flat mount retinas from BL6;Cx3cr1^{+/EGFP} chimeras 24 hours after corneal suture injury, penetrating keratoplasty (PK), and PK with globe (Globe) trauma. **E:** Quantification of the relative number of CX3CR1⁺ monocyte infiltration into the retina. Concomitant inhibition of TNF- α and IL-1 β inhibitors have a significant effect in suppressing peripheral CX3CR1⁺ monocyte entry into the retina following surgical or globe trauma. After TNF- α and IL-1 β inhibition, the number of peripheral CX3CR1⁺ cells in the retina is similar to that of naive mice. **F:** Schematic representation of flat mount retinas, showing the optic nerve (central white circle), the retinal vessels (red lines), and the infiltrating CX3CR1⁺ monocytes (green oval). **E:** Analysis of variance analysis with Tukey correction for multiple comparison. $n \geq 8$ mice per group (**E**). * $P < 0.05$, **** $P < 0.0001$. Scale bars = 100 μ m (**A–D**). Anak, anakinra (anti-IL-1 β); Ctrl, naive control; EGFP, enhanced green fluorescent protein; Infix, infliximab (anti-TNF- α).

Figure 4 Ocular surgical trauma causes prompt up-regulation of key inflammatory mediators in the retina. **A–I:** Corneal suture, penetrating keratoplasty (PK), and more severe globe trauma cause tumor necrosis factor- α (Tnf; **A–C**), Il1b (**D–F**), and Il6 (**G–I**) mRNA up-regulation in the retina within 24 hours. The magnitude of this up-regulation depends on the severity of the injury, with globe trauma causing the strongest response, whereas the corneal suture was the weakest. Prompt inhibition of TNF- α or IL-1 β after the injury suppresses the inflammatory expression in the retina; however, concomitant TNF- α and IL-1 β inhibition is synergistic in Il1b suppression after severe globe trauma. In contrast, systemic administration of dexamethasone (Dex) has limited effect in suppression of retinal inflammation and is therapeutically inferior to TNF- α or IL-1 β inhibitors. **J** and **K:** Injection of recombinant IL-1 β (1 μ g in 1 μ L) in the anterior chamber causes significant up-regulation of Il1b, Tnf, and Il6 mRNA (**J**) and infiltration of bone marrow-derived C-X3-C motif chemokine receptor 1-positive (CX3CR1⁺/EGFP) monocytes/macrophages in the retina within 24 hours (**K**). **A–I:** Analysis of variance analysis with Tukey correction for multiple comparison. **J:** Independent *t*-test. **L:** Schematic representation of flat mount retinas, showing the optic nerve (central white circle), the retinal vessels (red lines), and the infiltrating CX3CR1⁺ monocytes (green oval). *n* = 4 C57BL/6 mice per group (**A–J**). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Scale bar = 100 μ m (**K**). Anak, anakinra (anti-IL-1 β); A.U., arbitrary unit; Ctrl, naive control; EGFP, enhanced green fluorescent protein; Inf, infliximab (anti-TNF- α); NoTx, no treatment control; rIL-1 β , recombinant interleukin 1 beta.

Flat Mount Retinal Imaging

Eye were enucleated 24 hours after the injury and fixed in 4% paraformaldehyde solution for 1 hour at 4°C. Cornea and retina tissues were carefully dissected, washed three times for 5 minutes in phosphate-buffered saline solution at 4°C, and mounted using VECTRASHIELD mounting medium (H-1000; Vector Laboratories, Burlingame, CA) and a coverslip. Confocal images were taken using the Leica SP8 confocal microscope.

Statistical Analysis

The normality of continuous variables was assessed using the Kolmogorov-Smirnov test.³⁶ Quantitative variables were expressed as means $\pm$ SD, and qualitative variables were expressed as frequencies and percentages. Multiple comparison tests were performed using one-way analysis of variance, with Tukey correction. All tests were two tailed, and statistical significance was determined at $P < 0.05$. Results were

analyzed with Prism version 8.2.1 (GraphPad Software Inc., San Diego, CA).

Results

Ocular Surgery or Trauma Causes Monocyte Infiltration into the Retina within 24 Hours, Introducing a New Pathway to Further Neuroretinal Degeneration

CX3CR1 is a chemokine receptor expressed primarily by bone marrow–derived macrophages and microglia.³² The effect of ocular injury (surgical or trauma) on retinal innate immune response was evaluated using C57BL/6 mice transferred with CX3CR1^{+/EGFP} bone marrow cells following busulfan myelodepletion (Figure 1, A–D). It has been previously shown that busulfan myelodepletion does not cause blood retinal barrier disruption, and hence, peripheral monocyte infiltration into the retina would be the result of experimental injury.^{31–33}

Herein, it is shown that all types of ocular injuries [corneal suture, penetrating keratoplasty (PK), and globe trauma]

Figure 5 Tumor necrosis factor- α (TNF- α) and IL-1 β inhibition prevents retinal damage and ganglion cell loss after ocular surgical trauma. **A–E**: Retinal cell apoptosis 24 hours after penetrating keratoplasty (PK), using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (apoptotic cells: red). **F**: Quantification of relative cell apoptosis using pixel density analysis. **G**: Quantification of retinal Thy-1 cell surface antigen (Thy1⁺) ganglion cell number at 7 days using flow cytometry. **A** and **F**: PK injury causes retinal cell apoptosis within 24 hours of surgery, especially in the peripheral retina. **B** and **F**: Systemic administration of dexamethasone (Dex) has limited effect on retinal protection. **C–F**: In contrast, systemic administration of TNF- α or IL-1 β inhibitors abrogates retinal cell apoptosis, whereas concomitant inhibition of TNF- α and IL-1 β provides similar protection of the retinal cells as monotherapy. **G**: At 7 days after the injury, the number of Thy1⁺ retinal neuron cells exhibits a significant reduction. This loss of Thy1⁺ cells cannot be inhibited by dexamethasone administration (at clinically acceptable doses), but can be prevented using TNF- α or IL-1 β inhibitors (at clinically acceptable doses). Concomitant TNF- α and IL-1 β inhibition confers similar protection to Thy1⁺ cells as monotherapy. **F** and **G**: Analysis of variance analysis with Tukey correction for multiple comparison. $n = 5$ C57BL/6 mice per group (**F**); $n = 4$ Thy1^{YFP/YFP} mice per group (**G**). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Images obtained with a 25 $\times$ objective (**A–E**). Anak, anakinra (anti-IL-1 β); Ctrl, naive control; GCL, ganglion cell layer; Inf, infliximab (anti-TNF- α); INL, inner nuclear layer; ONL, outer nuclear layer; NoTx, no treatment control; YFP, yellow fluorescent protein.

cause significant infiltration of CX3CR1⁺ bone marrow–derived monocytes into the retina within 24 hours (Figure 1, E–J). The density of infiltrated cells is highest around the optic nerve head (Figure 1, E–H). Globe trauma caused significantly more peripheral CX3CR1⁺ monocyte infiltration into the retina, as opposed to corneal suture injury, which caused the least (Figure 1I). Schematic representation of flat mount retinal images is given (Figure 1J).

Further analysis was performed using flow cytometry of *Cx3cr1*^{+/EGFP} reporter mice after PK injury. At 24 hours after the injury, the retina exhibited significant increase in CX3CR1⁺ CD45⁺ monocytes. This increase was attributed to CD45^{hi} bone marrow–derived monocyte infiltration rather than CD45^{lo} yolk sac–derived microglia proliferation. Further phenotype analysis of the cells showed that most (75%) of the CX3CR1⁺ cells had peripheral origin (CX3CR1^{lo}) as opposed to naive mice, where 100% of the monocytes were CX3CR1^{hi} yolk sac–derived microglia (Figure 1K).

TNF- α or IL-1 β Inhibition Prevents Peripheral CX3CR1⁺ Monocyte Infiltration into the Retina

It was previously shown that infiltration of peripheral monocytes into the retina is a major mediator of long-term retinal inflammation and degeneration following chemical insult.^{32,33} Conversely, preventing these cells from entering into the retina is neuroprotective.^{32,33} Herein, systemic administration of TNF- α or IL-1 β inhibitor prevented peripheral CX3CR1⁺ monocyte infiltration into the retina after PK. Concomitant inhibition of TNF- α and IL-1 β had an additive effect, causing further reduction in peripheral monocyte infiltration (Figure 2). In contrast, systemic administration of corticosteroids (dexamethasone) had only a minimal effect and was significantly inferior to TNF- α or IL-1 β inhibition in suppressing monocyte infiltration after ocular trauma (Figure 2).

Further investigation of the effect of combined TNF- α and IL-1 β inhibition on peripheral immune cells was performed in C57BL/6 mice transferred with CX3CR1^{+/EGFP} bone marrow cells. Concomitant inhibition of anti-TNF- α and IL-1 β significantly suppressed CX3CR1⁺ monocyte entry into the retina following surgical or globe trauma (Figure 3, A–E). After TNF- α and IL-1 β inhibition, the number of peripheral CX3CR1⁺ cells in the retina was similar to that of naive mice (Figure 3E). Schematic representation of flat mount retinal images is given (Figure 3F).

Surgical Injury and Globe Trauma Cause Rapid Retinal Inflammation and Cell Apoptosis that Can Be Ameliorated by TNF- α and IL-1 β Inhibitors

It has been previously shown that ocular chemical trauma and corneal surgery cause increased TNF- α and IL-1 β expression in the retina.^{18,19,21} This study expands on those findings to show that corneal suture, PK, and more severe globe trauma all cause Tnf, Il1b, and Il6 mRNA up-regulation in the retina

within 24 hours (Figure 4, A–I). The magnitude of this up-regulation depended on the severity of the injury, with globe trauma causing the strongest response, and corneal suture the weakest (Figure 4, A–I). Prompt inhibition of TNF- α or IL-1 β after the injury suppressed inflammatory gene expression in the retina (Figure 4, A–I), and concomitant TNF- α and IL-1 β inhibition was better in suppressing retinal Il1b expression after severe globe trauma (Figure 4F). In contrast, systemic administration of dexamethasone had a limited effect in suppression of retinal cytokine expression, and was therapeutically inferior to TNF- α or IL-1 β inhibitors (Figure 4, A–I). To assess the role of cytokine diffusion from the injured cornea back into the retina and its effect on retinal inflammation and monocyte infiltration, anterior chamber injection was performed with recombinant IL-1 β . At 24 hours after the injection, the retina exhibited significant up-regulation of Il1b, Il6, and Tnf mRNA levels (Figure 4J) and marked infiltration of bone marrow–derived CX3CR1^{+/EGFP} cell (Figure 4K). Schematic representation of flat mount retinal images is given (Figure 4L).

Retinal cell apoptosis was previously identified as an early marker of retinal damage after chemical injury.^{18,20} In the current study, PK also causes retinal cell apoptosis at 24 hours of surgery, especially in the peripheral retina. This was abrogated with prompt administration of TNF- α or IL-1 β inhibitors (Figure 5, A and F). In contrast, administration of dexamethasone had a limited protective effect and was therapeutically inferior in retinal protection compared with TNF- α or IL-1 β inhibitors (Figure 5, B and F). Concomitant inhibition of anti-TNF- α and IL-1 β provided similar protection to the retinal cells as monotherapy (Figure 5, C–F). Thy1 is a marker of retinal neuron cells, used for the quantification of retinal ganglion cells.³⁷ At 7 days after PK, the number of Thy1⁺ retinal ganglion cells was significantly reduced. This loss of Thy1⁺ cells was not inhibited by dexamethasone administration (20 mg/kg), but was prevented using TNF- α or IL-1 β inhibition (Figure 5G). Concomitant TNF- α and IL-1 β inhibition conferred protection of Thy1⁺ cells similar to either agent alone (Figure 5G).

Discussion

Herein, we first confirm and expand on previous findings that mechanical trauma or ocular surgery can cause retinal damage, including ganglion cell apoptosis, through an inflammatory mechanism.^{18–20,38} To this end, three different ocular injury models (namely, a full-thickness corneal suture, PK, and PK with globe trauma) were employed to show that all injuries, even a single corneal suture, can cause not only rapid neuroglia changes in the retina but also retinal ganglion cell death.

Thus, there is robust evidence for the concept of TNF- α , IL-1 β , and other cytokines causing direct damage to the ganglion cells, but not necessarily mediated by IOP increase.²² Such ganglion cell attrition is known to lead to

glaucoma.³⁹ Anti-TNF- α monoclonal antibodies, such as infliximab or adalimumab, are markedly effective in retinal ganglion cell protection and can therefore be expected to prevent the time bomb of glaucoma.^{18,20,21,38} These findings may lead to effective prophylaxis against glaucoma after trauma or surgery.

However, recent discoveries suggest that there may be additional pathways to the ganglion cell attrition and delayed glaucoma due to trauma.²⁰ Monocytes/macrophages rapidly infiltrate the retina after eye trauma and by TNF- α secretion or other mechanism prolong and perpetuate the retinal damage. This new potential pathway to glaucoma has been the focus of the present study. Thus, CX3CR1⁺ monocytes were found to infiltrate the retina of mice within 24 hours after the trauma, thereby increasing the number of CD45⁺ myeloid cells in the retina. Because the retina is populated exclusively by nonperipheral, yolk sac-derived microglia,^{30,40} the presence of peripheral monocytes is deemed pathologic^{32,34,41} and likely contributes to neurodegeneration, as previously shown in chemical burns.^{31–33} Although TNF- α released from the injured cornea of mice ceases within weeks of the injury,²⁰ local retinal TNF- α secretion from engrafted monocytes continues for years,³¹ and is thereby expected to contribute to an autonomous, long-lasting degeneration of the retina. Trigeminal nerve stimulation from the injured cornea may also contribute to the neuroinflammatory signal to the retina, as previously shown for the brain.⁴² In this study, we provide compelling evidence to support that cytokine diffusion from the injured cornea to the retina is the most likely signaling mechanism of neuroretinal inflammation and monocyte infiltration; injection of recombinant IL-1 β in the anterior chamber causes similar inflammatory response and monocyte recruitment by the retina as in corneal surgical or traumatic injury. The detrimental role of monocyte infiltration into the retina was also confirmed in a light-inducible mouse model of photoreceptor degeneration,³⁴ corroborating earlier findings that engrafted monocytes are involved in long-term neurodegeneration of the retina.^{31,33}

More important, it is shown that blockade of TNF- α or IL-1 β prevents CX3CR1⁺ monocyte infiltration into the retina. Somewhat surprisingly, systemic dexamethasone had minimal effect. The limited immunosuppressive effect of dexamethasone, especially in severe ocular injuries, is consistent with previous observations²⁰ and underlines the therapeutic importance of targeted immunomodulation over steroids. This is likely attributed to other molecules, that are simultaneously released by the injured tissue, such as interferon- γ , which can inhibit the function of glucocorticosteroids.⁴³ This has practical implications for future efforts to prevent glaucoma after ocular trauma and suggests that systemic dexamethasone, although it reduces clinically evident anterior segment inflammation, does not provide adequate protection of the retinal ganglion cells. Antibodies targeting inflammatory cytokines seem to work well on multiple pathways against glaucoma.²²

Regardless of the mechanism of damage to the retinal ganglion cells, whether triggered by IOP increase or directly effected by cytokines, or indirectly via invading monocytes, prophylaxis with anti-TNF- α or IL-1 β antibodies appeared in the current study models to result in significant protection.

The effect of TNF- α inhibition on IL1b mRNA expression has been previously reported^{20,44} and is likely mediated by signaling cross talk and effects on the induction of IL-1 β rather than on the secretion or processing. Monocytes/macrophages/microglia secrete TNF- α , IL-1 β , and IL-6, and these cytokines share common intracellular signaling pathways, such as STAT3.⁴⁵ Neutralization of one of the proteins can therefore affect downstream signals through STAT3 and ultimately alter the phenotype of the cell. All these changes are expected to affect the regulation of multiple genes through autocrine/paracrine signaling.⁴⁴

Acute IOP elevation is still considered the major risk factor for post-traumatic glaucoma, and maximal IOP lowering therapy should be given, as outlined in earlier work.¹⁷ IOP levels were not assessed in this study, but the possible impact of IOP elevation in the mouse eye after corneal suture, PK, or globe trauma was negated by prompt treatment with either anti-TNF- α or anti-IL-1 β . Further studies are needed to assess the relationship between IOP and inflammation in the pathogenesis of post-traumatic glaucoma.

Although in traumatic ocular injuries, the above described events cannot be prevented, prompt intervention with antibodies may minimize the damage, suppress inflammation, and protect the remaining neurons. In elective procedures, however, such as PK or cataract, pre-emptive administration of inhibitors may prevent these events, protect the neurons, and minimize the known post-surgery complication of glaucoma. Future clinical studies will help assess the potential benefits of such therapy.

In conclusion, acute globe trauma, whether accidental or due to surgery, can cause indirect damage to the retina and result in glaucoma through release of inflammatory cytokines. Monocyte infiltration into the retina after an injury as minimal as corneal suture suggests previously unappreciated links between the anterior and posterior eye, mediated by the expression of inflammatory mediators. Both inflammatory gene expression and monocyte infiltration were prevented by TNF- α or IL-1 β inhibitors. Thus, targeted immunomodulation with TNF- α or IL-1 β blockade, combined with IOP reduction, deserves further study in the prevention of post-traumatic glaucoma.

Author Contributions

X.C., F.L., and C.Z. performed experiments, acquired, and analyzed data; J.C., L.W., and Y.H. critically reviewed the manuscript; C.H.D. and E.I.P. designed experiments and wrote the manuscript.

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