AD_____

Award Number: W81XWH-12-1-0255

TITLE: Genetic Networks Activated by Blast Injury to the Eye

PRINCIPAL INVESTIGATOR: Eldon E. Geisert

CONTRACTING ORGANIZATION:
University of Tennessee Health Science Center
Memphis TN, 38163

REPORT DATE: August 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| August 2013 | Annual | 15 July 2012 – 14 July 2013 |

**4. TITLE AND SUBTITLE**
Genetic Networks Activated by Blast Injury to the Eye

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-12-1-0255

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)** Eldon E. Geisert

E-Mail: egeisert@uthsc.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Tennessee Health Science Center
Memphis TN, 38163

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

**Purpose:** The present research project is designed to define the overall change in gene expression in the eye following a blast injury to the eye. In this process the genetic networks activated by injury will be defined along with biological markers of retinal injury. **Scope:** The proposal will examine the changes in gene expression that occurs in a mouse genetic reference panel, the BXD recombinant inbred (RI) strain set. This analysis will define genomic loci modulating the response of the eye to a blast injury and the genetic networks activated by the injury. **Major Finding:** A new microarray platform, Affymetrix GeneChip ST 2.0 Mouse Array, with over 28,000 protein-coding genes and 7,000 non-coding RNAs will be used to define the genetic response of the eye to blast injury. This has required the incorporation of a completely new annotation platform in our website GeneNetwork.org to accommodate the new DoD TATRC Retina Database. We will release the first version of the normal data within the next few months. We have defined a novel marker for retinal ganglion cell injury Sox11. This work has led to a series of investigations to define the molecular networks activated by Sox11.

**15. SUBJECT TERMS**
Genomics, Blast Injury, Eye, Retina

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 26 | 19b. TELEPHONE NUMBER *(include area code)* |

**Table of Contents**

## Introduction

Our group has developed a mouse model of blast injury to the eye, which accurately mimics the traumatic blast injury increasingly suffered by warriors under current battlefield conditions (Hines-Beard et al., 2012). We are taking full advantage of this mouse model in combination with a powerful combination of systems biology, microarray analysis, expression genetics, and bioinformatics. At the heart of our approach is a genetic reference panel of mice—the unique resource of BXD RI strain mouse panel. The set of RI strains was produced from a genetic cross between the C57BL/6J mouse and the DBA/2J mouse. Using 60 BXD strains provides a new and powerful method to defining elements in the genome regulating the response of the eye to blast injury. This allows us to generate specific, testable hypotheses to define the pathways that regulate the response of the eye to blast injury and reactive responses in the retina. As more diverse gene expression data sets become available, comparison of gene expression and regulation in different biological contexts will help identify the regulatory elements controlling the injury response of the eye and the retina. We will identify genetic networks activated by blast injury and the genomic loci modulating these genes. In addition, we hope to identify new markers for retinal injury as well as potential targets for intervention.

**Body**

The project is currently on track with data collections proceeding at a consistent rate. There were several issues that developed within the first few months of the project that have affected the overall design and procedures used. Some of the modified protocols affect a single task, and those will be discussed in relationship with that task. One affected all tasks and will be addressed first. All of the issues and modifications to the tasks were discussed with Mr. Robert Reed and presented to TATRC in quarterly reports.

Before beginning the massive data collection on all 60 of the BXD strains, we examined the proposed protocols using only the DBA/2J (a parental strain for the BXD RI strain set). Three arrays were run for each of 5 groups: control retina, retina 6 hours after blast, retina 1 day after blast, retina 2 days after blast and retina 5 days after blast. For these studies we used a 26-psi blast delivered directly to the eye. The retinas were harvested, the RNA was isolated, we ran quality control on all RNA samples, prepared the samples and ran them on Affymetrix microarrays. The samples were then normalized and placed into a File-Maker Pro database. When the data from all 15 arrays was examined we could not identify any statistically significant changes in gene expression that was due to the 26-psi blast. This included all of the time points after the blast was delivered to the eye. We also looked at specific genes that are known to be upregulated following injury, including Gfap (upregulated in reactive gliosis) and Aif1 (expressed by activated microglia). None of these genes were upregulated following the 26-psi blast injury. It became obvious that the changes seen by others represent an injury that includes approximately 10% of the retina and those changes were in a relatively small number of cells. Thus, we believe that the portions of the retina that were not injured with a 26-psi blast are masking the changes reported by others.

The results from our first microarray experiment demonstrated that we needed to make a more substantial blast injury to the retina if we were to examine the changes occurring in the retina with microarray technology. We modified the blast gun putting in clear plastic tubes so we can accurately align the eye to the barrel of the gun. We then increased the pressure delivered to the eye to eventually reach 50-psi. With the eye properly aligned we can deliver a 50-psi blast without muscle tears or death to the animal. In the process of modifying the blast to the eye we began to look for good markers for retina injury and we have identified new markers (see below Task 3). We repeated the blast injuries on the DBA/2J mice and now can see our marker being upregulated following blast injury. We are now examining the retina 5 days after a 50-psi blast injury. We are characterizing the blast wave at the 50-psi blast and the effects of this injury on the eye.

**Task 1) Quantify the strain-to-strain differences in the severity of blast-induced ocular pathologies, using a set of 60 BXD RI mouse strains and map the genomic loci that regulate the response of the eye to blast injury.**

In this Task we were measuring intraocular pressure (IOP), central corneal thickness (CCT) and visual acuity. We proposed to use the optomotor head-turning to measure visual acuity in the BXD RI strains. This is the only way to get a reasonable measurement of acuity in the moue. We were not able to measure visual acuity using the optomotor head-turning reflex in the BXD RI strains. The optomotor head-turning reflex cannot be measured in the DBA/2J strain. A recent publication from the Simon John Lab (Barabas et al., 2011) reveals that the DBA/2J mouse (one of the parental strains of the BXD strain set) does not have the appropriate head-turning reflex. Thus, this particular strain carries a mutation that inhibits our ability to measure acuity using optomotor responses. If we were to measure visual acuity across the BXD RI strains the only genomic loci that we would be able to map is associated with the lack of head-turning the DBA/2J allele. This would not tell us anything about the effects of blast injury on visual acuity, which was the goal in Task 1. This makes using changes in visual acuity no longer technically feasible. Thus, we eliminated this test from our characterization of phenotypic changes in the present study.

Progress: We are measuring ocular phenotypes in the BXD RI strains. At the present time we have measured IOP and CCT on 22 BXD strains (79 mice). There are an additional 11 strains (22 normal mice) that are the appropriate age for measurement this month and are currently being analyzed. We have measured the changes occurring following 50-psi blast injury to the eye of 10 BXD Strains (23 mice). We have an additional 11 strains (44 mice for blast injury) that will be included in the dataset in the near future. These data will be used to define quantitative trait loci, in the mouse, that control the response of the eye to blast injury.

**Task 2) Define the genetic networks activated by blast injury in the eye and in the retina, using transcriptome-wide profiling across the BXD RI strain set.**

Between the submission of this grant to the DOD and the funding of the grant, we received notification that the microarray platform (Illumina Bead Station) would no longer be supported by the manufacturer. Staying with the old Illumina microarray platform would mean that by the end of the study the microarray data would be run on an array (if they were even available) that would obsolete, and if our equipment failed there was no guarantee that parts or service would be available. Both of these potential problems would make completing the project difficult. Furthermore, anyone wanting to replicate any of the data might not be able to find the equipment or arrays to do so.

Solution: We have selected a new array from Affymetrix. It is the GeneChip Gene 2.0 ST Mouse Array. This array was released recently (2012) and has many features that are not found in any other array. Changing to the Affymetrix GeneChip Gene 2.0 Mouse Array means that the control dataset (HEI normal retina) that was run on the Illumina platform would no longer be an appropriate control set for the blast study. We are creating an entire new normal array dataset using the Affymetrix GeneChip Gene 2.0 Mouse Array. In addition to having to create a new Normal Retina Database our website, GeneNetwork.org did not contain the annotation for the new Affymetrix array. We have just finished the new annotation for the Affymetrix GeneChip ST 2.0 Mouse Array and are in the final stages of testing this version of annotation. This was a massive undertaking annotating over 28,000 protein-coding genes and 7,000 non-coding RNAs. We have created two separate dataset annotations. The first will be at the gene level, averaging the expression from each of the probes for each gene. The second will be at the exon level, providing the expression for each region of the gene that represents the DNA stretches that collectively makes up a single gene. This effort was well beyond the scope of the DoD grant but was necessary to make the data from the new Affymetrix microarrays accessible to the public.

There were several major benefits to using the new Affymetrix array. Specifically, there are probes for 7,000 non-coding RNAs (RNA that is not converted to protein but does affect the functioning of the cell). We are now finding out the many of these non-coding RNAs play extremely important roles in the body. Within these 7,000 probes, 588 encode microRNAs (small RNAs that regulate protein expression). These microRNAs play an astounding role in regulating the translation of RNA into protein. The new Affymetrix array will allow us for the first time to fully understand RNA expression in the retina and the changes that occur following blast injury.

Progress: We have set up the website for the DoD TATRC along with all of the annotation with the new GeneChip Gene 2.0 Mouse Array. We currently have arrays for 19 normal strains (43 microarrays from BXD retinas) in the DoD TATRC Retina Database on GeneNetwork (GeneNetwork.org). Data from an additional 32 arrays is ready to load into the database. All of the data will go through our rigorous quality control and normalization. Once this data is added it will bring the total number of strains to 22 and we will open the dataset to the public. For the blast injury eyes we have collected data from 50-psi blast retinas for 9 strains (23 arrays from retina that have received a 50-psi blast) and there are 6 additional strains (24 retinas) currently being processed.

**Task 3) Define biomarkers that can predict the severity of injury and eventual outcomes.**

This portion of our study was to begin in the latter years of the grant (Months 40 to 48). Due to lack of detectable changes in the 27-psi blast, it was necessary to modify the blast procedures and we needed good markers of retinal injury. We examined our optic nerve crush database on GeneNetwork to define a number of potential candidate genes for detecting changes occurring in the blast injury model. We began our search for a marker of retinal injury by examining changes in gene expression after optic nerve crush in the mouse. Sox11 was one of the genes with the largest change in expression two days after optic nerve crush. In the normal retinal dataset (genenetwork.org) the mean expression for Sox 11 (detected by Illumina probe ILMN_1235647) across the BXD RI strains was 8.4 on our 2 Z + 8 $Log_2$ scale (this is just above mean detection level of mRNA on our array which is set to 8). In the B6 parental strain the expression level in the normal retina was 8.59 and for the D2 strain the mean expression level was 8.54 (Figure 1). Two days after optic nerve crush there was a dramatic increase in the level of Sox11 expression, with the mean expression across the BXD strains being 11.03 (over a 4 fold increase). The same increase was observed in individual strains. The B6 strains had an expression level of 11.33 after optic nerve crush and the D2 strain increased to 11.44, over a four-fold increase for both strains. These data indicate that Sox11 is dramatically upregulated after a specific injury to the ganglion cell axons within the optic nerve. The initial studies on this project work were presented at the ARVO meeting in Seattle 2013.

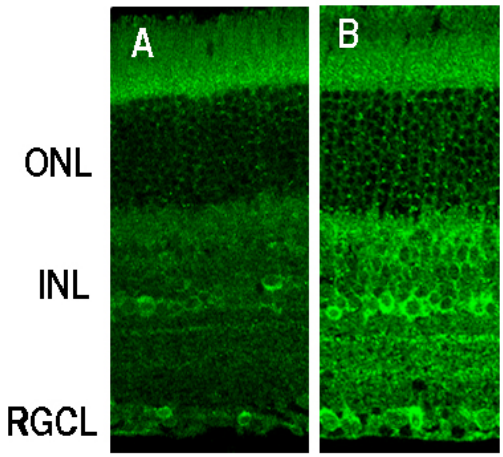


Figure 1. Sections of the normal retina (A) and the retina after a 45-psi blast (B) were stained for SOX11. SOX11 is an excellent marker for injury in the retina. Both pictures were taken at the exact same settings on the confocal microscope. Notice the dramatic upregulation of SOX11 in the inner retina, specifically in the retinal ganglion cells and the amacrine cells. The retinal layers are indicated: ONL (outer nuclear layer), INL (inner nuclear layer) and RGCL (retinal ganglion cell layer.

The best marker is SOX11 (see Figure 1). We are using this to characterize the 50-psi blast injury in advance of resuming the blast microarray study on the BXD RI strain set. Immunostaining sections of retina revealed that SOX11 was upregulated in the neurons of the inner retina following blast. SOX11 labeled cells in the ganglion cell layer and the inner nuclear layer. In the ganglion cell layer SOX11 labeled a majority of the cells, indicating that it was labeling most ganglion cells and displaced amacrine cells (Figure 2). Amacrine cells in the inner nuclear layer were also lightly labeled by SOX11. On immunoblots there was approximately a 2-fold increase in the intensity of the SOX11 band. This work was

presented at the ARVO meeting in Seattle 2013 (Hart et al., 2013). We have completed collecting data specifically related to SOX11 and are in the final stages of preparing the manuscript describing these exciting results.

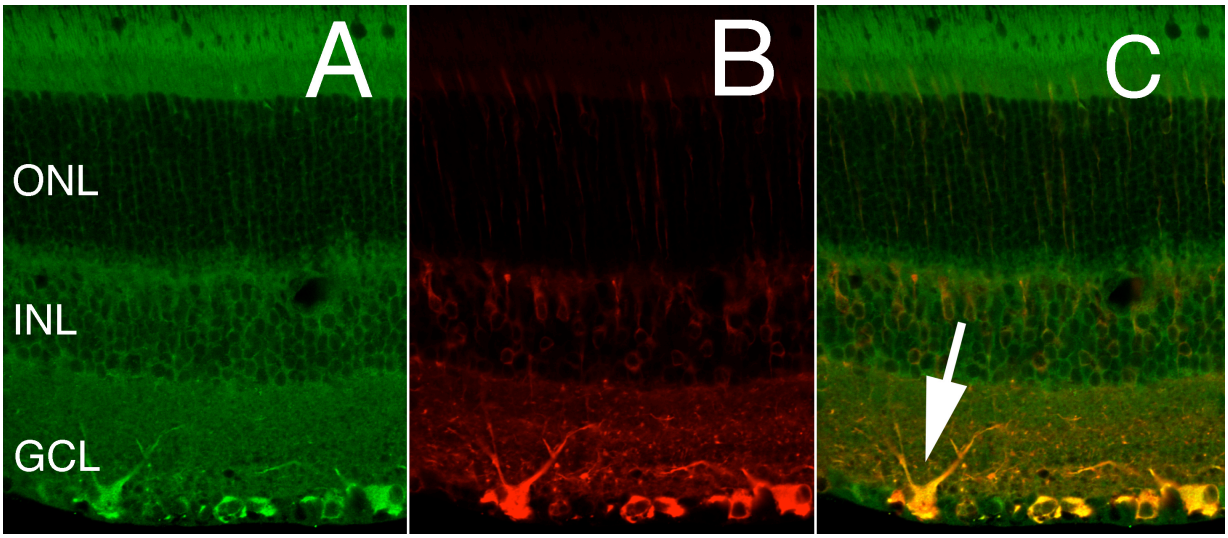


Figure 2. Sections of the injured retina stained for SOX11 (A), Class III Beta Tubulin (a retinal ganglion cell marker, B) and a merged image (C). Notice the intense SOX11 labeling of the retinal ganglion cells (Class III Beta tubulin positive) in the merged image (C) stained for SOX11 (arrow). SOX11 is an excellent marker for injury in the retina. All photomicrographs were taken at the exact same magnifications on the confocal microscope. Notice the dramatic upregulation of SOX11 in the inner retina, specifically in the retinal ganglion cells and the amacrine cells. The retinal layers are indicated: ONL (outer nuclear layer), INL (inner nuclear layer) and GCL (retinal ganglion cell layer).

We have also examined the role of the innate immune system in the retina and the first paper describing an innate immune network in the retina was recently published (Templeton et al., 2013). This is one of the networks that will be used to identify biomarkers of blast injury to the retina.

## Key Research Accomplishments

1. We have collected microarray data for 22 strains, run the quality control and prepared the scaled and normalized data for posting on Genenetwork.
2. We are identifying phenotypic changes that occur in the eye following blast injury.
3. We have prepared the annotation for all the genes on the new Affymetrix Microarray and installed it onto the GeneNetwork website.
4. We have identified a new marker for retinal ganglion cell injury, SOX11 (the paper describing this finding is currently in preparation).

## Reportable Outcomes

Abstracts

Bricker-Anthony, Courtney, Jessica Hines-Beard, Lauren D'Surney, Eldon E Geisert, Tonia S Rex, Ocular Blast Trauma in the DBA/2J Mouse, Poster abstract submitted for the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, May 5-9, 2013, Seattle, Washington.

Hart, Steven G; Wang, XiangDi; Rex, Tonia S.; Geisert, Eldon E. Biomarkers for Neuronal Injury Following Blast Trauma to the Eye. Poster abstract submitted for the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, May 5-9, 2013, Seattle, Washington.

Publications

Templeton JP, Freeman NE, Nickerson JM, Jablonski MM, Rex TS, Williams RW, Geisert EE. Innate Immune Network in the Retina Activated by Optic Nerve Crush. Invest Ophthalmol Vis Sci. 2013 54:2599-2606.

**Conclusions:**

We are now using a new microarray platform, Affymetrix GeneChip ST 2.0 Mouse Array platform that can monitor the expression of 28,000 protein-coding genes and 7,000 non-coding RNAs. This will allow us to define the full genetic response of the eye to blast injury. This has required the incorporation of a completely new annotation platform in our website GeneNetwork.org to accommodate the new DoD TATRC Retina Database. We will release the first version of the normal data within the next few months. We have defined a novel marker for retinal ganglion cell injury SOX11. This work has led to a series of investigations to define the molecular networks activated by SOX11.

**Reference**

Barabas P, Huang W, Chen H, Koehler CL, Howell G, John SW, Tian N, Rentería RC, Krizaj D., (2011) Missing optomotor head-turning reflex in the DBA/2J mouse. Invest Ophthalmol Vis Sci. 52:6766-73

Hart, Steven G; Wang, XiangDi; Rex, Tonia S.; Geisert, Eldon E. Biomarkers for Neuronal Injury Following Blast Trauma to the Eye. Poster abstract submitted for the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, May 5-9, 2013, Seattle, Washington.

Hines-Beard J, Marchetta J, Gordon S. Chaum E, Geisert EE and Res TS. A mouse model of ocular blast injury that induces closed globe anterior and posterior pole damage. Exp Eye Res. 99 (2012) 63-70.

Templeton JP, Freeman NE, Nickerson JM, Jablonski MM, Rex TS, Williams RW, Geisert EE. Innate Immune Network in the Retina Activated by Optic Nerve Crush. Invest Ophthalmol Vis Sci. 2013 54:2599-2606.

**List of Appendices:**

1) Hart, Steven G; Wang, XiangDi; Rex, Tonia S.; Geisert, Eldon E. Biomarkers for Neuronal Injury Following Blast Trauma to the Eye. Poster abstract submitted for the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, May 5-9, 2013, Seattle, Washington.

2) Hines-Beard J, Marchetta J, Gordon S. Chaum E, Geisert EE and Res TS. A mouse model of ocular blast injury that induces closed globe anterior and posterior pole damage. Exp Eye Res. 99 (2012) 63-70.

3) Templeton JP, Freeman NE, Nickerson JM, Jablonski MM, Rex TS, Williams RW, Geisert EE. Innate Immune Network in the Retina Activated by Optic Nerve Crush. Invest Ophthalmol Vis Sci. 2013 54:2599-2606.

**ABSTRACT TITLE**

**TITLE:** Ocular Blast Trauma in the DBA/2J Mouse

**PROGRAM # (Final ID)**

**ABSTRACT FINAL ID:** 728 - D0285

**SESSION TYPE:** Poster Session

**POSTER BOARD # (DOI)**

**DIGITAL OBJECT IDENTIFIER (DOI):** D0285

**PRESENTATION START/END**

**SESSION ABSTRACT START TIME:** 10:30 AM

**SESSION ABSTRACT END TIME:** 12:15 PM

**SESSION # (Abbreviation)**

**SESSION ABBREVIATION:** 132

**SESSION TITLE:** Retinal and Photoreceptor Degeneration I: Cell Biology

**SESSION DAY & DATE:** Sunday, May 5, 2013

**SESSION START TIME:** 10:30 AM

**SESSION END TIME:** 12:15 PM

**AUTHORS (LAST NAME, FIRST NAME):** Bricker-Anthony, Courtney[2]; Hines-Beard, Jessica[2]; D'Surney, Lauren[1]; Geisert, Eldon E.[1]; Rex, Tonia S.[2]

**INSTITUTIONS (ALL):** 1. Ophthalmology, University of Tennessee Health Science Center, Memphis, TN, United States.

2. Ophthalmology, Vanderbilt University, Nashville, TN, United States.

**Study Group:**

**ABSTRACT BODY:**
**Purpose:** To characterize ocular blast injury in the DBA/2J mouse.

**Methods:** We exposed the eyes of 3-month-old DBA/2J mice to a single overpressure air wave (blast) produced by a modified paintball marker. Output pressure was measured using a pressure transducer. The mice were placed in a protective housing chamber with only the left eye exposed. Mice experienced a 23, 26, or 30 psi blast. Assessments of intraocular pressure, gross pathology, and visual acuity were performed at 0, 3, 7,14, and 28 days following blast exposure. Optical coherence tomography was performed at 3 days post-blast. Cryo embedded eyes were labeled for anti-GFAP, TUNEL, and anti-Nitrotyrosine. Eyes and optic nerves were embedded in resin and sections were stained with toluidine blue or p-Phenylenediamine, respectively.

**Results:** Frequency of anterior pole damage increased with time and blast pressure. Commonly observed gross pathologies included corneal edema, corneal neovascularization, and traumatic cataract. Optical coherence tomography imaging revealed retinal detachments at 3 days post-injury. The average percent of retina length with TUNEL positive cells was 24% and 11% after exposure to a 26 psi blast at 3 and 7 days, respectively. Affected cell populations included photoreceptor cells, inner nuclear layer cells, and retinal ganglion cells. GFAP upregulation was observed throughout the retina at both 3 and 7 days. Positive staining for anti-nitrotyrosine was noted in the inner retina of 3 day blast exposed retinas. Additionally, in resin embedded eyes, pyknosis, immune infiltrate, retinal detachments, epiretinal membranes, and subretinal debris were observed. Degenerating axons were present in the optic nerves 28 days post-injury.

**Conclusions:** The DBA/2J mice are more vulnerable to anterior and posterior pole damage than the C57Bl/6 and Balb/c mice. Differences in responses to blast exposure among inbred mouse strains may be indicative of genetic susceptibility. Immune infiltration was primarily observed in the DBA/2J and is likely due to Anterior Chamber-Associated Immune Deviation, which may contribute to the severity of the phenotype. This mouse model recapitulates commonly observed ocular pathologies in blast-exposed Veterans and may prove useful for testing of potential therapeutics in vivo.

(No Image Selected)

**Commercial Relationship(s) Disclosure:**

Courtney Bricker-Anthony: Commercial Relationship: Code N (No Commercial Relationship)

Jessica Hines-Beard: Commercial Relationship: Code N (No Commercial Relationship)

Lauren D'Surney: Commercial Relationship: Code N (No Commercial Relationship)

Eldon Geisert: Commercial Relationship: Code N (No Commercial Relationship)

Tonia Rex: Commercial Relationship(s);PCT/US2012/021247:Code P (Patent)

**Grant Support:** Yes

**Support Detail:** W81XWH-10-1-0528

**Clinical Trial Registration:** No

**Other Registry Site:**

**Registration Number:**

---

**ABSTRACT TITLE**

**TITLE:** Biomarkers for Neuronal Injury following Blast Trauma to the Eye

**PROGRAM # (Final ID)**

**ABSTRACT FINAL ID:** 1583 - D0010

**SESSION TYPE:** Poster Session

**POSTER BOARD # (DOI)**

**DIGITAL OBJECT IDENTIFIER (DOI):** D0010

**PRESENTATION START/END**

**SESSION ABSTRACT START TIME:** 8:30 AM

**SESSION ABSTRACT END TIME:** 10:15 AM

**SESSION # (Abbreviation)**

**SESSION ABBREVIATION:** 235

**SESSION TITLE:** Ocular Disease Expression, Proteomics, Biomarkers, and Polymorphisms

**SESSION DAY & DATE:** Monday, May 6, 2013

**SESSION START TIME:** 8:30 AM

**SESSION END TIME:** 10:15 AM

**AUTHORS (LAST NAME, FIRST NAME):** Hart, Steven G.[1]; Wang, XiangDi[1]; Rex, Tonia S.[2]; Geisert, Eldon E.[1]

**INSTITUTIONS (ALL):** 1. Ophthalmology, University of Tennessee Health Science Center, Memphis, TN, United States.

2. Ophthalmology, Vanderbilt University, Nashville, TN, United States.

**Study Group:**

**ABSTRACT BODY:**
**Purpose:** The pathogenic pathways triggered by blast injury to the eye and biomarkers that reflect the activation of these pathways are largely unknown. The present study is the initial attempt to define potential biomarkers that reflect the severity of the retinal injury.

**Methods:** Blast injuries to the eye were produced by a paintball gun fitted with a shortened and narrowed barrel and an integrated pressure regulator. The mice were deeply anesthetized and secured in a PVC pipe. A 45-psi overpressure wave was delivered selectively to the eye of C57BL/6 mice and DBA/2J mice. The animals were then sacrificed at 2 or 5 days after the blast injury. To aid in our initial survey of potential biomarkers of retinal injury, we examined our Optic Nerve Crush Microarray Dataset and compared it to our Normal Retinal Microarray Dataset in GeneNetwork.org. This work led to six potential biomarkers for blast injury, *Gfap*, *Iba1*, *C1q*, *Aqp4*, *Sox11* and *Hsp25*. One set of retinas were removed and stained by indirect immunohistochemical methods to assess the distribution and intensity of the staining compared to uninjured control retinas. For a second set of retinas, the animals were anesthetized; the retinas were removed and placed in sample buffer. The level of protein expression was determined by semi-quantitative immunoblot methods.

**Results:** Immunostaining sections of retina revealed that two of the markers, SOX11 and HSP25, were upregulated in the neurons of the inner retina following blast. Both SOX11 and HSP25 labeled cells in the ganglion cell layer and the inner nuclear layer. In the ganglion cell layer SOX11 labeled approximately 90% of the cells, indicating that it was labeling most ganglion cells and displaced amacrine cells. Furthermore, amacrine cells in the inner nuclear layer were labeled by SOX11. The intensity of staining increased dramatically after blast injury and on immunoblots there was approximately a 2-fold increase in the intensity of the SOX11 band. A similar pattern of staining was observed with HSP25. The increased staining after blast injury did not appear to be as dramatic as it was for SOX11. On immunoblots, there was also an observed increase in the intensity of the HSP25 band following injury.

**Conclusions:** SOX11 and HSP25 are markers for blast injury to the retina, labeling both retinal ganglion cells and amacrine cells. The better of the two markers appears to be SOX11.

(No Image Selected)

**Commercial Relationship(s) Disclosure:**

Steven Hart: Commercial Relationship: Code N (No Commercial Relationship)

XiangDi Wang: Commercial Relationship: Code N (No Commercial Relationship)

Tonia Rex: Commercial Relationship(s);PCT/US2012/021247:Code P (Patent)

Eldon Geisert: Commercial Relationship: Code N (No Commercial Relationship)

**Grant Support:** Yes

**Support Detail:** DOD-TATRC W81XWH-12-1-0255; Unrestricted Grant from Research to Prevent Blindness, Inc.

**Clinical Trial Registration:** No

**Other Registry Site:**

**Registration Number:**

# Innate Immune Network in the Retina Activated by Optic Nerve Crush

Justin P. Templeton,[1] Natalie E. Freeman,[1] John M. Nickerson,[2] Monica M. Jablonski,[1] Tonia S. Rex,[3] Robert W. Williams,[4] and Eldon E. Geisert[1]

[1]Department of Ophthalmology, University of Tennessee Health Science Center, Memphis, Tennessee
[2]Department of Ophthalmology, Emory University, Atlanta, Georgia
[3]Department of Ophthalmology and Visual Sciences, Vanderbilt University, Nashville, Tennessee
[4]Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, Tennessee

Correspondence: Eldon E. Geisert, Department of Ophthalmology, University of Tennessee Health Science Center, Memphis, TN 38163; egeisert@uthsc.edu.

Submitted: October 19, 2012
Accepted: March 4, 2013

Citation: Templeton JP, Freeman NE, Nickerson JM, et al. Innate immune network in the retina activated by optic nerve crush. *Invest Ophthalmol Vis Sci*. 2013;54:XXX–XXX. doi:10.1167/iovs.12-11175.

**Purpose.** Innate immunity plays a role in many diseases, including glaucoma and AMD. We have used transcriptome profiling in the mouse to identify a network of genes involved in innate immunity that is present in the normal retina and that is activated by optic nerve crush (ONC).

**Methods.** Using a recombinant inbred (RI) mouse strain set (BXD, C57BL/6 crossed with DBA/2J mice), we generate expression datasets (Illumina WG 6.2 arrays) in the normal mouse retina and 2 days after ONC. The normal dataset is constructed from retinas from 80 mouse strains and the ONC dataset is constructed from 62 strains. These large datasets are hosted by GeneNetwork.org, along with a series of powerful bioinformatic tools.

**Results.** In the retina datasets, one intriguing network involves transcripts associated with the innate immunity. Using *C4b* to interrogate the normal dataset, we can identify a group of genes that are coregulated across the BXD RI strains. Many of the genes in this network are associated with the innate immune system, including *Serping1*, *Casp1*, *C3*, *Icam1*, *Tgfbr2*, *Cfi*, *Clu*, *C1qg*, *Aif1*, and *Cd74*. Following ONC, the expression of these genes is upregulated, along with an increase in coordinated expression across the BXD strains. Many of the genes in this network are risk factors for AMD, including *C3*, *EFEMP1*, *MCDR2*, *CFB*, *TLR4*, *HTA1*, and *C1QTNF5*.

**Conclusions.** We found a retina-intrinsic innate immunity network that is activated by injury including ONC. Many of the genes in this network are risk factors for retinal disease.

Keywords: innate immunity, genetic networks, retinal injury

The innate immune system plays many different roles in the central nervous system (CNS)[1] during development[2,3] and disease.[4–7] The primary component of innate immunity is the complement system, and its involvement in CNS disorders is well documented. For example, members of the complement cascade are associated with the pathological changes occurring in Alzheimer disease[8–11] and spinal cord injury.[12,13] Within the past decade, the significance of the complement system was dramatically demonstrated by its role in AMD, where recent studies have shown that common variants of complement factor H (*CFH*) represent major risk factors for AMD.[14–17] Many of the components of the complement system are also found in drusen that accumulate in AMD, examples of which include CFH,[15] complement factor B (CFB),[18] complement receptor 1,[19] C3,[19] C5,[20] C6–C9,[21] and members of the terminal pathway, vitronectin, and clustrin.[22] As it turns out, these same genes are also risk factors for AMD (for review see Anderson et al.[23]), including *CFB*,[18] *C2*,[18] *C3*,[23] and *SERPING1*.[24]

The complement components also play a significant role in animal models of glaucoma.[25–29] One of the most accepted murine models of naturally occurring glaucoma is the DBA/2J mouse. This mouse carries two mutations that result in a progressive iris stromal atrophy and pigment dispersion.[30,31] The dispersed pigment blocks the trabecular meshwork causing an elevation in IOP, and a phenotype similar to pigmentary dispersion glaucoma.[30] Studies using the DBA/2J model[26–28] examined the changes in gene expression during the progression of the disease.[26,28] In this mouse model, many components of the complement system increased in expression as the disease process progressed, specifically including *C1q*, *C3*, and *Cfi* (see Fig. 5 in Howell et al.[28]). The upregulation of these components of the complement system suggests that the innate immune system plays an important role in the immune-privileged environment of the retina and specifically in the response of the retina to disease. The role of complement in glaucomatous damage was tested by Howell et al.[28] by knocking out *C1q* on the DBA/2J background. These *C1q* knockout mice developed iris disease and elevated IOP similar to the normal DBA/2J mice; however, knocking out *C1q* reduced and delayed the loss of axons in the optic nerve,[28] which suggests that interrupting the complement cascade in DBA/2J mice may alter disease severity and progression.

The present study investigates the regulation of the innate immune system in the retina, and identifies changes in global expression of the complement system after optic nerve crush (ONC). We created two comprehensive and complementary expression data sets for the retina of the BXD mouse strains. One dataset was generated using untreated control retinas and

the other was generated using the same genotypes of mice but 2 days after ONC. By examining the changes that occur in the gene expression profiles, we have been able to define an innate immune network active in the normal retina that is upregulated by the ONC.

## Materials and Methods

### Animals: Strains, Sex, and Age

The full Hamilton Eye Institute (HEI) Retina Database contains the data analysis of 346 Illumina Sentrix Mouse WG-6 v2.0 arrays (Illumina, San Diego, CA).[32] Eighty strains of mice are represented, including 75 BXD RI strains with 307 independent retinal samples (for details see GeneNetwork.org). In the ONC HEI Retina Database, there are 62 strains (57 BXD RI strains, C57BL/6J, DBA/2J, and the reciprocal F1 crosses) with 184 independent biological samples run on the Illumina Sentrix Mouse WG-6 v2.0 array (for detailed method of ONC see Templeton and Geisert 2012[33]). Samples were collected for microarray analyses 2 days after ONC. Based on our findings from C57BL/6 and DBA/2J mice,[34] this is the time point when we are able to see early response genes upregulated and the changes that reach maxima at later time points, such as the upregulation of *Gfap*. Mice were either purchased from Jackson Laboratory (Bar Harbor, ME)[35,36] or were from the breeding colonies of Robert Williams and Lu Lu at the University of Tennessee Health Science Center.[37] All of the animals were young adults between 60 and 120 days of age. The housing room was maintained on a 12 hour on, 12 hour off light cycle. Expression data were obtained from independent biological samples from both sexes. For details regarding the animals in each of the datasets see Freeman et al.[32] or the Info file on GeneNetwork.org for each of the datasets. All protocols used in this study were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center and were in accordance with the Institute for Laboratory Animal Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Sample Processing and RNA Isolation

All mice were deeply anesthetized with a mixture of 13 mg/kg of xylazine (Rompun) and 87 mg/kg of ketamine (Ketalar) and killed by rapid cervical dislocation. The retinas were removed immediately. Two retinas per mouse were immersed in RNALater (Qiagen, Germantown, MD) and stored in a single tube overnight at 4°C. Total RNA was prepared from the retinal tissue with RNA-Stat-60 (Tel-Test, Friendswood, TX). The quality and purity of RNA were assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA) to assess the relative quantities of 18S and 28S RNA, as well as the RNA integrity.

### Sample Preparation and Hybridization

Total RNA (150 ng) was processed with the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Foster City, CA) to produce biotinylated cRNAs. The concentration of the cRNA solution was determined by measuring the absorbance at 260 nm and 280 nm using the NanoDrop 1000A Spectrophotometer (Thermo Scientific, West Palm Beach, FL). The biotinylated cRNAs (1.5 μg/sample) were hybridized to the Illumina Sentrix Mouse WG-6 v2.0 arrays for 19.5 hours at 58°C according to the manufacturer's instructions (Illumina).

### Quality Control of Array Data

Quality control analysis of the raw image data was performed using the Illumina BeadStudio software. Rank invariant normalization with BeadStudio software was used to calculate the data. Once these data were collected, they were globally normalized in a four-step process: (1) Compute the log base 2 of each raw signal value. (2) Calculate the mean and SD of each Mouse WG-6 v2.0 array. (3) Normalize each array using the formula 2 ($z$ score $\text{Log}_2$ [intensity]) + 8. The result is to produce arrays that have a mean of 8 and an SD of 2. (4) Compute the mean of the values for the set of microarrays for each strain. This process produces a distribution of probe labeling that is $z$-scaled with a mean probe labeling set to 8 on a log base 2 scale. Probes with scores below 8 are either expressed at extremely low levels or are not detected (i.e., absent) from the tissue.

## Results

### The Innate Immune System in the Retina

To begin our analysis of the innate immunity in the retina, we searched the Full HEI Retina Database in GeneNetwork to find mRNAs that correlate with the levels of *C4b* mRNA, a well-established marker of innate immunity. In the Illumina dataset, there are seven probes that target different exons in *C4b*. For this analysis, we chose the probe with the highest expression (ILMN_1215092 coding exon 39). All seven probes that recognize different exons within the gene covary (the lowest of 21 pairwise correlations among these probes was greater than 0.77). As a next step in the analysis, we examined genes within the Full HEI Retina Dataset that had a similar pattern of expression across the BXD RI strains (Fig. 1A). Using the correlation program within GeneNetwork, we found 100 genes that had a Pearson correlation greater than $r = 0.73$ (within this dataset six strains are present that display retinal degeneration and elevated glial fibrillary acidic protein [GFAP]). In this list of genes, many are associated with the innate immune network (see Table 1), including genes such as *C4*, *C3*, *Cd1q*, and *Serping1*.

### Reactive Gliosis as a Potential Confound

In the list of C4b correlates from the Full Retina Dataset were a group of genes associated with reactive gliosis, including *Gfap*, *Stat3*, *Egrf*, *Cp*, and *Cd74*. Examination of *Gfap* expression across all of the strains in the HEI Retinal Dataset (Fig. 1B) reveals that some of the strains express very high levels of *Gfap*. For example, BXD24 expresses high levels of *Gfap* and also expresses *C4b* at a 9-fold higher level than BXD22. During the inbreeding process, BXD24 acquired a mutation in *Cep290* that results in early-onset photoreceptor degeneration.[38] This degeneration results in reactive gliosis throughout the retina and clearly an elevation in *C4b*. When we checked the expression of *Gfap* and *C4b* across the BXD strains (Figs. 1A, 1B, respectively) we observed that some strains in the Full HEI Retinal Dataset (BXD24, BXD32, BXD49, BXD70, BXD83, and BXD 89) have extremely high levels of these two genes. Immunostaining for GFAP showed that all strains with elevated levels of *Gfap* message displayed reactive gliosis, with high levels of GFAP in both Müller cells and astrocytes (data not shown). We assume that either a mutation occurred during the breeding process necessary to produce an inbred strain (as is the case for BXD24) or the specific constellation of genes in these particular strains cause reactive gliosis. To eliminate the potential confounding influence due to reactive gliosis, we created a second "normal" retina dataset (Normal HEI Retina), eliminating strains with elevated levels of *Gfap* (i.e., BXD24, BXD32, BXD49, BXD70, BXD83, and BXD89). We also examined the effects of ONC using the ONC HEI Retina

Investigative Ophthalmology & Visual Science

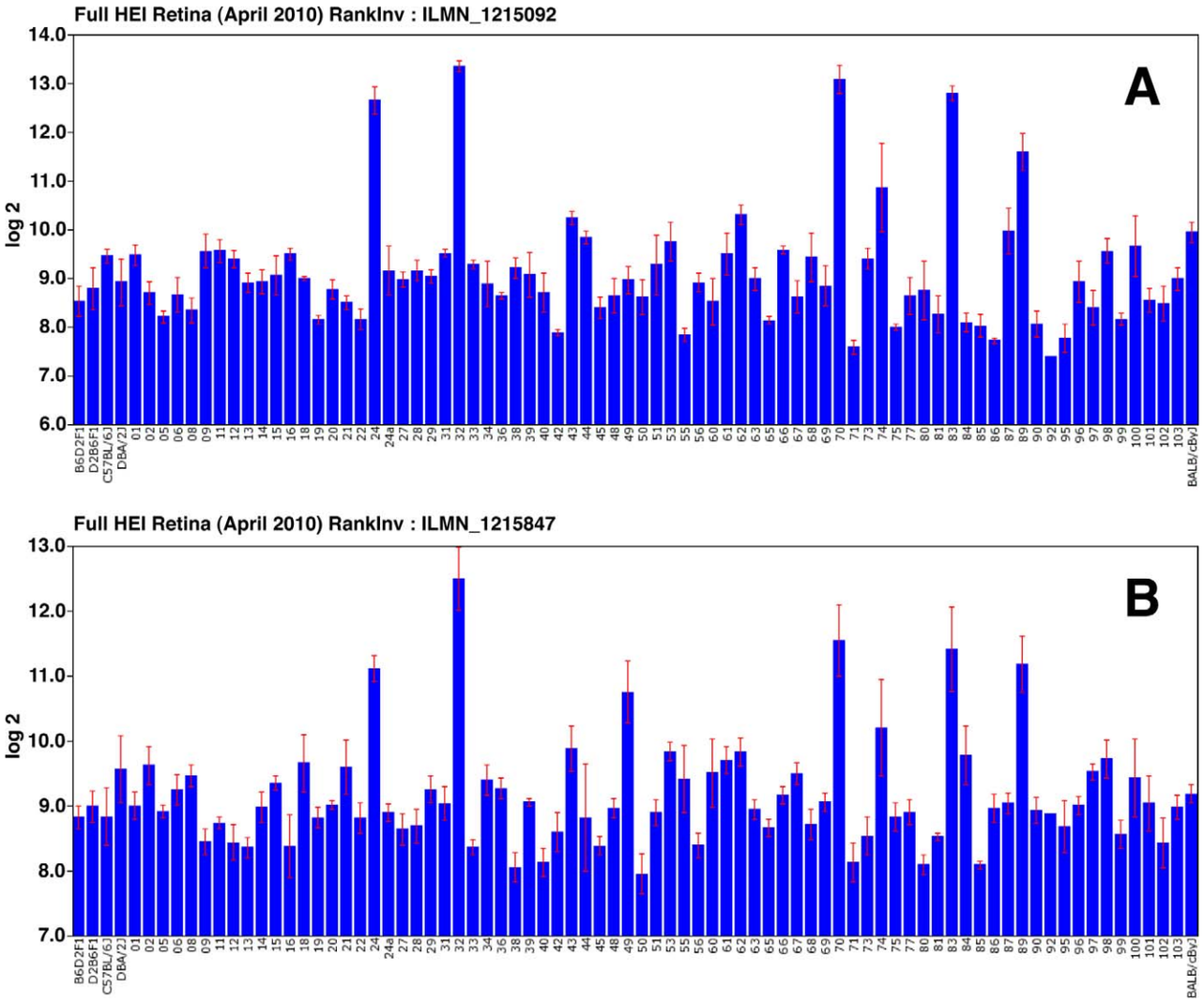


**Figure 1.** The expression of *C4b* (**A**) and *Gfap* (**B**) is shown across all of the BXD RI strains in the Full HEI Retina Datasets. The *scale* to the left is expression from the microarray studies expressed in log base 2 with the mean expression of mRNA across the microarray set to 8. Notice that the strains expressing high levels of *C4b* also express high levels of *Gfap*, suggesting that the elevation in *C4b* expression may be associated with reactive gliosis. These high *Gfap* strains were removed from this dataset to form the Normal HEI Retina Dataset.

**Table 1.** The Selected Genes in the Innate Immune System Are Presented Along With Their Illumina ID, Chromosomal Location, and Mean Expression Level in the Normal Retina Database

| Symbol | Description | Record | Location, Chr, Mb | Mean Expression Level |
|---|---|---|---|---|
| C4b | Complement component 4B | ILMN_1215092 | Chr17: 34.866133 | 9.10 |
| Serping1 | Serine peptidase inhibitor, clade G, member 1 | ILMN_2913166 | Chr2: 84.605753 | 9.66 |
| C3 | Complement component 3 | ILMN_2759484 | Chr17: 57.343430 | 7.97 |
| Casp1 | Caspase 1 | ILMN_1247592 | Chr9: 5.306713 | 7.11 |
| Icam1 | Intercellular adhesion molecule 1 | ILMN_2896601 | Chr9: 20.832882 | 7.32 |
| Tgfbr2 | Transforming growth factor, beta receptor 2 | ILMN_2762979 | Chr9: 115.997139 | 8.86 |
| Cfi | Complement component factor i | ILMN_2631704 | Chr3: 129.577959 | 7.44 |
| Clu | Clusterin | ILMN_2727153 | Chr14: 66.600224 | 15.85 |
| C1qg | Complement component 1, q subcomponent, gamma | ILMN_2715840 | Chr4: 136.446132 | 8.18 |
| Aif1 | Allograft inflammatory factor 1 | ILMN_2804487 | Chr17: 35.308081 | 7.52 |
| Cd74 | CD74 antigen | ILMN_1221817 | Chr18: 60.971927 | 7.39 |
| Cp | Ceruloplasmin | ILMN_2520239 | Chr3: 19.90862 | 11.27 |
| Gfap | Glial fibrillary acidic protein | ILMN_1215847 | Chr11: 102.748858 | 9.15 |
| Stat3 | Signal tranducer and activator of transcription 3 | ILMN_2698046 | Chr11: 100.749555 | 11.22 |

All of these genes are expressed in the retina.

Investigative Ophthalmology & Visual Science

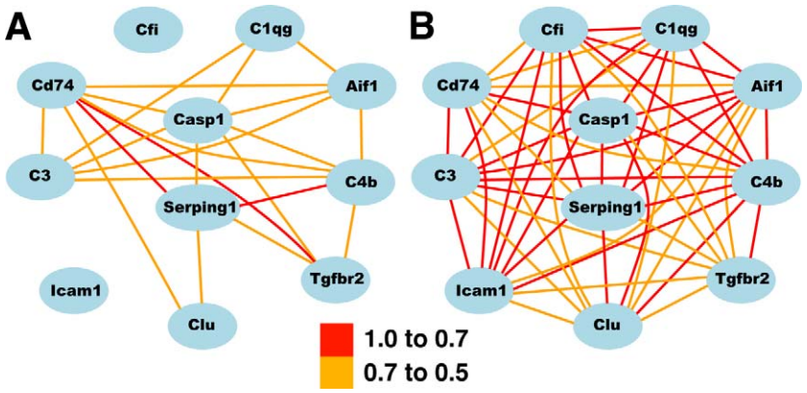


FIGURE 2. The increased correlation after ONC among selected genes in the innate immune network. The correlation among genes in the Normal Retina Database is shown in (**A**). The increased correlation among these genes following ONC is clearly demonstrated in (**B**). The *scale* indicates the correlation between genes (Pearson). See Table 1 for a complete list of gene symbols and descriptions.

Database. All of these datasets are presented on our GeneNetwork Web site (genenetwork.org).

## Network Maps of Innate Immunity

One way to illustrate the changes occurring in the network is to generate network maps using the Normal HEI Retinal Dataset (without the six animals with elevated *Gfap*) and comparing the correlations with a map generated from the ONC Retina Dataset (Fig. 2). When the top 100 correlates of C4b were analyzed using the Gene Set Analysis Toolkit V2 (Vanderbilt University, Nashville, TN; http://bioinfo.vanderbilt.edu/webgestalt), 30 of the 100 probes (25 of the 82 genes) were associated with the immune system process (see Supplementary Table S2). In the analysis of biological processes, there were no other significant enrichments of genes in any other functional category. Thus, immune system genes dominate this network, and examining the list of genes within the network reveals that most are associated with the innate immune system. We selected a small group of genes from this list (*C4b*, *Serping1*, *Casp1*, *C3*, *Icam1*, *Tgfbr2*, *Cfi*, *Clu*, *C1qg*, *Aif1*, *Cd74*) to illustrate effectively the relationships of the innate immune network in the retina. These genes were used to generate the two network maps in the normal retina and in the retina following ONC (Fig. 2).

The innate immune system network is present within the normal retina (see Table 1). This small set of genes was selected to highlight genes associated with the innate immune system and to illustrate the changes occurring in a large group of genes within the retinal innate immune network (Supplementary Table S1 lists the top 100 genes in this network). The correlates of *C4b* in the Full Retina Dataset, the Normal Retina Dataset, and the ONC Dataset have a considerable overlap. All three correlate lists that contain the top 200 correlates share 66 genes (Fig. 3), represent 78 probes on the Illumina microarray. Among these 78 probes, the correlation values increase following ONC, with virtually all of the genes having a correlation above 0.7 (see Fig. 2B and Table 2). In addition to the increases in correlation values across the BXD RI strains, the expression of these genes was significantly upregulated following optic nerve injury (Table 2). Relative to the average expression of these genes in the normal retina, there was an average of a 3-fold increase in expression 2 days after ONC. Taken together, these data demonstrate that there is an existing innate immune network in the normal retina and that network is activated by crushing the axons within the optic nerve.

## QTL Mapping of the Innate Immune Network

The finding that the innate immune system in the retina is so tightly correlated indicates that there are common regulatory mechanisms in the retina that are activated by the ONC injury. To define genomic loci that modulate the alterations in gene expression following ONC, we examined the changes occurring in the quantitative trait locus (QTL) map. The normal QTL Map was built using the top 40 correlates of *C4b*, and the same genes were used for the optic nerve injury heat map (Fig. 4). In the heat maps displayed in Figure 4, the regions of the genome that modulate the expression of the members of the innate immune network are highlighted (for a full description see Geisert et al.[39]). When the QTLs extend across the entire group of genes within the network (indicated by arrowheads in Fig. 4), we call these "signature QTLs" because they represent regions of the genome that affect the entire network. Within these signature QTLs are cis-acting loci (cis-QTLs) that are candidates for loci that modulate the innate immune network. When comparing the QTL maps from the normal retina database and ONC dataset, it is obvious that the signature QTL patterns change. The signature QTLs (marked with upward-pointing blue arrowheads in the lower part of Fig. 4) identify regions of the genome that modulate the innate immune network in the normal retina. Following ONC, the pattern of signature QTLs changes with most of the bands in the normal

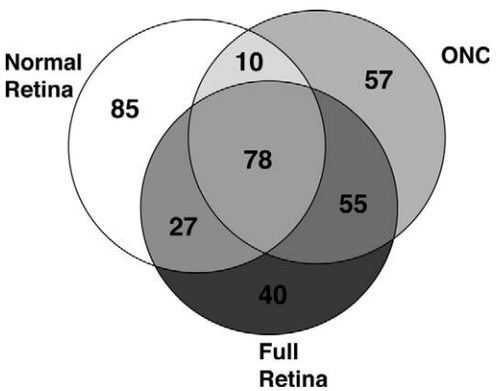


FIGURE 3. The Venn diagram reveals the overlap in the top 200 correlates of *C4b* in the normal retina dataset (Normal Retina), the ONC Dataset (ONC), and the Full Retinal Dataset (Full Retina). Of the top 200 correlates from each dataset, 78 probes (representing 66 different genes) are in common among the three experimental groups.

**Table 2.** The Mean Expression Level of the Genes and Correlation to C4b for a Select Group of Genes in the Innate Immune Network Is Shown for the Normal HEI Retinal Database (Control Retina), the ONC HEI Retinal database (ONC), and the Meta-Analysis of the Howell et al.[28] DBA/2J data (D2 Glaucoma)

| Symbol | Control Retina Expression | ONC Expression | D2 Glaucoma Expression | Control Retina Correlation | ONC Correlation | D2 Glaucoma Correlation |
|---|---|---|---|---|---|---|
| C4b | 8.75 | 10.23 | 8.85 | 1 | 1 | 1 |
| Serping1 | 8.48 | 10.62 | 9.50 | 0.768 | 0.811 | 0.924 |
| C3 | 8.14 | 8.54 | 7.83 | 0.636 | 0.779 | 0.88 |
| Casp1 | 6.52 | 7.44 | 7.03 | 0.646 | 0.768 | 0.704 |
| Icam1 | 7.66 | 7.50 | 7.26 | 0.231 | 0.749 | 0.807 |
| Tgfbr2 | 8.21 | 8.99 | 8.83 | 0.644 | 0.723 | 0.69 |
| Cfi | 12.45 | 7.65 | 7.38 | 0.23 | 0.72 | 0.692 |
| Clu | 11.19 | 16.42 | 15.78 | 0.406 | 0.719 | 0.835 |
| C1qg | 6.91 | 9.02 | 8.08 | 0.481 | 0.708 | 0.818 |
| Aif1 | 6.40 | 7.93 | 7.47 | 0.432 | 0.667 | 0.723 |
| Cd74 | 6.22 | 7.66 | 7.29 | 0.721 | 0.657 | 0.628 |
| Cp | 8.96 | 11.93 | 11.59 | 0.169 | 0.603 | 0.741 |
| Gfap | 7.41 | 10.31 | 8.96 | 0.308 | 0.45 | 0.883 |
| Stat3 | 9.28 | 11.58 | 11.13 | −0.137 | 0.437 | 0.774 |
| Mean | 8.33 | 9.70 | 9.07 | 0.47 | 0.70 | 0.79 |

Notice that in the case of naturally occurring glaucoma and after ONC there is an increase in gene expression and an increased correlation among genes in this network.

retina dataset diminishing in influence and two new prominent signature QTLs appearing in the ONC dataset (upper part of Fig. 4). The two major new signature bands (downward-pointing red arrowheads in Fig. 4) are on chromosome 6 at 116-127 Mb and chromosome 16 at 80-95 Mb. These two genome loci contain elements that potentially modulate the response of the innate immune network to ONC. Within these regions, cis-acting QTLs[39] are putative candidate genes that modulate the innate immune network. In the chromosome 6 signature locus there are 9 genes with significant cis-QTLs: *Adipor2*, *Cops7a*, *Dcp1b*, *Ing4*, *Lrrc23*, *Phc1*, *Slc6a12*, *Tmcc1*, and *Afp9*. Within the signature band on chromosome 16, three genes are present with significant cis-QTLs: *Cbr3*, *Sfrs15*, and *Tiam1*. These genes represent putative upstream modulators in the activation of the innate immune network that occurs during optic nerve injury.

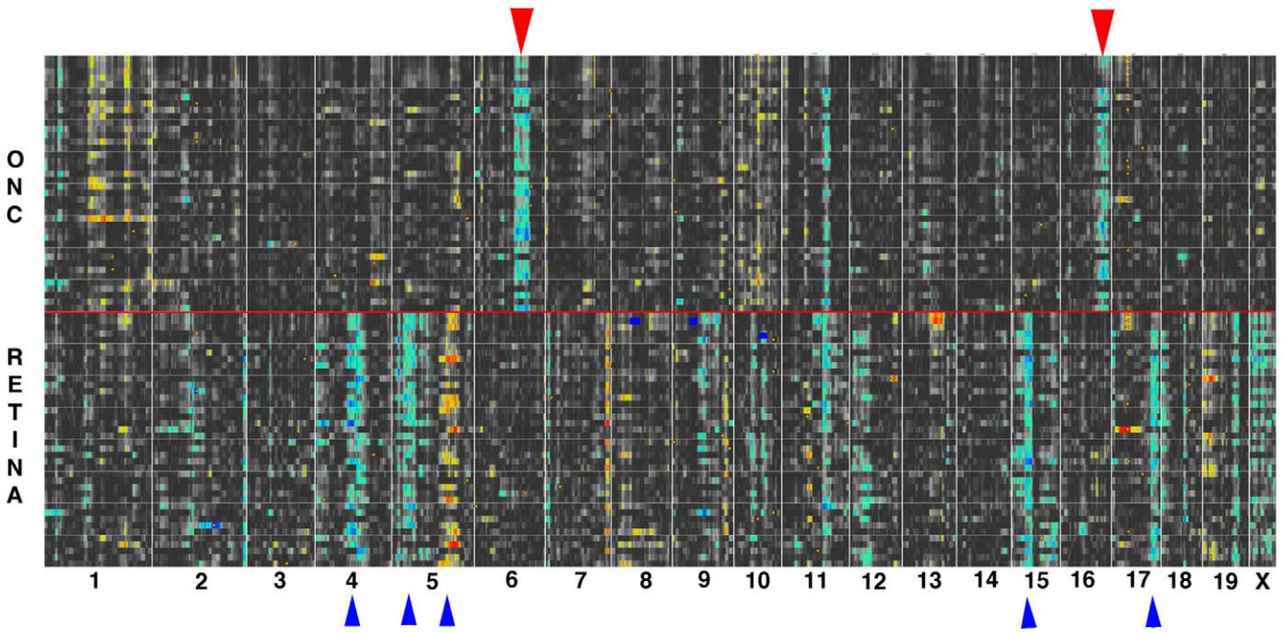


**Figure 4.** The genomic regions modulating the change in the innate immune network following ONC are shown by the differential QTL maps from the ONC HEI Database and the Normal Retinal Database (RETINA). The upper heat map was generated from the top 40 correlates of *C4b* found in the ONC HEI Database. The same genes (Illumina probes) were used to generate the lower heat map for the normal retina (RETINA). The upper map of the ONC data is separated from the lower normal retina data by a *red line*. The individual QTL maps are stacked on top of each other to create heat maps revealing signature QTLs for each dataset. Notice the two prominent new bands on Chr 6 and Chr 16 in the ONC heat map (*red downward arrows*). These represent regions of the genome that modulate the activation of the innate immune network following ONC. Note the loss of several cis-QTLs in the normal retina on Chr 4, Chr 5, Chr 15, and Chr 17, indicated by the *blue arrowheads*.

Investigative Ophthalmology & Visual Science

### A Replication Dataset

To examine the possibility that the innate immune network is activated in a naturally occurring model of glaucoma, we examined data from the DBA/2J mouse. We analyzed the data of Howell et al.[28] to explore this model of glaucoma using the GeneNetwork suite of tools. Howell and coworkers[28] had examined changes in gene expression in the DBA/2J mouse using Affymetrix microarrays (Santa Clara, CA). In their study, they related changes in the transcriptome of retinas from DBA/2J mice directly to the severity of the glaucoma (as defined by determining the degree of degeneration in the optic nerve). After processing their microarray data (which they had deposited in the public domain at GEO under accession number GSE26299) in GeneNetwork, we plotted C4b expression levels from all of the individual animals and severity groups (Fig. 5). After identifying the top 100 correlates of C4b within the DBA/2J dataset,[28] we compared these correlates with the list of correlates of *C4b* in the HEI Retina Dataset. Approximately one-quarter of the probe sets (26%), were in common, and these genes form the core of the innate immune network. The ONC network we derived in our study (Fig. 2), reveals that a similar set of genes in the innate immune network is activated in the DBA/2J model of glaucoma and the degree of activation is proportional to the degree of optic nerve damage.[28] Howell et al.[28] also identified members of the complement cascade that are upregulated. We observed similar findings after ONC in our database (Table 2). Howell et al.[28] found that the innate immune network was upregulated in early stages of glaucoma in their optic nerve head dataset. The data from their optic nerve head study[28] was placed in GeneNetwork and the variation across individual animals was analyzed. It appears that the members of the innate immune system are upregulated relatively early in the optic nerve head sample; however, using our approach, it was difficult to separate the changes occurring in the nerve head from the changes occurring in an adjacent portion of retina included in the microarray sample.

## Discussion

The single most novel and exciting aspect of the present study is the identification of a genetic network in the retina that modulates innate immunity in the retina. The internal environment of the retina and optic nerve are protected from outside influences by the blood–brain and blood–retina barriers, creating an immunologically partially privileged environment where peripheral blood components do not enter the parenchyma of the retina or optic nerve. Within this immunologically privileged environment, we demonstrated that many components of the innate immune system are expressed intrinsically by retinal cells (Retinal Database, GeneNetwork.org[32,40]). Others find that components of the complement cascade are expressed in human RPE.[5,23] The genes within this internal retinal network are activated by injury. This innate immune network is activated by ONC[34] and in mouse models of glaucoma.[28] In the ONC database, the top 100 correlates of C4b have correlation values greater than 0.66. When we examined the DBA/2J glaucoma database in a similar manner (meta-analysis of the Howell et al.[28] data), we find the top 100 correlates for C4b to have correlation values above 0.78. Many innate immune system genes are in both of the ONC and glaucoma innate immune networks. Of the top 200 correlates from each dataset, 78 probes (representing 66 different genes) are in common among the three experimental groups (Fig. 3), and they include *Serping1*, *C3*, *Tlr4*, *H2-Q2*, *H2-Tk23*, *CD74*, *C1q*, and *Cfi*. Thus, different types of insults to the retina activate this same innate immune network.

Members of this innate immune network play significant roles in the development of the brain. One specific example of the functional role of the innate immune system is best illuminated by a recent developmental study of the lateral geniculate nucleus (LGN).[3,41] In the developing brain, complement components are involved in synaptic elimination,[3] marking the specific synaptic contacts that are disposed to elimination. During development, as the LGN receives input from both eyes, there is significant overlap in projections from

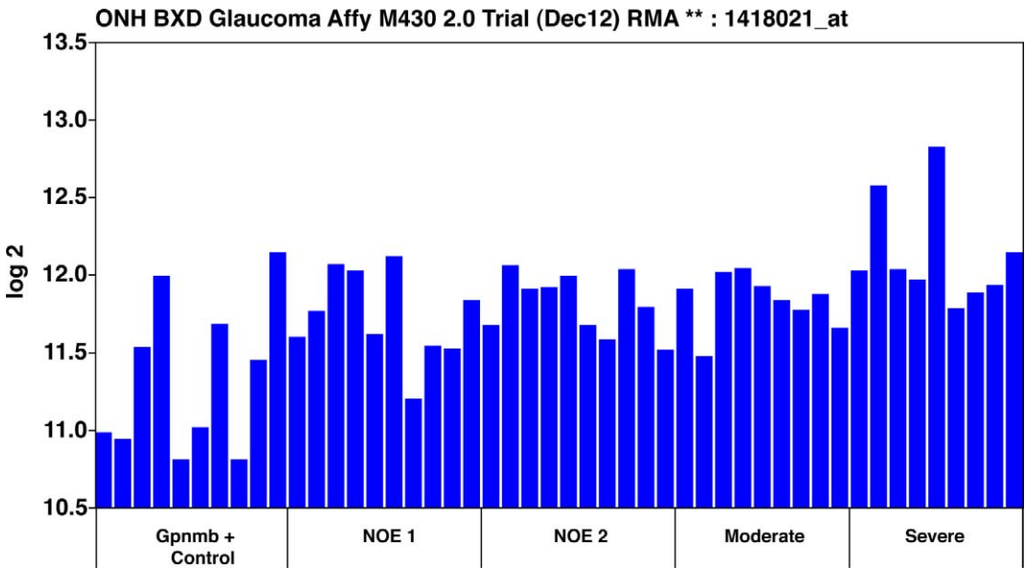


**Figure 5.** *C4b* RNA levels. The levels of *C4b* in a meta-analysis of mouse pigmentary glaucoma dataset from Howell et al.[28] The ordinate represents the *C4b* mRNA level from microarrays, expressed in log base 2, scaled with the mean set to 8. The mice were classified as wild-type $Gpnmb^{+/+}$ controls (Gpnmb + Controls), no detectable glaucoma 1 (NOE 1), no detectable glaucoma 2 (NOE 2), moderate glaucoma (Moderate), and severe glaucoma (Severe). Other than the wild-type group, all the groups were $Gpnmb^{-/-}$. There were no average differences among the ages of the mouse groups, and the reasons that the $Gpnmb^{-/-}$ were different in severity are not obvious. But it is essential to note that the expression of *C4b* in the retina increases as the severity of glaucoma increases.

the two eyes.[42] As the visual system matures, the nucleus goes from an intermixing of the retinal inputs to a complete segregation of the inputs of one eye to the other eye. One layer of the LGN receives inputs exclusively from one eye, whereas, the other layer receives input exclusively from the other.[42] The synapses that are to be eliminated express C3 and in animals in which this gene is knocked out, segregation does not completely occur.[3] Thus, members of the complement cascade form part of the system by which elements of the developing CNS are tagged for elimination.

In the adult retina, the *C4b* network, as defined by the expression pattern in the 80 BXD RI mouse strains, is an intrinsic network within retinal cells. The mRNA expression levels are upregulated by ONC (above the mean expression levels of mRNA in the retina as measured by our microarray platform). Since we have not found invading peripheral blood components in the retinas after ONC,[34] these high levels of message must represent an increased expression in intrinsic retinal cells. In addition, several cellular markers are associated with the innate immune network, including astrocyte and Müller cell markers such as *Gfap*, *Stat3*, and *Cp*. Microglial signature genes are also present within the network, including *Aif1*, *Cd68*, and *Cd74*. This indicates that at least glial cells intrinsic to the retina are associated with this innate immune network. In the brain, others have found that glial cells are sources of many members of the complement cascade.[1,43,44] All of these data indicate that, within the immunologically privileged environment of the retina and other CNS structures, the components of the complement cascade are produced and play a functional role. We are currently conducting experiments to define the specific cell types expressing the major members of the innate immune network in the retina.

An examination of the genes within the innate immune network points not only to genes associated with a response to injury, but also reveals many genes that are risk factors for retinal disease. There is a particularly strong association of the members of the complement system to human disease, specifically AMD. Many of the allelic variants of the components of the complement system are risk factors for AMD.[1,14,15,23,45] Many of these complement proteins form deposits that are found within drusen, a pathological deposit that characterizes AMD.[7,21,30,46]

The associations of the complement system with neuronal diseases are not restricted to the retina. The role of the complement cascade in the brain is relatively well documented (for review see Refs. 11, 47). In the brain, the complement cascade is strongly associated with Alzheimer's disease.[8,10] Members of this innate immune system are active after brain trauma.[47,48] The innate immune system is associated with Parkinson's disease[49] and multiple sclerosis[29] in the CNS. Not only are individual members of the complement system associated with these diseases, it may be the case that the entire innate immune network is activated in each of these disease processes. One of the most instructive disease models is experimental autoimmune encephalomyelitis, a model for multiple sclerosis. In this disease model, the terminal pathway involving the membrane attack complex is not critical for the development of the disease.[50] However, mice deficient in either *C3* or *Cfb* displayed markedly reduced disease response and severity.[29,50] These data suggest that the components of the complement system are not interacting with exactly the same components of the traditional complement pathways.

In the retina, these same components of the innate immune system are expressed and, as we have shown here, form a coregulated group of transcripts that form a novel genetic network. When members of this network carry mutations, the network can become dysregulated; it can drive the retina to accumulate drusen, and it can result in AMD (for review see Ref. 5). Clearly, the specific interactions of the innate immune network and its role in injury and disease are fertile grounds for future studies.

## Acknowledgments

Supported by Grants R01 EY017841 (EEG), University of Tennessee Health Science Center Center for Integrative and Translational Genomics, U01 AA016662 (RWW), U01 AA013499 (RWW), R01 EY021200 (MMJ), unrestricted funds from Research to Prevent Blindness (University of Tennessee, Emory University, Vanderbilt University), R01 EY016470 (JMN); R01 EY021592 (JMN); R01 EY0211200 (MMJ); P30EY006360 (Emory University); R01EY022349 (TSR); and Department of Defense W81XWH-10-1-0528.

Disclosure: **J.P. Templeton**, None; **N.E. Freeman**, None; **J.M. Nickerson**, None; **M.M. Jablonski**, None; **T.S. Rex**, None; **R.W. Williams**, None; **E.E. Geisert**, None

## References

1. Veerhuis R, Nielsen HM, Tenner AJ. Complement in the brain. *Mol Immunol*. 2011;48:1592–1603.
2. Prasad SS, Cynader MS. Identification of cDNA clones expressed selectively during the critical period for visual cortex development by subtractive hybridization. *Brain Res*. 1994;639:73–84.
3. Stevens B, Allen NJ, Vazquez LE, et al. The classical complement cascade mediates CNS synapse elimination. *Cell*. 2007;131:1164–1178.
4. Anderson DH, Radeke MJ, Gallo NB, et al. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. *Prog Retin Eye Res*. 2010; 29:95–112.
5. Sparrow JR, Ueda K, Zhou J. Complement dysregulation in AMD: RPE-Bruch's membrane-choroid. *Mol Aspects Med*. 2012; 33:436–445.
6. Bonifati DM, Kishore U. Role of complement in neurodegeneration and neuroinflammation. *Mol Immunol*. 2007;44:999–1010.
7. Alexander JJ, Anderson AJ, Barnum SR, Stevens B, Tenner AJ. The complement cascade: Yin-Yang in neuroinflammation—neuro-protection and -degeneration. *J Neurochem*. 2008;107: 1169–1187.
8. Rogers J, Schultz J, Brachova L, et al. Complement activation and beta-amyloid-mediated neurotoxicity in Alzheimer's disease. *Res Immunol*. 1992;143:624–630.
9. Jiang H, Burdick D, Glabe CG, Cotman CW, Tenner AJ. Beta-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J Immunol*. 1994;152:5050–5059.
10. Bradt BM, Kolb WP, Cooper NR. Complement-dependent proinflammatory properties of the Alzheimer's disease beta-peptide. *J Exp Med*. 1998;188:431–438.
11. Stoltzner SE, Grenfell TJ, Mori C, et al. Temporal accrual of complement proteins in amyloid plaques in Down's syndrome with Alzheimer's disease. *Am J Pathol*. 2000;156:489–499.
12. Anderson AJ, Najbauer J, Huang W, Young W, Robert S. Upregulation of complement inhibitors in association with vulnerable cells following contusion-induced spinal cord injury. *J Neurotrauma*. 2005;22:382–397.
13. Anderson AJ, Robert S, Huang W, Young W, Cotman CW. Activation of complement pathways after contusion-induced spinal cord injury. *J Neurotrauma*. 2004;21:1831–1846.
14. Edwards AO, Ritter R III, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005;308:421–424.
15. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/

CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2005;102:7227–7232.

16. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science*. 2005;308:419–421.
17. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385–389.
18. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet*. 2006;38:458–462.
19. Johnson LV, Leitner WP, Staples MK, Anderson DH. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Exp Eye Res*. 2001;73:887–896.
20. Mullins RF, Johnson LV, Anderson DH, Hageman GS. Characterization of drusen-associated glycoconjugates. *Ophthalmology*. 1997;104:288–294.
21. Crabb JW, Miyagi M, Gu X, et al. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2002;99:14682–14687.
22. Hageman GS, Mullins RF, Russell SR, Johnson LV, Anderson DH. Vitronectin is a constituent of ocular drusen and the vitronectin gene is expressed in human retinal pigmented epithelial cells. *Faseb J*. 1999;13:477–484.
23. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat Genet*. 2007;39:1200–1201.
24. Ennis S, Jomary C, Mullins R, et al. Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. *Lancet*. 2008;372:1828–1834.
25. Ahmed F, Brown KM, Stephan DA, Morrison JC, Johnson EC, Tomarev SI. Microarray analysis of changes in mRNA levels in the rat retina after experimental elevation of intraocular pressure. *Invest Ophthalmol Vis Sci*. 2004;45:1247–1258.
26. Steele MR, Inman DM, Calkins DJ, Horner PJ, Vetter ML. Microarray analysis of retinal gene expression in the DBA/2J model of glaucoma. *Invest Ophthalmol Vis Sci*. 2006;47:977–985.
27. Fan W, Li X, Wang W, Mo JS, Kaplan H, Cooper NG. Early involvement of immune/inflammatory response genes in retinal degeneration in DBA/2J mice. *Ophthalmol Eye Dis*. 2010;1:23–41.
28. Howell GR, Macalinao DG, Sousa GL, et al. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. *J Clin Invest*. 2011;121:1429–1444.
29. Bo L, Vedeler CA, Nyland HI, Trapp BD, Mork SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. *J Neuropathol Exp Neurol*. 2003;62:723–732.
30. Anderson MG, Smith RS, Hawes NL, et al. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. *Nat Genet*. 2002;30:81–85.
31. Howell GR, Libby RT, Marchant JK, et al. Absence of glaucoma in DBA/2J mice homozygous for wild-type versions of Gpnmb and Tyrp1. *BMC Genet*. 2007;8:45.
32. Freeman NE, Templeton JP, Orr WE, Lu L, Williams RW, Geisert EE. Genetic networks in the mouse retina: growth associated protein 43 and phosphatase tensin homolog network. *Mol Vis*. 2011;17:1355–1372.
33. Templeton JP, Geisert EE. A practical approach to optic nerve crush in the mouse. *Mol Vis*. 2012;18:2147–2152.
34. Templeton JP, Nassr M, Vazquez-Chona F, et al. Differential response of C57BL/6J mouse and DBA/2J mouse to optic nerve crush. *BMC Neurosci*. 2009;10:90.
35. Morse HC III, Chused TM, Hartley JW, Mathieson BJ, Sharrow SO, Taylor BA. Expression of xenotropic murine leukemia viruses as cell-surface gp70 in genetic crosses between strains DBA/2 and C57BL/6. *J Exp Med*. 1979;149:1183–1196.
36. Taylor BA, Wnek C, Kotlus BS, Roemer N, MacTaggart T, Phillips SJ. Genotyping new BXD recombinant inbred mouse strains and comparison of BXD and consensus maps. *Mamm Genome*. 1999;10:335–348.
37. Peirce JL, Lu L, Gu J, Silver LM, Williams RW. A new set of BXD recombinant inbred lines from advanced intercross populations in mice. *BMC Genet*. 2004;5:7.
38. Chang B, Khanna H, Hawes N, et al. In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse. *Hum Mol Genet*. 2006;15:1847–1857.
39. Geisert EE, Lu L, Freeman-Anderson NE, et al. Gene expression in the mouse eye: an online resource for genetics using 103 strains of mice. *Mol Vis*. 2009;15:1730–1763.
40. Rosen GD, Chesler EJ, Manly KF, Williams RW. An informatics approach to systems neurogenetics. *Methods Mol Biol*. 2007;401:287–303.
41. Boulanger LM, Shatz CJ. Immune signalling in neural development, synaptic plasticity and disease. *Nat Rev Neurosci*. 2004;5:521–531.
42. Godement P, Salaun J, Imbert M. Prenatal and postnatal development of retinogeniculate and retinocollicular projections in the mouse. *J Comp Neurol*. 1984;230:552–575.
43. Walker DG, Kim SU, McGeer PL. Complement and cytokine gene expression in cultured microglial derived from postmortem human brains. *J Neurosci Res*. 1995;40:478–493.
44. Trouw LA, Blom AM, Gasque P. Role of complement and complement regulators in the removal of apoptotic cells. *Mol Immunol*. 2008;45:1199–1207.
45. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med*. 2007;357:553–561.
46. Johnson PT, Betts KE, Radeke MJ, Hageman GS, Anderson DH, Johnson LV. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. *Proc Natl Acad Sci U S A*. 2006;103:17456–17461.
47. Alexander JJ, Anderson AJ, Barnum SR, Stevens B, Tenner AJ. The complement cascade: Yin-Yang in neuroinflammation—neuro-protection and -degeneration. *J Neurochem*. 2008;107:1169–1187.
48. Charbel Issa P, Chong NV, Scholl HP. The significance of the complement system for the pathogenesis of age-related macular degeneration—current evidence and translation into clinical application. *Graefes Arch Clin Exp Ophthalmol*. 2011;249:163–174.
49. Yamada T, McGeer PL, McGeer EG. Lewy bodies in Parkinson's disease are recognized by antibodies to complement proteins. *Acta Neuropathol*. 1992;84:100–104.
50. Weerth SH, Rus H, Shin ML, Raine CS. Complement C5 in experimental autoimmune encephalomyelitis (EAE) facilitates remyelination and prevents gliosis. *Am J Pathol*. 2003;163:1069–1080.

An intrinsic innate immune network was identified in the mouse retina. This network is activated by injury and mutations in members of this network result in disease, including AMD.

ED: Please verify the accuracy of any edits made to the article summary below.

Investigative Ophthalmology & Visual Science