

Innate Immune Network in the Retina Activated by Optic Nerve Crush

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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 *Serp1g1*, *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.⁴), 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 2012³³). 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 $2(z \text{ score } \text{Log}_2[\text{intensity}] + 8)$. 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 *Serp11*.

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

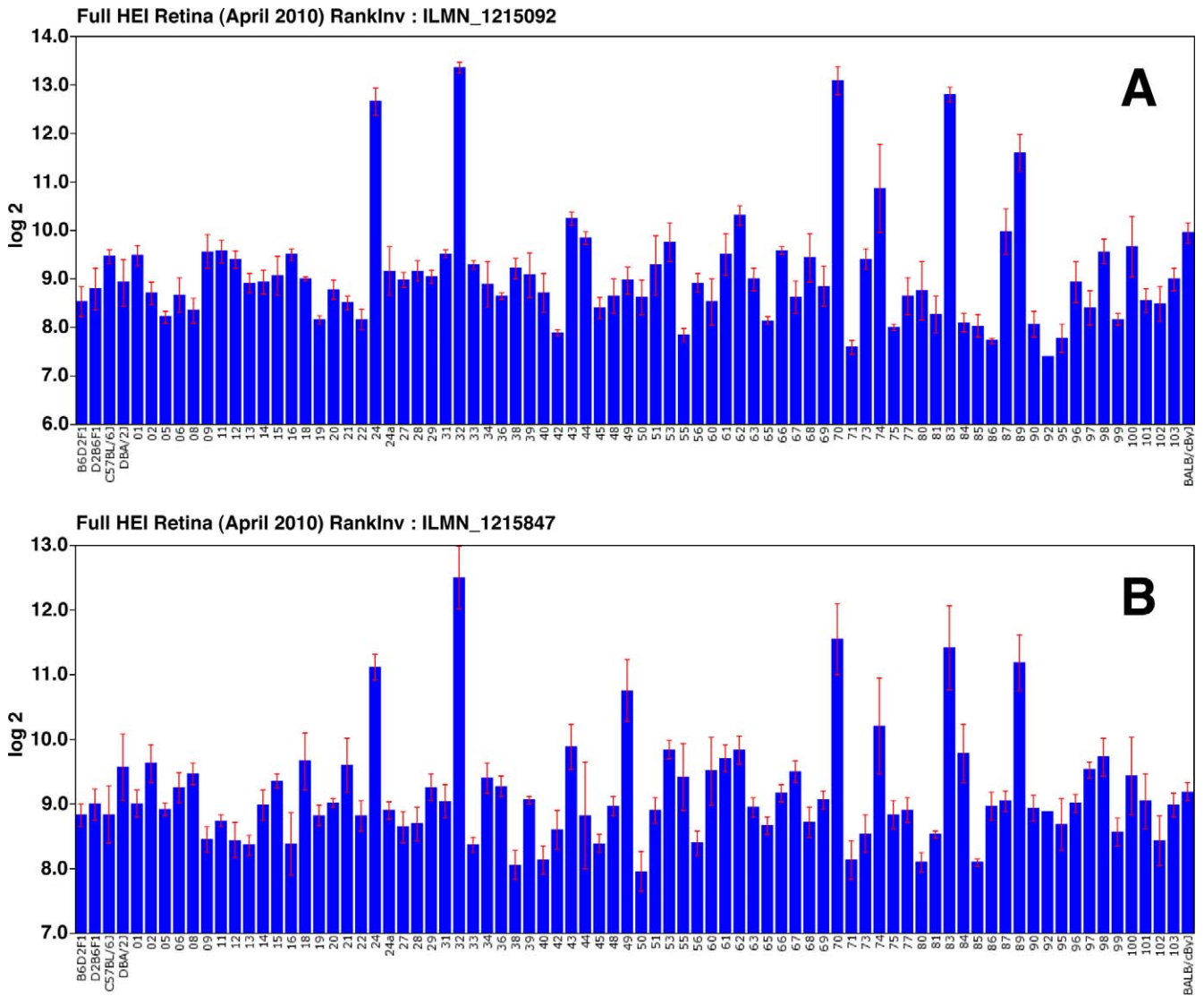


FIGURE 1. The expression of *C4b* (A) and *Gfap* (B) is shown across all of the BXD RI strains in the Full HEI Retina Datasets. The *scale* to the left is expression from the microarray studies expressed in log base 2 with the mean expression of mRNA across the microarray set to 8. Notice that the strains 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
Tgfb2	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 transducer and activator of transcription 3	ILMN_2698046	Chr11: 100.749555	11.22

All of these genes are expressed in the retina.

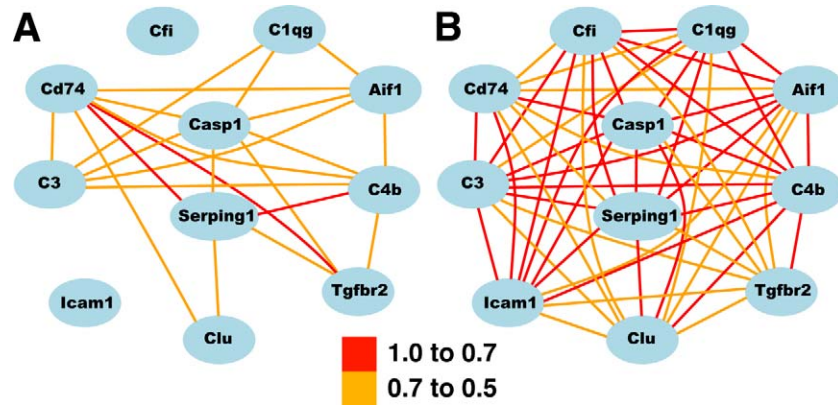


FIGURE 2. The increased correlation after ONC among selected genes in the innate immune network. The correlation among genes in the Normal Retina Database is shown in (A). The increased correlation among these genes following ONC is clearly demonstrated in (B). The *scale* indicates the correlation between 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

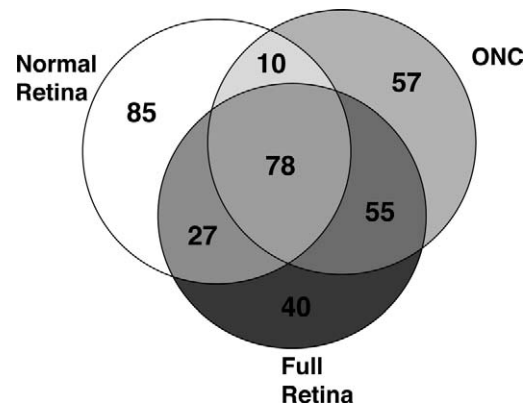


FIGURE 3. The Venn diagram reveals the overlap in the top 200 correlates of *C4b* in the normal retina dataset (Normal Retina), the ONC Dataset (ONC), and the Full Retinal Dataset (Full Retina). Of the top 200 correlates from each dataset, 78 probes (representing 66 different genes) are in common among 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
Tgfb2	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 QTLs³⁹ 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*, *Pbc1*, *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.

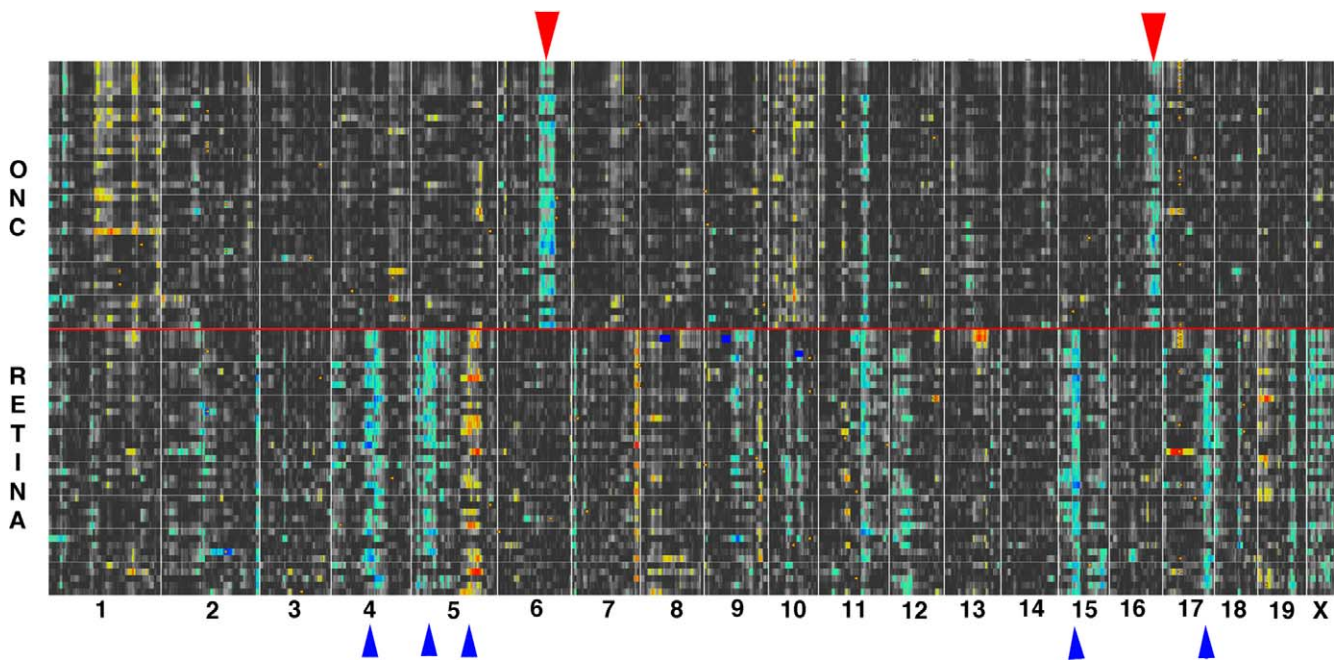


FIGURE 4. The genomic regions modulating the change in the innate immune network following ONC are shown by the differential QTL maps from the ONC HEI Database and the Normal Retinal Database (RETINA). The upper heat map was generated from the top 40 correlates of *C4b* found in the ONC HEI Database. 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.

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 disease⁴⁹ and multiple sclerosis²⁹ 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.

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