Lack of Immunoglobulins Does Not Prevent C1q Binding to RGC and Does Not Alter the Progression of Experimental Glaucoma

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Purpose. The degeneration of retinal ganglion cells (RGC) in the glaucomatous retina is accompanied by activation of the classical complement cascade. The purpose of this study was to evaluate whether complement component C1q binding and activation of the complement cascade in the glaucomatous retina requires the presence of immunoglobulins.

Methods. Experimental glaucoma was induced in normal mice and those carrying a targeted deletion of the *RAG1* gene. Binding of C1q to RGC and accumulation of *C3* and C5b-9 was investigated using immunohistochemical and proteomic approaches. Damage to the optic nerve and RGC was determined and compared between the two strains. Complement activation and accumulation were also evaluated in vitro using dissociated retinal cell cultures.

Results. C1q was detected in the RGC layer in both *RAG1*$^{-/-}$ and control mice with elevated IOP, but not in mice with normal IOP. Proteomic analysis of retinal membrane fractions indicated that C1q and *C3* are membrane bound to a similar degree in *RAG1*$^{-/-}$ and control mice with elevated IOP. The absence of Ig does not affect the rate of axonal damage or RGC loss. Furthermore, cultured RGC maintained in serum-free media are also C1q and *C3* immunoreactive, demonstrating that Ig is not required for C1q binding to damaged RGC.

Conclusions. Our data demonstrate that lack of immunoglobulins and mature T/B cells does not influence the progression of glaucoma. Furthermore, immunoglobulins do not appear to be required for C1q binding and complement cascade activation on damaged RGC. These findings suggest that C1q recognizes an alternative binding partner expressed by stressed RGC. (*Invest Ophthalmol Vis Sci.* 2012;53:6370–6377) DOI: 10.1167/iovs.12-10442

Glaucoma is a leading cause of irreversible blindness worldwide.[1] The disease is characterized by the degeneration of retinal ganglion cells (RGC) and their axons, which comprise the optic nerve, and eventually leads to the loss of vision.[2,3]

Local synthesis and deposition of components of the complement cascade is a common feature of neurodegenerative diseases. The innate immune response, in addition to mediating host immunity to invading pathogens, also participates in the removal of dying host cells and it is thought that this process is designed to prevent autoimmunity, minimize tissue inflammation, and support the reestablishment of tissue homeostasis.[4,5] Neuroinflammation and complement activation is frequently observed following retinal injury and has been described not only in glaucoma, but also in response to other injuries including ischemia/reperfusion, retinal degeneration, and mechanical injury.[6–8] Acute neuroinflammation is typically a beneficial process that results in the efficient removal of apoptotic cell debris, supports the reestablishment of tissue homeostasis, and avoids a long-term immunologic response. However, under chronic conditions, the sustained release of proinflammatory mediators such as TNF, IL-1β, and IFN-gamma can create a neurotoxic environment that can induce additional neuronal damage, leading to a self-propagating cycle of injury. Such mechanisms have been described in other neurodegenerative condition such as Alzheimer and Parkinson disease[9] and likely also occur in glaucoma, which involves progressive RGC and axonal loss over many years.

C1q, the initiator of the classical complement cascade, is a crucial component of this opsonin-mediated phagocytotic process and loss of C1q results in delayed clearance of apoptotic cell debris.[10] Genetic deficiencies in C1q and other early components of the classical complement cascade result not only in enhanced susceptibility to infection, but have also been strongly implicated in the development of systemic lupus erythematosus.[11–13]

The C1q complex is a soluble serum component and does not typically bind directly to cells. Rather it becomes fixed on the cell surface through interaction with other molecules. Initially these binding partners were thought to be exclusively immunoglobulins (Ig), but it has now become clear that a number of other molecules can fulfill this role, including α2β1 integrin,[14] beta-amyloid,[15] Clq receptor (calreticulin),[5] and the Receptor for Advanced Glycation End Products (RAGE)[16] and other molecules.

The retinal synthesis and deposition of components of the classical complement pathway is an aspect of the pathophysiology of glaucoma that has been demonstrated both in human postmortem tissue and in animal models of the disease.[6,17–20] Longitudinal studies using mouse models deficient in C1q and C3 have suggested that blocking this pathway does not ultimately result in rescue of RGC but rather delays RGC loss.[6,21] One interpretation of these findings is that complement actively contributes to the rapid degradation of damaged, but temporarily still viable, RGC through C5b-9–mediated lysis. This process may ultimately benefit the individual as it reduces the period of

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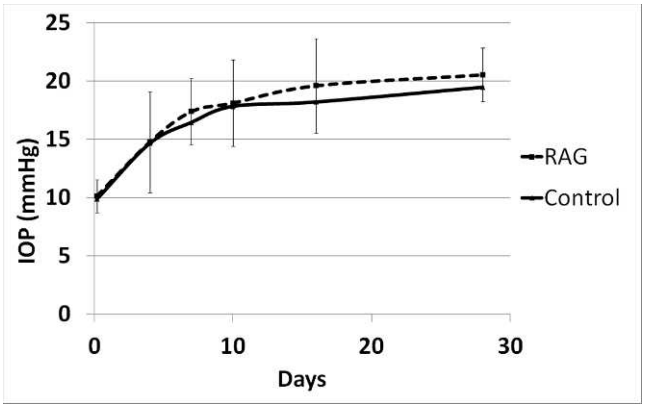

Figure 1. Assessment of IOP elevation in study eyes after microbead injection. A single injection of microbeads induced an elevation of IOP that lasted for more than 4 weeks ($n = 24$ eyes/strain). Values are mean ± standard deviation.

active neuroinflammation in the retina and may help prevent the formation of autoantibodies directed against RGC epitopes that may subsequently lead to IOP-independent RGC loss.

Much of the etiology of glaucoma remains unresolved and there has been speculation that the humoral immune response contributes to the pathophysiology of glaucoma.[22,23] Abnormal retinal autoantibody profiles have been observed in glaucoma patients and it has been proposed that these autoantibodies may exacerbate RGC damage in the disease.[24–26] Modeling an autoimmune response through injection of antibodies directed against HSP60, one of the antigens detected in the serum of glaucoma patients, into rats resulted in IOP-independent RGC loss, providing functional evidence that the development of autoimmunity could indeed lead to RGC damage.[27,28] One plausible mechanism to explain these observations is that RGCs are lysed and opsonized following activation of the classical complement cascade after autoantibody binding of these cells. However, as pointed out above, C1q binding is not strictly dependent upon the presence of Ig and may be initiated through other molecules.

The goal of this study was to evaluate whether activation of the classical complement cascade, as evidenced by C1q binding and cleavage of *C3*, in the glaucomatous retina is dependent upon the presence of Ig and to evaluate if the presence of immunoglobulins alters the rate of RGC degeneration. Toward this end, we induced RGC degeneration through elevation of IOP in normal mice and congenic mice homozygous for the Rag1^{tm1Mom} mutation (RAG$^{-/-}$).[29] These animals lack the recombination activating gene 1 that plays a crucial role in the rearrangement and recombination of the immunoglobulin and T cell receptor molecules during the process of VDJ recombination. As a result, RAG$^{-/-}$ mice produce no mature T cells or B cells and thus are incapable of producing antibodies. Immunohistochemical and proteomic methods were employed to determine the presence of complement components in the retina of these animals in the presence or absence of elevated IOP. Finally, glaucomatous damage was assessed through the determination of RGC density and evaluation of optic nerve damage.

Materials and Methods

Animal Model

Mice. Experimental glaucoma was induced in 6 to 8-week old C57BL/6J mice (control) and the RAG1 deficient congenic strain B6.129S7-Rag1^{tm1Mom}/J (RAG1$^{-/-}$) obtained from The Jackson Laboratory (Bar Harbor, ME). The experimental procedures and use of animals were approved and monitored by the Institutional Animal Care and Use Committee of the University of Iowa and conformed to the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of Elevated IOP. Mice were anesthetized using a Ketamine/Xylazine mix and body temperature was maintained using a heat pad. Forceps were used to expose and grip the conjunctiva and a 30½-gauge needle attached to a10 μL syringe (Hamilton, Reno, NV) was used to create a corneal incision through the limbus and aqueous humor was allowed to discharge. The needle was then reinserted through the same incision into the anterior chamber. A 3-μL injection of 15 μm green fluorescent polystyrene microspheres (FluoSpheres; Invitrogen, Grand Island, NY) was delivered to the anterior chamber. The needle was held in place for 30 seconds to allow for dispersion of the microspheres. The needle was then removed and held on the incision site for an additional 30 seconds to prevent discharge of the microspheres. Mice were observed until fully recovered from anesthesia. IOP was monitored using a rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH) as described previously.[30]

Mice were sacrificed 14 and 28 days after induction of elevated IOP.

Assessment of Glaucomatous Damage

RGC Counts. RGC counts were carried as reported previously.[6,17] Briefly, retinas were fixed for 2 hours in 4% paraformaldehyde, dissected, flat mounted onto slides, and incubated with rabbit anti-gamma synuclein primary antibody (1:300 dilution, Abcam, Cambridge, MA) and secondary antibody goat anti-rabbit conjugated to a thiol-reactive dye (Alexa Fluor 546 1:200; Invitrogen, Carlsbad, CA). For each eye, a Z-series was taken from six randomly chosen locations using a confocal microscope at 600× magnification (Nikon Eclipse 80i; Nikon Instruments Inc., Elgin, IL). Images were imported into an image editing program (ImageJ; National Institutes of Health, Bethesda, MD) and stacked. Cells were counted using the "Cell Counter" Plug-in.

Optic Nerve Damage. Mouse optic nerves were fixed in 20% paraformaldehyde, 5% glutaraldehyde, 0.25% CaCl2, 0.1 M cacodylate at pH 7.4, osmicated, dehydrated with 25% acetone, 50% acetone, 75% acetone, 95% acetone, and 100% acetone, and embedded in Eponate resin. Sections of 1 μm were cut from each optic nerve with a ultramicrotome (Leica EM UC7; Leica Microsystems, Buffalo Grove, IL). The sections were stained with 1% paraphenylenediamine (PPD) and pictures were taken at 200× and 400× magnification using a microscope (Olympus BX41; Olympus, Center Valley, PA). Optic nerves were evaluated by three independent investigators in a masked fashion and assigned a damage score as previously reported.[6,17] The damage scores are defined as follows: 1 = no damage: no PPD stained axons, no gliosis; 2 = very mild damage: <40 axons stained with PPD, no gliosis; 3 = mild damage: frequent PPD stained axons, no gliosis; 4 = moderate damage: many PPD stained axons, gliosis apparent in some portions of the optic nerve; 5 = severe damage; gliosis apparent throughout the optic nerve, few surviving axons, many staining with PPD. Examples of these are given in Figure 2C. Differences between the groups were statistically evaluated using the Wilcoxon Rank Sum Test.

Immunohistochemistry

Mouse eyes were fixed in 4% paraformaldehyde immediately after enucleation, infiltrated with increasing amounts of sucrose, and embedded in optimal cutting temperature (OCT) compound. Following embedment, blocks were sectioned on a cryostat (HM 505E Microtome; Microm International, Walldorf, Germany) using a thickness setting of 7 μm. Sections were collected on Superfrost plus slides and stored at −80°C. The sections were incubated with primary antibody against C1q (1:100 dilution, Abcam) or C5b-9 (1:250 dilution; Abcam) and secondary antibodies conjugated either to one of two

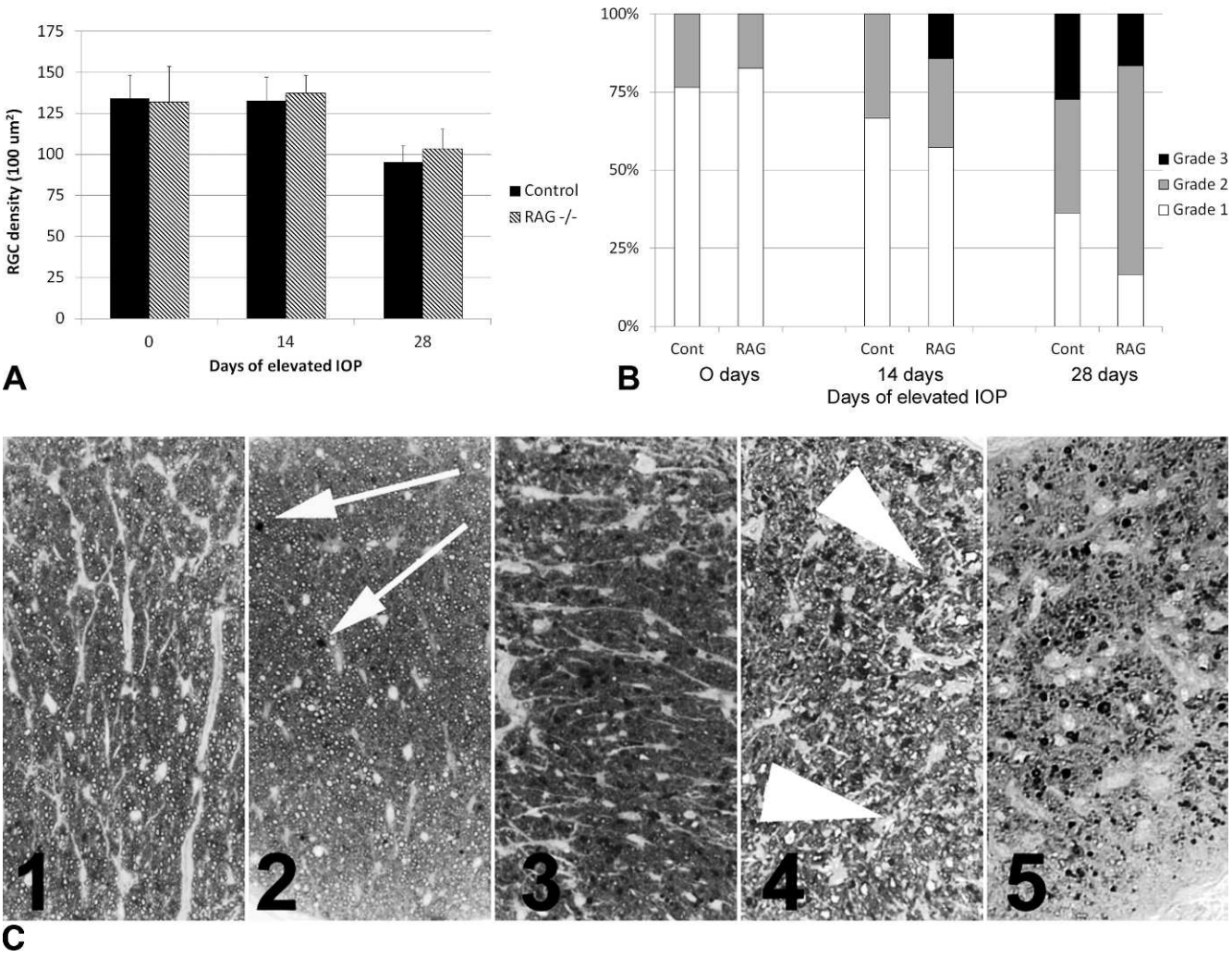

Figure 2. Evaluation of RGC loss and optic nerve damage in mice with elevated IOP. (**A**) RGC densities in control and RAG$^{-/-}$ mice prior to induction of elevated IOP (0 days) and 14 and 28 days after microbead injection. RGC density is unchanged 14 days after induction of elevated IOP, but was significantly decreased by 28 days ($N = 12$ for each group and timepoint. Error bars represent standard deviation.) (**B**) Distribution of optic nerve damage grades assigned to RAG$^{-/-}$ (RAG) and normal control (Cont) mice prior to induction of elevated IOP (0 days), and 14 days, and 28 days after induction. Mild damage progressively develops in both mouse strains. ($N = 12$ for each group and timepoint). (**C**) Example of the grading scheme used to evaluate damage in traverse sections of optic nerve. Damage grades range from 1 (*left panel*), indicating a healthy optic nerve, to 5 (*right panel*) indicating severe degeneration of the optic nerve. Increasing axonal damage is revealed by increasing numbers of PPD stained axons (*arrow in 2*) and the appearance of gliosis (*arrowheads in 4*) in the optic nerve. Magnification: 400×.

thiol-reactive dyes (Alexa Fluor 488 or Alexa Fluor 546, both at 1:200 dilution; Invitrogen). DAPI was included to facilitate orientation on the slide. The microphotographs were taken using an Olympus BX41epi-fluorescence microscope.

Proteomic Analyses

Cell Membrane Preparations. Fresh retinas from 10 mouse eyes were carefully dissected, pooled, and homogenized using a laboratory homogenizer (Tissuemiser; Fisher Scientific, Pittsburgh, PA) in PBS containing a protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenate (H) was centrifuged at 10,000*g* for 10 minutes and the pellet (P1) was discarded. The supernatant (S1) was centrifuged at 80,000*g* for 1 hour. The ultra-centrifugation supernatant (S2) containing the soluble protein fraction was removed and the pellet containing cell membranes (P2) was dissolved in PBS. All steps were carried out at 4°C. Total protein concentration was evaluated at A280.

Western Blot Analysis. A total of 25 μg of protein per sample was loaded on TGX 4% to 20% SDS-PAGE gels (BioRad, Hercules, CA) and separated at 200V for 30 minutes. Proteins were transferred to a PVDF membrane at 100V for 1 hour. Blots were blocked in 5% non-fat dry milk (NFDM) at room temperature, and then incubated with primary antibodies against anti-β-tubulin (1:200 dilution; Santa Cruz Biotech, Santa Cruz, CA); anti-Glucose transporter 1 (1:100 dilution; Abbiotech, San Diego, CA); anti-C1q (1:200 dilution; Abcam); or anti-C3 antibody (1:200 dilution; Calbiochem) in 5% NFDM at 4°C overnight. After extensive washing, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody, and immunoreactive bands were visualized using chemiluminescent detection (Immu-star Western Kit; BioRad). Images were captured using an imaging system (Versadoc; BioRad).

Retinal Cell Culture

Retinas from 6 to 8-week-old normal control mice with normal IOP were carefully dissected and dissociated with collagenase (2 mg/mL) at 37°C for 1 hour. The cells were seeded in NeuroBasal serum-free media into 24-well plates covered with glass slips and incubated at 37°C, 5% CO_2 and 90% humidity. The media was exchanged every other day, and cells were harvested after 6 days by fixation in 4% paraformaldehyde.

Cells were incubated with goat primary antibodies directed against C1q (1:200 dilution; Abcam) and double labeled with rabbit antibody against neurofilament heavy chain (1:100 dilution; Chemicon). Binding was visualized using the secondary antibodies anti-rabbit IgG (Alexa Fluor 546 conjugate) and anti-goat IgG and (Alexa Fluor 488 conjugate, both at 1:200 dilution; Invitrogen).

Results

Elevated IOP was induced in RAG$^{-/-}$ and control mice by injection of polystyrene microbeads into the anterior chamber as described previously by others.[31–33] In our hands, the injections resulted in increased IOP that developed within 1 week in approximately 70% of treated eyes, although the magnitude of the observed IOP changes varied between individuals. Overall, individuals in the RAG$^{-/-}$ group tended to respond to this treatment with slightly higher elevation of IOP than normal mice. In the interest of yielding comparable data, eyes were selected from each strain to yield groups with similar IOP prior to subsequent analyses of the induced glaucomatous changes ($N = 12$ per genotype and for each time point, 72 eyes total). The observed IOP increased from 9.9 and 10.1 mm Hg prior to microbead injection, to 14.7 and 14.8 mm Hg after 4 days, and continued to increase to 19.5 and 20.6 mm Hg by the end of the experiment in the normal and the RAG$^{-/-}$ mice, respectively (Fig. 1). The IOP differences between the groups are statistically not significant ($P = 0.81$, 0.24, and 0.45 on days 10, 16, and 28, respectively).

To confirm the development of glaucomatous damage and to assess any potential differences between normal and RAG$^{-/-}$ experimental glaucomatous change, RGC density was determined following immunohistochemical identification of γ-synuclein positive RGC (Fig. 2A). As expected, continued exposure to elevated IOP resulted in progressive RGC loss and optic nerve damage (Fig. 2A). While eyes obtained from both RAG$^{-/-}$ and control mice 14 days after induction of elevated IOP display essentially normal RGC densities ($P = 0.46$ and 0.76, respectively), retinas harvested 28 days after induction of elevated IOP exhibit 21.5% (RAG$^{-/-}$) and 29.6% (control) loss of RGC ($P = 3.66 \times 10^{-06}$ and 2.06×10^{-08}; respectively). The differences between the RAG$^{-/-}$ and control mice in the number of surviving RGC are not statistically significant ($P = 0.42$ and 0.07 after 14 and 28 days of elevated IOP, respectively).

The degree of axonal damage in the optic nerve was determined using a grading scheme as previously described.[6,17] Increasing axonal damage is indicated by a rise in the number of PPD stained axons and increasing amounts of gliosis. Optic nerves evaluated are assigned to one of five damage categories ranging from 1 (no damage) to 5 (severe damage) as illustrated in the examples in Figure 2C. Our data demonstrate that 2 weeks after induction of elevated IOP damage to the optic nerve was not significant in either group ($P = 0.66$ in control mice and 0.24 in RAG1$^{-/-}$; Fig. 2B). However, 28 days after IOP elevation, evidence of mild optic nerve damage was frequently evident and a fraction of samples displayed signs of advanced damage as indicated by PPD staining of numerous axons and clear signs of gliosis ($P = 0.03$ in control mice and 0.0005 in RAG1$^{-/-}$ compared with baseline values). Congruent with our data indicating a similar degree of RGC loss in RAG$^{-/-}$ and normal mice, we did not observe a statistically significant difference in the extent of optic nerve damage between the two groups ($P = 0.73$ after 14 days and 0.78 after 28 days).

The development of RGC damage is accompanied by increased immunohistochemical labeling of C1q and the C5b-9 complex (Fig. 3). Four weeks after microbead injection, immunohistochemical detection of C1q and C5b-9 revealed increased labeling in the ganglion cell layer (GCL) in both normal and RAG$^{-/-}$ mice in animals with elevated IOP. Other layers of the retina are not complement-immunoreactive, although occasionally weak C1q binding was observed in the outer plexiform layer (OPL). Immunoreactivity to complement components can also frequently be observed within the retinal vasculature (arrowhead in Fig. 3F) presumably due to the presence of complement components in the serum. In contrast, retinal labeling is absent in both mouse strains at normal IOP. Eyes that received microbead injections, but failed to develop elevated IOP, did not display C1q or C5b-9 immunoreactivity (Figs. 3C, 3F).

In order to demonstrate that the complement components detected are fixed on the cell membranes, we separated soluble retinal protein from the cell membrane fraction and analyzed the latter by Western blot analyses. As described in the "Methods" section, our experimental approach yields fractions of insoluble cellular components (P1) as well as a fraction of cellular constituents that remain soluble after centrifugation at 10,000*g* (S1). This fraction S1 can further be divided by ultracentrifugation into soluble components (S2) and a pellet enriched in cell membranes and cell-membrane associated proteins (P2). Figures 4A and 4B demonstrate the effectiveness of our separation method. Glucose Transporter 1 (*Glut1*), a membrane spanning protein, is retained in the membrane fraction P2 (Fig. 4A). Conversely, β-tubulin, a biomarker for soluble protein, is detected in the soluble protein fraction S2, but is removed from the membrane enriched fraction P2 (Fig. 4B).

Retinal membrane preparations (P2) were then obtained from RAG$^{-/-}$ and normal mice with and without elevated IOP and evaluated for the presence of C1q and *C3* by Western blot analyses (Figs. 4C, 4D). Membrane associated dimers of C1q (66 kDa) are readily detectable in the membrane enriched retinal fraction obtained from mice with elevated IOP (Fig. 4C). Low levels of C1q are also occasionally observed in control mice with normal (low) IOP. Importantly, membrane-associated C1q can also be demonstrated in mice lacking RAG1, suggesting antibody independent fixation of C1q in the glaucomatous retina.

Western blot analysis of the same protein fractions for the presence of C3 also revealed an accumulation of this protein in retinas obtained from mice with elevated IOP but not those with normal IOP (Fig. 4D). A major band with an apparent molecular weight of 63 kDa, as well as minor bands at 75 kDa and 101 kDa representing iC3bα, iC3bβ, and the uncleaved C3α' chains, respectively, are present. Congruent with our findings for C1q, membrane-associated *C3* was observed in control and RAG$^{-/-}$ mice, indicating that C1q fixation led to activation of the complement cascade in both mouse strains.

Finally, C1q fixation on the surface of RGC in the absence of antibodies can also be demonstrated in a primary cell culture system of mouse retinal cell suspensions. RGC maintained in cell culture exhibit considerable cell stress and typically die within days or weeks. During this period, the accumulation of complement components can be observed. Here we maintained RGC in cell culture medium without serum to rule out an exogenous source of immunoglobulins. Adult mouse retinae were enzymatically dissociated and maintained in serum free cell culture media. After 6 days in culture, numerous RGC were identified immunohistochemically by the presence of neurofilaments heavy chain (NFH). Many of these cells are also immunoreactive with antibodies directed against C1q (Fig. 5). These findings not only further indicate that immunoglobulins are not required for C1q fixation on RGC, but also indicate that both C1q and the ligand fixing it to these cells are not serum derived and are consequently synthesized by retinal cells, possibly RGC themselves.

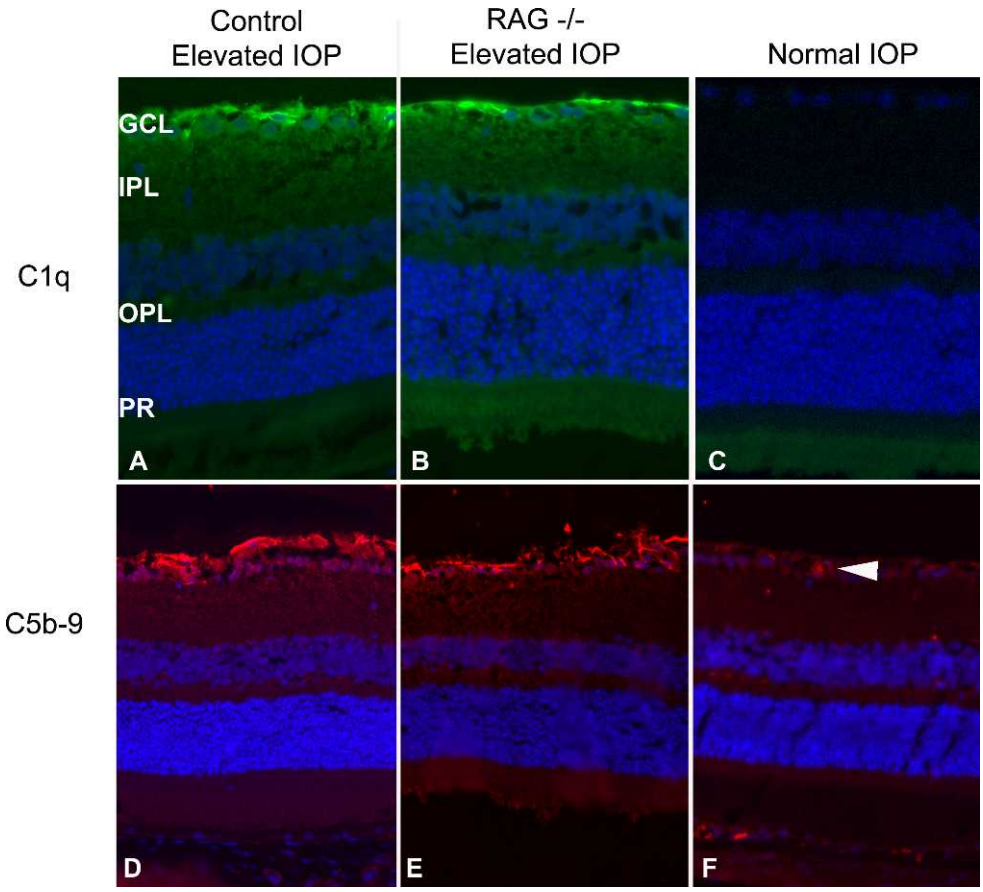

Figure 3. Immunohistochemical detection of complement components in the retina of control mice (**A**, **D**) and RAG$^{-/-}$ mice (**B**, **E**) with elevated IOP as well as control mice that received injections of microbeads but failed to develop elevated IOP (**C**, **F**). Immunoreactivity to C1q (**A–C**) and C5b-9 (**D–F**) can be detected in the nerve fiber and GCL of the animals with high IOP, but not in normotensive controls. Complement immunoreactivity can also occasionally be observed in association with retinal capillaries (*arrowhead* in **F**), presumably due to the presence of serum remaining in these vessels. Nuclei are stained blue with DAPI to facilitate orientation. IPL, inner plexiform layer; PR, photoreceptor layer. Magnification: 200×.

Discussion

The susceptibility of an individual to develop glaucoma and the rate at which the disease progresses are influenced by multiple factors, many of which remain incompletely understood. While the development of elevated IOP is clearly a risk factor for this disease,[34] other, IOP independent, factors are involved in its pathophysiology. These factors include anatomical features, such as the diameter of an individual's optic nerve head,[35,36] but may also involve factors that influence the ability of a patient's RGC to withstand cell stress brought about by the disease. Regardless of the underlying etiology, glaucoma invariably involves the degeneration of RGC and triggers a neuroinflammatory response in the retina that includes the release of pro-inflammatory mediators and activation of the complement cascade.[21,37–40] It is conceivable that an overly vigorous, or poorly controlled, activation of complement could result in the premature degeneration of stressed cells or in loss of additional cells through C5b-9 mediated bystander lysis.

Under normal physiological conditions the retina is considered to be immunologically privileged. Antigen presentation is actively suppressed, glial cells are maintained in the quiescent state, and immune components are excluded from the retina by the blood brain barrier.[41] Yet in the glaucomatous retina, the synthesis of molecules mediating antigen presentation is significantly increased, glia become reactive, and there are reports of increased levels of autoantibodies directed against retinal antigens in the serum of glaucoma patients.[38,42,43] These findings raise the possibility that autoantibodies directed against RGC could lead to complement activation and cell lysis resulting in IOP-independent degradation of these cells. However, fixation of C1q on the cell surface can also be mediated by a number of other molecules or the presence of phosphatidylinositol within the cell membrane. This study was designed to investigate the interactions between the humoral immune response in complement activation in glaucoma. In particular, we sought to assess if antibodies are required to fix C1q to the surface of damaged RGC.

Immunohistochemical analysis for C1q and *C3* deposition in the retina of mice with induced elevated intraocular pressure confirmed our earlier findings that these molecules accumulate primarily in the ganglion cell layer, but cannot be detected in the retinas of eyes with normal IOP.[6,17] The observed labeling pattern did not reveal any noticeable differences between normal mice and those lacking RAG, suggesting that autoantibodies are not required for C1q fixation on RGC in the glaucomatous retina. To confirm these findings and to ensure that the detected complement components are tightly associated with the retinal cell membranes, we isolated cell membrane preparations to remove any soluble complement components that may be present in the retinal vasculature. Data obtained not only confirm antibody independent fixation of C1q in RAG$^{-/-}$ mice, but also indicate that the formed C1q

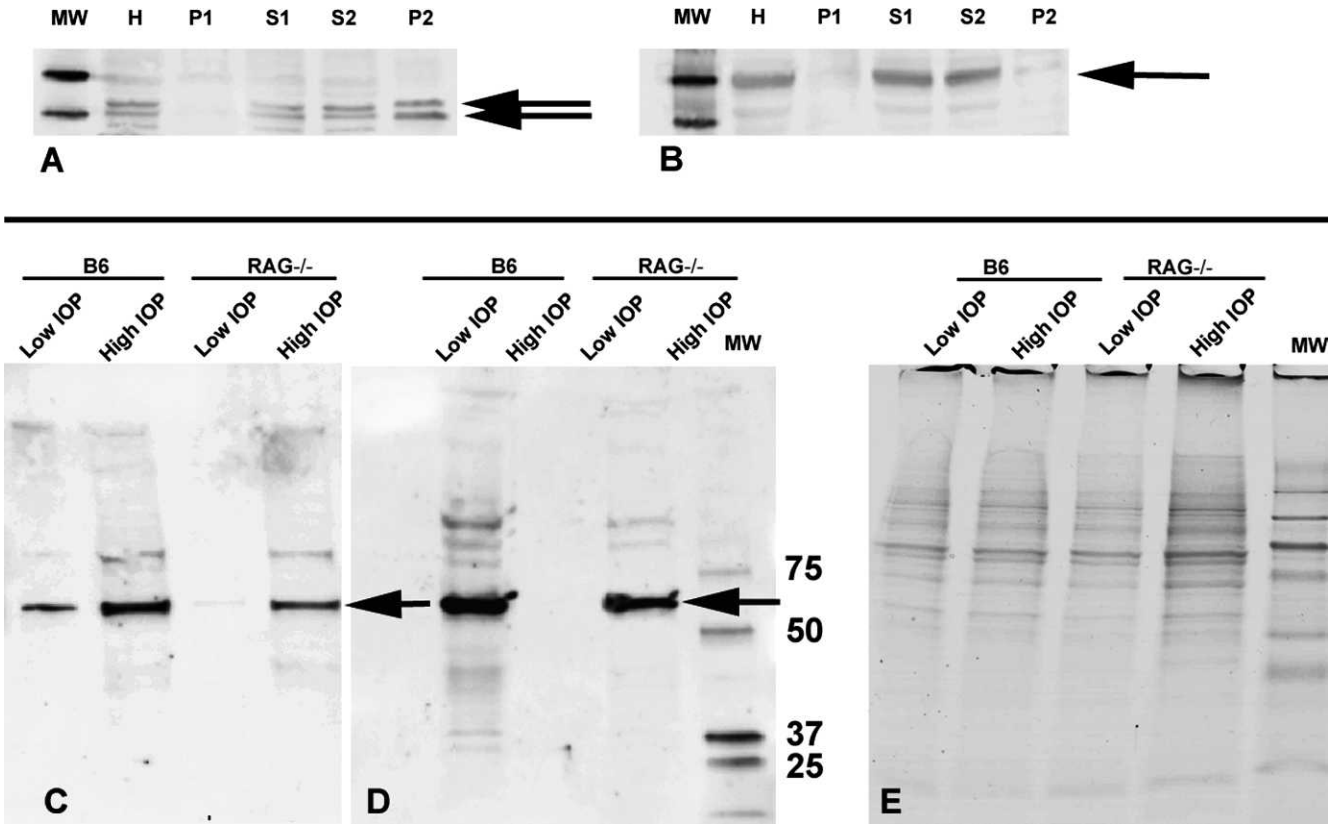

Figure 4. Western blot analysis of retinal protein fractions. (**A**) glucose transporter 1. (**B**) β-tubulin as biomarkers for membrane bound and soluble proteins, respectively. The cell membrane fraction P2 contains glucose transporter 1 (double band indicated by *arrows* in **A**) but β-tubulin has been removed (expected size indicated by *arrow* in **B**). MW, molecular weight standard; H, unfractioned cell homogenate. (**C**, **D**) Mouse retinal membrane preparations (P2) probed with an anti-C1q antibody (**C**) or anti-C3 antibody (**D**). Membrane preparations from control mice (B6) and RAG1$^{-/-}$ (RAG$^{-/-}$) display prominent labeling to bands of 66 kDA (*arrow* in C) and 63 kDa (*arrow* in D), indicative of the presence of C1q dimmers and iC3bα, respectively. (**E**) Coomassie Blue stained gel containing the same fractions of proteins analyzed in (**C**) and (**D**).

complexes are biologically active and result in surface bound and processed C3. The apparent molecular weight for the detected C3 suggested that the vast majority of the protein is present as iC3b. Under most conditions, surface-bound C3b is rapidly converted to iC3b, which can remain bound to the cell surface. While this moiety is no longer able process C5 and participate in the propagation of complement activation,[44] iC3b interaction with the CR3 receptor (CD11b/CD18) does greatly enhance phagocytosis of iC3b-coated target cells or particles.[45] As such, the presence of iC3b may be a crucial aspect in mediating the ability of CR3 positive retinal glia to phagocytose damaged or dying RGC.

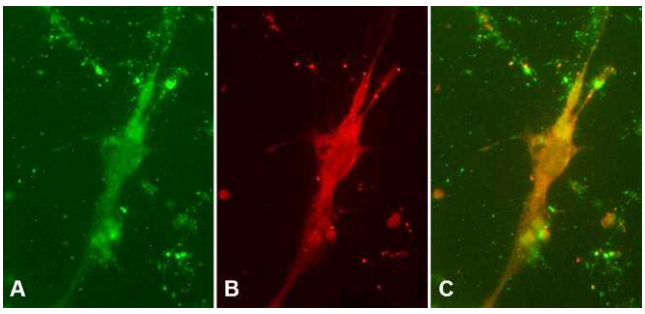

Figure 5. Immunohistochemical detection of complement components associated with retinal ganglion cells in mouse retinal dissociation cultures. C1q (**A**) can be detected on cells that also bind the RGC marker neurofilaments heavy chain (**B**). (**C**) Merged image of (**A**) and (**B**). Magnification: 400×.

Finally, our finding of immunoglobulin-independent C1q fixation on degenerating RGC is further supported by our in vitro data indicating the presence of C1q associated with RGC in a serum-free cell culture system. The occurrence of this response not only indicates the presence of a distinct C1q ligand, but also suggests that this complement component, as well as the ligand, can be synthesized by retinal cells and are not recruited from the bloodstream.

Serum autoantibodies directed against retinal epitopes are frequently observed in healthy older human subjects, but a number of reports have suggested that they occur at increased frequency and elevated titers in retinal diseases associated with advanced age, such as AMD or glaucoma.[22,24–26,46] In our experiments, progressive degeneration of RGC and associated damage of the optic nerve, the hallmark features of glaucoma, were observed both in control and RAG$^{-/-}$ mice following the induction of elevated IOP. The differences between the RAG$^{-/-}$ and control mice in either the number of surviving RGC or the degree of optic nerve degeneration are statistically nonsignificant, indicating that the absence of antibodies does not influence the rate or degree of glaucomatous damage. However, our data do not indicate that antibodies directed against RGC antigens are incapable of initiating a classical complement response. We previously speculated that the occurrence of elevated titers of autoantibodies directed against retinal antigens may be the result of insufficient clearance of cellular debris during phases of rapid RGC degeneration in

glaucoma.[38] Here the induced retinal damage was relatively mild and the experiment was terminated four weeks after the elevation of IOP. It is possible that this period was not long enough or that RGC damage was not sufficiently pronounced to allow the formation of a significant autoimmune response. A longer study might reveal differences between $RAG^{-/-}$ and control mice.

Taken together, our data demonstrate that C1q binding to RGC in the glaucomatous retina does not require the presence of antibodies, relying on a yet unidentified molecule to mediate fixation on the cell surface of damaged RGC. Our data also indicate that the absence of antibodies is not protective in glaucoma.

Increased synthesis of complement components is one of the earliest molecular indications of glaucomatous damage in the retina. Complement targeting of damaged RGC appears to be highly specific and consequently the identification of the recognition molecule mediating this event may provide insight into the very early cellular events that occur in this blinding disease.

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