Glaucoma

T and B Lymphocyte Deficiency in Rag1−/− Mice Reduces Retinal Ganglion Cell Loss in Experimental Glaucoma

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PURPOSE. We previously demonstrated that passive transfer of lymphocytes from glaucomatous mice induces retinal ganglion cell (RGC) damage in recipient animals, suggesting a role for immune responses in the multifactorial pathophysiology of glaucoma. Here we evaluate whether absence of an adaptive immune response reduces RGC loss in glaucoma.

METHODS. Elevated intraocular pressure (IOP) was induced in one eye of C57BL/6j (B6) or T- and B-cell–deficient Rag1−/− knockout mice. After 16 weeks RGC density was determined in both the induced and the normotensive contralateral eyes. Data were compared to mice having received injections of “empty” vector (controls). The number of extravascular CD3+ cells in the retinas was determined using FACS.

RESULTS. Retinas of eyes with elevated IOP contain significantly more extravasated CD3+ cells than control retinas (46.0 vs. 27.1, P = 0.025). After 16 weeks of elevated IOP the average RGC density in B6 mice decreased by 20.7% (P = 1.9 × 10−4). In contrast, RGC loss in Rag1−/− eyes with elevated IOP was significantly lower (10.3%, P = 0.006 vs. B6). RGC loss was also observed in the contralateral eyes of B6 mice, despite the absence of elevated IOP in those eyes (10.1%; P = 0.008). In Rag1−/− loss in the contralateral eyes was minimal (3.1%) and significantly below that detected in B6 (P = 0.02).

CONCLUSIONS. Our findings demonstrate that T Rag1−/− mice are significantly protected from glaucomatous RGC loss. In this model, lymphocyte activity contributes to approximately half of all RGC loss in eyes with elevated IOP and to essentially all loss observed in normotensive contralateral eyes.

Keywords: glaucoma, RGC, autoimmune response/disease, intraocular pressure
glaucomatous mice into naive animals leads to progressive RGC loss in the recipients without an associated increase in IOP.23 These findings were subsequently confirmed by others24 and strongly indicate that elevated IOP and glaucoma leads to the establishment of an immune response that is sufficiently pronounced to cause the death of RGC in an otherwise healthy eye. However, it was unclear whether immune-mediated processes alter the rate of RGC degeneration or vision loss during the course of the disease in affected eyes.

In this study we compare RGC loss in recombination activating 1 knock out mice (Rag1−/-) with elevated IOP to that of control animals. Our studies were aided by a refined approach to inducing elevated IOP that enabled us to exert this stress factor to one eye of each animal for 16 weeks. Our findings demonstrate an increase of extravasated CD3+ cells in eyes with elevated IOP and that Rag1−/- mice are partially protected from RGC loss in eyes with elevated IOP. We further present data demonstrating that the contralateral eye in normal mice experiences significant RGC loss, which is not observed in Rag1−/-.

**Materials and Methods**

**Animals**

Mice used in this study were either normal C57BL/6J (B6), or B6.129S7-Rag1<sup>tm1Domm</sup>/J (Rag1−/-) mice. These mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at the University of Iowa for no more than four generations to avoid genetic drift. Healthy Rag1−/- mice have an identical RGC density to B6 mice (see Supplemental Figure S1). In addition, 11- to 13-month-old myocilin Y437H transgenic mice,25 as well as nontransgenic littermates, were used for flow cytometry studies. Both male and female animals were used for experiments. All studies were carried out according to the ARVO statement for the use of Animals in Ophthalmology and Vision Research and were approved by the University of Iowa Committee for Animal Care and Use.

**Induction of Elevated IOP**

Elevated IOP was induced using an adenoviral vector expressing a pathogenic form of human myocilin Ad5RsvMyocilin<sup>Y437H</sup>Flag (Ad5myoc) as described by others.24,25 Control animals received an equal amount of an Ad5 vector not expressing exogenous genes (Ad5empty). Both vectors were obtained from the University of Iowa Viral Vector Core, Iowa City, IA, USA. Newborn (P2-P4) B6 and RAG mice received a subcutaneous injection of Ad5myoc (3 × 10^9 PFU) to induce tolerance to the vector and prevent ocular inflammation. When mice reached the age of eight weeks, they were sedated using xylazine and ketamine (12.5 mg/mL and 87.5 mg/kg, respectively) and 9 × 10^9 PFU virus in 3 mL PBS were delivered to the anterior chamber of the left eye (OS) by transcorneal injection.

For IOP measurements mice were anesthetized with 2.5% isoflurane and a rebound tonometer (Tonolab, Colonial Medical Supply, Windham, NH, USA) was used as described earlier.26 IOP measurements were taken between 9 AM and 12 PM by an investigator blinded to the animals’ status. Animals that failed to develop IOP elevation of at least 7 mm Hg over baseline for a period of at least eight weeks were excluded from analysis.

**Determination of Retinal Ganglion Cell Density and Immunohistochemical Detection of T Cells**

Mice were sacrificed and immediately enucleated, and eyes were fixed for two hours in 4% paraformaldehyde. The retina was carefully dissected and incubated in PBS containing 0.3% Triton-X100 for six hours. After three freeze and thaw cycles, retinas were blocked for one hour at room temperature using 1% BSA/0.3% Triton-X100. Retinas were incubated with goat-anti Brn3a and Armenian hamster–anti-T-cell receptor primary antibodies (C20, Santa Cruz, TX, USA, and ab25336, Abcam, Cambridge, UK; diluted 1:200 in 1%BSA/0.3% Triton-X100/1% DMSO/PBS) at 4°C for two days on a rocker platform. After washes in PBS, binding was visualized following incubation in a donkey anti-goat Alexa Fluor 546 and rabbit anti-Armenian hamster FITC secondary antibody solution (both Invitrogen, Carlsbad, CA, USA, and Thermo, diluted 1:200 in PBS) for three hours at room temperature in the dark. Retinas were extensively washed in PBS and then mounted with Vectashield (Vector Laboratories, Burlingame, CA). Twenty-four images using a lens at magnification ×20 were taken at standardized locations of the central, mid-peripheral, and peripheral retina using a Zeiss Observer Z1 microscope. Each image represents 0.144 mm²; thus the total area examines equals 3.46 mm² or approximately 25% of the mouse retina. Brn3a positive cells were counted semiautomatically using ImageJ software.

**Flow Cytometry**

Mice were euthanized by CO₂ inhalation. Cardiac perfusion was done with 10 to 12 mL PBS containing heparin (2 U/mL). Retinas were removed by dissection from enucleated eyes and prepared for FACS analysis as previously described.27-30 Individual retinas were suspended in 0.5 mg/mL Liberase/TM (Roche, Indianapolis, IN, USA) and 0.01% to 0.05% DNase in DPBS and gently homogenized by trituration. Fluorescent-labeled antibodies (Thermo Fisher Scientific, Waltham, MA, USA) and viability dye (eFluor 780 Fixable Viability Dye, Thermo Fisher Scientific) were added to retina cell suspensions and incubated on ice for 30 minutes. Fluorescent proteins and other fluorophores were compensated using fluorescence minus one controls. Analysis of the flow cytometry data was done using FlowJo (Becton Dickinson, Ashland, OR, USA) software. Data collected from a single retina was treated as a single sample. Individual optic nerves, 4 to 5 mm in length, were similarly prepared and each was analyzed as a single sample. Our strategy for flow counting retina and optic nerve samples was based on populations gated for cell viability, doublet rejection and FSC/SSC scatter. See Supplemental Figure S2 for a representative example of the flow cytometric analysis of a single retina. Myeloid cells were then assessed by gating on CD45<sup>−/−</sup>CD11b<sup>−/−</sup>Ly6G<sup>−/−</sup> cells. Lymphocytes were distinguished by gating on CD45<sup>−/−</sup> CD11b<sup>−/−</sup> cells.

**Statistical Treatment of Data**

All IOP and RGC data are given as mean ± standard deviation (SD) and were tested for Gaussian distribution before statistical analysis. To test for pairwise significance between two groups, Student’s t-test was used and for statistics including more than two groups, P values are calculated using
Reduced RGC Loss in Glaucomatous RAG1−/− Mice

Figure 1. IOP (mm Hg) in mice following injection of Ad5myoc into the anterior chamber of the left eye (OS). (A) Elevated IOP was only observed in injected eyes. (B) Summation of IOP over the experimental period was similar in injected eyes between the two groups of mice (area under the curve: AUC).

ANOVA followed by Tukey's post hoc tests. All calculations were performed using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA) and P values < 0.05 were considered statistically significant.

Results

We previously described that transgenic mice expressing human myocilin with the pathogenic Y437H mutation develop elevated IOP and subsequent RGC loss.23 Here, MyocY437H was expressed after transfection of the anterior chamber and trabecular meshwork with an adenoviral vector, as previously described.24 Prior to experimentation we induced immunological tolerance to the adenoviral particle in experimental mice, which radically reduces the number of eyes experiencing inflammation. Using this approach, a single injection of 9 × 10⁷ PFU Ad5myoc results in an IOP increase of at least 7 mmHg for a period of at least 8 weeks in over 90% of injected eyes.

Ad5myoc particles were delivered to the anterior chamber of the left eyes (OS) of normal B6 mice, as well as Rag1−/− mice (n = 20/group) by transcorneal injection. Rag1−/− mice do not possess functional T- or B-lymphocytes but are otherwise healthy31,32 and display the same RGC density as immune-sufficient B6 mice (see Additional File 2). Injection of Ad5myoc results in an increase in IOP in the injected eyes, which was maintained for the 16-week duration of this study (Fig. 1A). No change in IOP was observed in the untreated contralateral eye (OD) and no differences in the IOP response of B6 and Rag1−/− mice were noted (Fig. 1B). In addition, a control group of B6 control mice received injections of adenoviral vectors lacking the myocilin construct ("empty" vectors), which did not alter IOP in treated eyes.

Sixteen weeks after Ad5myoc injection mice were sacrificed and the RGC density of the central, midperipheral, and peripheral retina was determined after Brn3a labeling in all groups (Fig. 2, Table 1). As expected, chronic elevation of IOP in the left eye (OS) results in a significant decrease of RGC density in all three retinal areas when compared with eyes from control animals (central: 3233 vs. 4150, P = 1.6 × 10⁻⁵; midperipheral: 2878 vs. 3706, P = 5.3 × 10⁻⁶; and

Table 1. RGC Density in Mouse Eyes With Unilaterally Elevated IOP (OS) and in Contralateral Fellow Eyes (OD)

<table>
<thead>
<tr>
<th>Control</th>
<th>B6</th>
<th>RAG</th>
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<tr>
<td>Periphery</td>
<td>OU</td>
<td>OS</td>
</tr>
<tr>
<td></td>
<td>2005 ± 256</td>
<td>1618 ± 234</td>
</tr>
<tr>
<td>Mid-Periph.</td>
<td>3706 ± 360</td>
<td>2878 ± 363</td>
</tr>
<tr>
<td>Central</td>
<td>4150 ± 377</td>
<td>3233 ± 509</td>
</tr>
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</table>

Values were obtained in normal B6 mice as well as Rag1−/− animals and compared to those observed in either eye (OU) of control mice having received Ad5empty.
Reduced RGC Loss in Glaucomatous RAG1−/− Mice

FIGURE 3. RGC density in contralateral eyes in control mice (hashed bars), B6 (white bars), and RAG1−/− (gray bars). Each dot represents one eye. Values are obtained in the retinal periphery, midperiphery (Mid) and centrally (Central). *: \( P < 0.05 \), **\( P < 0.01 \), ns = not significant, \( n = 15 \) group.

In contrast, eyes of Rag1−/− mice experience only a small decline of RGC density in eyes with chronically elevated IOP when compared to control eyes (central: 3700 vs. 4150, \( P = 0.012 \); midperipheral: 3273 vs. 3706, \( P = 0.011 \); and peripheral: 1880 vs. 2005, \( P = 0.277 \)). These decreases correspond to 10.8%, 11.7%, and 6.2% RGC loss, respectively. Furthermore, the RGC densities in the eyes of Rag1−/− mice after 16 weeks of elevated IOP are significantly higher than those observed in immune sufficient B6 mice that experienced a similar IOP elevation (central: \( P = 0.043 \), midperipheral: \( P = 0.028 \), and peripheral: \( P = 0.032 \)).

We then quantified the RGC density in the contralateral eyes (OD), which did not receive injections of Ad5myoc and did not develop elevated IOP (Fig. 3). Our findings indicate that even in the absence of elevated IOP, significant RGC losses occur in the central and midperipheral retinal areas of B6 mice when compared with controls (central: 4150 vs. 3742, \( P = 0.008 \); midperipheral: 3706 vs. 3166, \( P = 3.9 \times 10^{-4} \); peripheral: 2005 vs. 1946, \( P = 0.609 \)). These values correspond to a loss of 9.6%, 14.6%, and 2.9% of RGC in these regions. Again, RGC loss was much less pronounced in Rag1−/− mice and a statistically significant reduction between Rag1−/− and control mice could only be demonstrated in the midperiphery (Central: 4060 vs. 4700, \( P = 0.53 \); midperiphery: 3473 vs. 3706, \( P = 0.09 \); periphery: 2005 vs. 2005, \( P = 0.88 \)). These numbers correspond to 2.2% and 6.3% losses in the central and mid-peripheral regions of Rag1−/− contralateral eyes, whereas RGC density in the periphery remains unchanged (+0.6%).

Takentogether,ourfindingsdemonstratethattheamount and duration of IOP stress used in this experiment, induces on average 20.7% loss of RGC in B6 mice. In contrast, Rag1−/− mice lose only 9.6% of RGC, suggesting that T- or B-cell-mediated mechanisms contribute to glaucomatous damage. Consistent with this notion we also observed a 10.1% decline in RGC density in the normotensive contralateral eye of B6 mice, whereas no significant RGC loss (3.1%) was apparent in the immune deficient animals.

We then sought to examine whether T-cells can be detected in the retina. For this purpose, eyes of small group of mice (\( n = 4 \)) with unilateral Ad5Myoc induced IOP elevation were harvested after eight weeks of ocular hypertension. Immunohistochemical examination of whole-mounted retinas indicates that a small number of extravasated cells immunopositive for the T-cell receptor (TCR) are present in all eyes of B6 mice (Fig. 4). These cells are not evenly distributed but rather tend to occur in clusters throughout the retina. We observed TCR-positive cells in the inner retinal cell layers of B6 mouse eyes both with and without elevated IOP.

To quantitatively determine the number of T-cells in glaucomatous eyes we used flow cytometry on preparations of dissociated retina and optic nerve. Toward this end, glaucomatous (\( n = 8 \)) and age matched naïve mice (\( n = 10 \)) were sacrificed and perfused with PBS containing heparin which effectively removes residual blood from the ocular vasculature. Retinas and optic nerves were then harvested and enzymatically dissociated. Data obtained confirm our observation that a small number of T-cells is invariably present in the retina of both control and glaucomatous mice (Table 2). However, eyes with elevated IOP contain significantly more CD3+ cells (69.5% increase, \( P = 0.026 \)). This increase appears to be largely due to higher numbers of CD4+ Helper T-cells (95% increase, \( P = 0.0023 \)). An increase
in the number of cytotoxic T-lymphocytes (CD3+, CD4−) was also noted, but statistical significance was not reached.

The analysis of the optic nerves of the same animals revealed the presence of only a very small number of CD45(+/−), CD3+ cells in the tissue. No statistically significant differences were observed when glaucomatous optic nerves to naïve controls are compared. In summary, our findings suggest that elevated IOP leads to a higher degree of T-cell extravasation into the retina and that lymphocyte mediated RGC loss significantly contributes to damage in mouse models of glaucoma.

**DISCUSSION**

Increased IOP is often observed in patients with glaucoma and in rodent models chronic, moderate elevation of IOP almost invariably leads to RGC loss and optic nerve damage. Yet it has become increasingly clear that glaucoma is a multifactorial, complex eye disease and that IOP is only one of the factors influencing the development of vision loss.34 We previously demonstrated that adoptive transfer of lymphocytes from glaucomatous mice to healthy animals results in progressive RGC loss in recipients, indicating that elevated IOP results in the development of an immune response targeting RGC or perhaps a loss of tolerance.22 Here we examined whether immune reactions also contribute to the rate of RGC degeneration in eyes with elevated IOP by comparing RGC loss in normal, immune competent mice to that observed in Rag1 knockout mice that lack functional T and B cells.31

Our data demonstrate that lymphocyte dependent processes strongly contribute to RGC loss in glaucoma that is evident in the presence of continuously elevated IOP. Rag1−/− mice retain significantly higher numbers of surviving RGC when compared to immune competent control mice after 16 weeks of elevated IOP. However, Rag1−/− retinas are not fully protected from RGC loss. This is not surprising and further underscores the multifactorial pathogenesis of glaucoma involving glial activation, metabolic stress, neuroinflammatory cytokine release, autophagy and other processes expected to occur in all glaucomatous retinas, including those of Rag1−/− mice.35,36

A second indication for immune-mediated processes in glaucoma is our finding that in this unilateral mouse model, fellow eyes of immune competent mice experience significant RGC loss even in the absence of elevated IOP, while RGC decline in fellow eyes of immunodeficient Rag1−/− mice is minimal. An effect on the fellow eye is consistent with data presented in a number of previous publications that have demonstrated neuroinflammatory changes in the second eye of animal models of glaucoma. These studies described activation of retinal microglia and macroglia, as well as enhanced MHC II reactivity in contralateral eyes.9,40,41 However, significant loss of RGC was not described by the investigators. One difference between these and our study is that here exposure to elevated IOP was maintained for much longer than in the earlier studies. We hypothesize that immune-mediated glaucomatous damage is a relatively slow process and consequently it is possible that earlier studies evaluated the retinas before noticeable RGC loss occurred. This sympathetic effect on the fellow eye is almost completely abolished in Rag1−/− animals, again indicating the activation of an immune response that not only damages eyes with elevated IOP but also the unperturbed contralateral eye. It must be cautioned that our data do not demonstrate direct lymphocytic cytotoxicity directed against RGC. It is conceivable that the development of an immune response serves to heighten glial hyperreactivity in the eye, leading to the observed RGC degeneration. Furthermore the absence Rag1 in zebrafish has been reported to lead to increased transcription of complement and coagulation genes,32 which could potentially account for some of the differences observed here.

Our data can also be used to estimate the effect size of the immune response on RGC survival. In immunocompetent eyes with elevated IOP 20.9% of all RGC are lost, whereas only 10.2% are lost in Rag1−/−, indicating that 10.7%, or approximately half, of all RGC are damaged by immune processes. Interestingly, a nearly equivalent amount of RGC loss (9.1%) was observed in the fellow eyes of B6 mice, but not Rag1−/−. These data suggest that immune mediated damage is the primary cause of RGC loss in these normotensive contralateral eyes.

Rag1−/− mice lack both T and B cells, and this study was not designed to resolve which of these cell types causes RGC damage in glaucoma. However, in our adoptive transfer studies transfer of T cells resulted in a much stronger response than transfer of B-cells, implicating a functional role for T rather than B cells. Similar findings have also been presented by others.16 A limited role for B lymphocytes is further supported by more recent studies demonstrating that use of the B lymphocyte inhibitor Belimumab does not provide RGC protection in a rodent model of glaucoma.11 This suggests that T-cell-mediated damage processes are likely involved in the pathogenesis of glaucoma and that the role of B cells is limited. Finally, a role of T cells in glaucomatous RGC loss is congruent with our earlier studies demonstrating that CD8 T cells are capable of degrading retinal neurons while causing minimal inflammation in the tissue.35,36

Many mechanistic aspects of these observations remain unresolved, including the site of the immune response. Chen et al.18 reported an increase in CD4+ T-cells infiltrating the retina of eyes with elevated IOP indicating that retinal epitopes are targeted. We also observed higher numbers of T-cells in eyes with glaucoma, although the overall numbers of cells are somewhat lower than those by Chen et al.16 In contrast, we identified only very few T cells in the optic nerve of mice with or without elevated IOP. This relatively small number of extravasated T cells in the retina is consistent with clinical findings indicating that glaucoma is not associated with significant infiltration of leukocytes into the retina. Furthermore, low-level but persistent immune activity

<table>
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<tr>
<th>TABLE 2. Number of T Cells</th>
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<tr>
<td>Retina</td>
</tr>
<tr>
<td>Glaucoma</td>
</tr>
<tr>
<td>Control</td>
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<td>Increase</td>
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<td>P value</td>
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<tr>
<td>Optic Nerve</td>
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<tr>
<td>Glaucoma</td>
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<tr>
<td>Control</td>
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<tr>
<td>Increase</td>
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<td>P value</td>
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Number of T-cells detected in the retina and optic nerve of glaucomatous (n = 8) and naïve (n = 10) mice.
agrees with the slow progression of RGC loss which occurs over many weeks or months. It is intriguing to speculate whether a similar effect also occurs in human glaucoma. Unilateral glaucoma after ocular trauma is perhaps the most unambiguous condition allowing an estimation of the effects on the fellow eye. In a patient cohort with traumatic angle cleavage evaluated by Tesluk and Spaeth, over half (55%) of patients developed either frank glaucomatous or suspicious visual field abnormalities in the fellow eye after several years. A more recent study of unilateral POAG, exfoliation, or pigmentary glaucoma found visual field loss in 21% of the first-affected eyes, and in approximately one third of these patients (6.2% of all eyes) vision loss was also noted in fellow eyes. Finally data from the Collaborative Initial Glaucoma Treatment Study demonstrate that 52.1% of fellow eyes were not treated at the beginning of the study, but 20.3% eventually required treatment, indicative of advancing disease. The effect on the fellow eye also appeared to be particularly pronounced in patients with rapid disease progression in the first eye. The factors leading to vision loss in the fellow eyes were not addressed in these clinical studies and could be explained by a number of biological processes. However, many glaucoma patients display T-cell responses to heat shock proteins that are highly expressed by stressed neurons, including RGC. Activated T cells may then induce RGC apoptosis along the inflammatory Fas/Fas ligand pathway. Indeed, overexpression of an inactive decoy form of Fas ligand antagonistically reduces Fas activation and prevents RGC loss in mouse eyes with elevated IOP.

Taken together, our findings suggest that glaucomatous damage to RGC is in part due to the development of a cellular immune response that, once established, can contribute to RGC degeneration in an IOP-independent fashion. Our data agree with other recent findings emerging from preclinical models and suggest that immune responses may contribute to visual loss in both bilateral and unilateral glaucoma in patients with inadequate immunoregulation.

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