Ocular inflammation in age-related eye diseases

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CD4+ T CELL RESPONSES MEDIATE PROGRESSIVE NEURODEGENERATION IN EXPERIMENTAL ISCHEMIC RETINOPATHY

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ABSTRACT

Retinal ischemic events as a result of occlusion of the ocular vasculature share similar etiologies of central nervous system (CNS) stroke and are among the most common cause of acute and irreversible vision loss in elderly patients. Currently, there is no established treatment, and the condition often leaves patients with seriously impaired vision or blindness. The immune system, particularly T cell-mediated responses, is known to be intricately involved, but its exact roles remain elusive. Here we showed that acute ischemia/reperfusion injury to the retina induced a prolonged phase of retinal ganglion cell (RGC) loss that continued to progress over 8 weeks post procedure. This was accompanied by microglial activation and T cell infiltration into the retina. Adoptive transfer of T cells isolated from diseased mice exacerbated RGC loss in mice with retinal reperfusion damage. Whereas, T cell deficiency or administration of T cell or interferon-γ neutralizing antibody attenuated RGC degeneration and retinal function loss after injury. These findings demonstrate a crucial role for T cell-mediated responses in the pathogenesis of neural ischemia. They point to novel therapeutic strategies of limiting or preventing neuron and function loss for currently untreatable conditions of optic neuropathy and/or CNS ischemic stroke.

INTRODUCTION

Ischemia, broadly defined as the loss of blood supply to tissues, leads to energy depletion and cell death. It is one of the key contributing factors to the pathophysiology of a variety of brain and retinal diseases, such as stroke[1], acute coronary syndrome[2], diabetic retinopathy, and central retinal artery occlusion[3, 4]. Acute retinal ischemia/reperfusion injury, which results in permanent loss of retinal ganglion cells (RGCs), is a common cause of severe impairment of vision and blindness in middle-aged and elderly patients.[5] Recent guidelines of the American Heart Association and American Stroke Association, as well as American Academy of Ophthalmology, have recognized acute retinal ischemia as a stroke equivalent and recommend urgent etiologic work-up, including brain imaging.[6, 7] However, no effective treatment is currently available for acute retinal ischemia, and the optimal management remains unknown because the underlying causes of neuron loss have not been fully understood.

Recent investigations reveal that the eye, while has been known for a long time as an immune-privileged site[8, 9], elicits immunological responses under pathophysiological stress. It was reported that ischemia-reperfusion injury results in sequestration of immune cells, including T cells and macrophages[10], and inflammatory mediators to the ischemic region, which in turn induces local inflammatory responses.[4, 11, 12] A previous study showed that $CD4^+$ T helper cells participate in ischemic neurodegeneration and that severe combined immune-deficient (SCID) mice lacking T and B lymphocytes developed less RGC death after retinal ischemic injury than wild-type (WT) mice.[13] In line with these observations, we showed recently that pathological stress such as that induced by elevated intraocular pressure (IOP) in glaucoma is sufficient to trigger $CD4^+$ T cell infiltration into the retina.[14] Heat shock proteins (HSP) were identified as pathogenic antigens of these T cells. Importantly, these T cells attacked RGCs by recognizing the surface HSPs that were induced following IOP elevation and contributed critically to the development of a prolonged phase of RGC and axon loss in glaucoma.[14] These findings suggest a critical involvement of adaptive immune responses in perpetuating neural damage following neural stress or injury.[15, 16]

As ischemic insult is reported to recruit T cells into the retina and upregulate HSPs in RGCs, we hypothesized that $CD4^+$ T cell-mediated responses also play an important role

in perpetuating retinal neurodegeneration in ischemic/reperfusion injury. In the present study, we sought to test this hypothesis by employing T cell deficient mice and adoptive T cell transfer and assessing T cell responses. Our study provided compelling evidence indicating that an acute ischemic event in the retina induced IFN-γ-secreting $CD4^+$ T helper cell infiltration and a prolonged phase of neurodegeneration over 8 weeks while administration of T cell blocking antibodies attenuated RGC and retinal function loss in an experimental model of retinal reperfusion injury. Our results suggest the existence of a therapeutic window and novel strategies for saving vision in retinal ischemia. Likely, a similar mechanism may be involved in ischemic stroke of the CNS.

METHODS

Mice

C57BL/6J wild-type (B6) mice and mice deficient for Rag1 ($Rag1^{-/-}$) or T cell receptor ($TCR^{-/-}$) between 12-16 weeks old were purchased from Jackson Laboratories, Bar Harbor, Maine. Animals were housed under a 12 h light/dark cycle and kept under pathogen-free conditions. All experimental procedures and the use of animals were approved and monitored by the Animal Care Committee of the Schepens Eye Research Institute/Massachusetts Eye and Ear, and performed according to the standards of the National Institute of Health and the Association for Research in Vision and Ophthalmology.

Acute retinal reperfusion injury

Retinal ischemia was induced in B6, $Rag1^{-/-}$, and $TCR^{-/-}$ mice as previously described.[17, 18] Mice were anesthetized with a mixture of 120 mg/kg Ketamine and 20 mg/kg Xylazine in sterile saline (1:1:6). Retinal ischemia was induced unilaterally in the right eye, while the contralateral eye served as a non-ischemic control. The pupil was dilated with 1% tropicamide (Bausch & Lomb Inc., Tampa, FL), and 0.5% Proparacaine Hydrochloride (Bausch & Lomb Inc., Tampa, FL) was applied topically onto the cornea. The cornea was gently punctured near the center using a 30.5-gauge needle to generate an easy entry for a glass micropipette, which was connected by polyethylene tubing and an intravenous tube set (Abbott Laboratories, North Chicago, IL) to a sterile physiological (0.9% sodium chloride) bag (Hospira, Inc., Lake Forest, IL). By elevating the saline bag up to 120 cm above the eye level, the intraocular pressure (IOP) was raised acutely to 90 mmHg. Whitening of the

fundus was observed to ensure the induction of retinal ischemia, followed by observation of corneal edema. After 60 minutes of a highly-elevated IOP, the saline bag was slowly lowered to the eye level, and the needle was withdrawn from the anterior chamber. Reappearance of vessels in the fundus was confirmed as a sign of reperfusion of the retina. In sham-operated mice, the right cornea was punctured near the center to generate an entry for the glass micropipette, but the saline bag was not raised above eye level so that no IOP elevation was generated in these mice. Mice were sacrificed at day 3, week 1, 2, 4, or 8 after injury.

Adoptive transfer of CD4⁺ T cells

Mouse spleens were dissected and mechanically homogenized, and cells were suspended in RPMI media (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine. Red blood cells (RBC) were lysed with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). $CD4^+$ T cells were purified using an automated MACS Separator and a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Briefly, $CD4^+$ T cells were negatively selected from splenocytes of B6 mice with induced retinal ischemia or sham-operated mice at 2 weeks post procedure by depletion with a mixture of lineage-specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119, and antibiotin-conjugated microbeads. The procedure yielded purity of over 90% $CD4^+$ T cells, as assessed by flow cytometry. The donor cells (2×10^8 cells in a volume of 200 µL sterile saline) were adoptively transferred into recipient $Rag1^{-/-}$ mice 2 weeks after the induction of retinal ischemia via tail vein injection. Same numbers of $CD4^+$ T cells isolated from sham-operated mice were injected to the control group of recipient $Rag1^{-/-}$ mice 2 weeks after the induction of retinal ischemia. All recipient mice were sacrificed 2 weeks after adoptive T cell transfer and quantified for RGC loss.

Immunohistochemistry and cell counts

As previously described,[19] mouse eyeballs were dissected and fixed in 4% paraformaldehyde (PFA) overnight, transferred to 20% sucrose for 2 h before embedded in Tissue-Tek (Sakura Finetek Inc., Torrance, CA). Transverse retinal sections (10 µm) or retinal flat-mounts were stained with a primary antibody against CD11b (Invitrogen) or CD4 (clone GK1.5, Abcam, Cambridge), followed by reaction with an Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Inc, West Grove, PA), and counterstained with the

nuclear marker 4', 6-diamidino-2-phenylindole (DAPI, Vector laboratories, Burlingame, CA). The numbers of $CD11b^+$ cells and $CD4^+$ T cells were counted under direct fluorescence microscopy (Olympus IX51). RGC loss was assessed quantitatively in retinal flat-mounts using a standard protocol as previously described by our laboratory[20] with minor modifications. In brief, eyeballs were fixed in 4% PFA for 3 hours at room temperature. Retinal flat-mounts were incubated with a primary antibody against an RGC specific-marker, β-III-tubulin[21, 22] (Tuj1; MAB5564, Millipore, Darmstadt, Germany), followed by a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Inc, West Grove, PA). Retinal flat-mounts were divided into quadrants: superior, temporal, nasal and inferior. Using the optic nerve head as the origin, four standard regions were selected from each quadrant: one peripheral, two intermediate, and one central (Fig. 1A). In total, 16 rectangular regions (each 193 mm x 193 mm) of each retinal flat-mount were photographed at 400x magnification with a confocal microscope (Leica TCS-SP5). The degree of RGC loss was assessed as previously described[20]. RGC densities were calculated, and the percentage of RGC loss was determined by dividing the RGC density from the retina with ischemic injury by that of the contralateral control retina of the same mouse. All quantification procedures were conducted by 2 investigators under a masked fashion.

RT-PCR to detect cytokine expression in ischemic retinas

Total RNA was extracted from mouse retina using RNAeasy Plus Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from total RNA using Supercript III First Strand Kit (Invitrogen, Carlsbad, CA). Reaction mixture of RT-PCR contained cDNA, 2x Master Mix from KAPA SYBR Fast qPCR kit and 10 mM of specific primers. Quantitative detection of specific mRNA transcript was carried out by RT-PCR using the Mastercycler ep realplex real-time PCR system (Eppendorf, Westbury, NY). The sequences of all primers are listed in Table 1. Relative amount of specific mRNA transcript was presented in fold changes by normalization to the expression level of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1. List of primer sequences used in real-time PCR

Gene	Foward	Backward
IFN-γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-17	TCAGCGTGTCCAAACACTGAG	TCTCGACCCTGAAAGTGAAGG
TGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Intravitreal administration of antibodies

Intravitreal injection procedure was as previously described.[23] Mice received intravitreal injections of antibodies on days 3, 7, 10, and 14 after induction of acute retinal ischemia. Control mice received intravitreal injections of sterile saline or isotype IgG. To control the small volume (2 µL) of intravitreal injection, a glass micropipette was connected to a Hamilton syringe. The right eye was gently punctured posterior to the limbus using a 30.5-gauge needle to generate access for the glass micropipette. Using this entry wound, 2 µL of Ultra-LEAF (Low Endotoxin, Azide-Free) purified anti-mouse CD4 (IgG2b, clone GK1.5, Biolegend, San Diego, CA), Ultra-LEAF purified anti-mouse interferon (IFN-γ) (IgG1, clone XMG1.2, Biolegend, San Diego, CA), Isotype IgG (Biolegend, San Diego, CA), or sterile saline was given intravitreally using a glass micropipette. Reports have shown that Ultra-LEAF anti-mouse CD4 blocked CD4-mediated cell adhesion and $CD4^+$ T cell activation, causing ***in vivo*** depletion of $CD4^+$ T cells.[24-29] Ultra-LEAF anti-mouse IFN-γ neutralizes the bioactivity of natural or recombinant IFN-γ.[30, 31]

Flow cytometry analysis for identifying $CD4^+$ T cell subsets

To define the subsets of T cells involved in the pathological process following acute retinal ischemia, we analyzed cytokine expression by T cells in the eye draining (superior cervical) lymph nodes (LNs). Superior cervical LNs were dissected, and cells were mechanically dissociated using two forceps. Cell aggregates were separated by filtration through a 70 µm nylon cell strainer (BD Falcon, San Jose, CA). For analyzing the frequencies of $CD4^+$ T cells that expressed IFN-γ (T_H1), IL-17 (T_H17), IL-4 (T_H2), or TGF-β (Treg), isolated lymphocytes were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) and ionomycine (Sigma-Aldrich, St. Louis, MO) in the presence of monensin (Biolegend, San Diego, CA). Cells were washed in IsoFlow (Beckman Coulter Inc, Brea, CA) and stained with surface FITC-conjugated anti-mouse CD4 antibody (IgG2b, clone GK1.5, Biolegend, San Diego, CA). Thereafter, cells were permeabilized with Perm/Wash buffer (BD biosciences, Frankin Lakes, NJ), and stained with PE-labeled anti-mouse IFN-γ antibody (IgG1, clone XMG1.2, Biolegend, San Diego, CA), PE-labeled anti-mouse IL-4 antibody (IgG1, clone 11B11, Biolegend), PE-labeled anti-mouse TH17A (IgG1, clone TC11-18H10.1, Biolegend), or PE-labeled anti-mouse TGF-β1 antibody (IgG1, clone TW7-20B9), detecting T_H1, T_H2, T_H17, and Treg cells, respectively. The antibody-stained cells were analyzed with BD LSR II Flow Cytometer (BD Biosciences), and data were analyzed using Summit Software v4.3 (Beckman Coulter Inc, Brea, CA).

Electroretinography

Animals were dark adapted for 5 hours prior to electroretinogram (ERG) recordings. All procedures were performed in a dark room under the dim red safety light. Mice were anesthetized with 120 mg/kg Ketamine and 20 mg/kg Xylazine, and the pupils were dilated with 1% tropicamide. Mice were placed in the sternal-abdominal position within the Ganzfield bowl. During recording, mouse body temperature was maintained at 37°C using a heating pad to prevent hypothermia. Recording gold lens electrodes were placed on both corneas; the reference and ground electrodes were placed subcutaneously in the mid-frontal head area and caudal area near the tail, respectively. Light stimulations were delivered with a Xenon lamp at 0.0002, 0.02, 2, 200, 600 cd·s/m^2 for dark-adapted tests. Thereafter, animals were subjected to 7-minute light adaptation with a light intensity of 50 cd·s/m^2 before initiating the light-adapted tests. The light-adapted tests were conducted by Xenon light at 600 cd·s/m^2, green light at 13 cd·s/m^2, and blue light at 1 cd·s/m^2 sequentially. Flickr test were executed with 6,500 K white light at 15 cd·s/m^2 and 3 different frequencies of 3, 10 and 15 Hz. Data were processed by the software included in the ERG recorder (Espion Electroretinography System; Diagnosys LLC, Lowell, MA). ERG a-waves were measured from the baseline to the cornea-negative peak, and b-waves from the cornea-negative peak to the major cornea-positive peak.

Statistical analysis

All statistical analyses were performed using GrapPad Prism for Windows, version 5.0 (GraphPad Software Inc, La Jolla, CA). The performed tests were two-sided and a value of $P < 0.05$ was considered as statistically significant. At least six animals were used for each experimental or control group. For the comparison between two groups, the Mann-Whitney test was performed, and for three or more groups the non-parametric Kruskal-Wallis test was employed.

RESULTS

Acute retinal ischemia induces a prolonged phase of progressive neurodegeneration

To investigate if acute ischemic injury in the retina induces a prolonged phase of neurodegeneration, transient retinal reperfusion injury was induced in adult B6 mice by

raising IOP to 90 mmHg for 60 minutes. RGC loss was quantified at day 3, and weeks 1, 4, and 8 after ischemic injury or at 4 weeks post procedure in sham-operated mice, using Tuj1 labeling in retinal flat-mounts as previously described (Fig. 1B)[20]. As expected, no significant difference of RGC counts was noted in uninjured contralateral eyes at all time points post procedure or in sham-operated control eyes (Supplementary Fig. 1). In contrast, starting from 3 days post injury, retinas subjected to reperfusion injury displayed progressive RGC loss (Fig. 1C, D and Supplementary Fig. 1). Significant less RGCs in the ischemic retinas (3,250 ± 87 cells/mm^2) was counted than that in sham-operated retina (3,831 ± 78 Cells/mm^2) at as early as 3 days post-procedure (Fig. 1C,D). Although the ischemic injury lasted for only 60 minutes, in the absence of any sustained injury progressive RGC loss continued to occur and 1,737 ± 94 cells/mm^2 were counted by 8 weeks post injury. This was equivalent to a 17.2% ± 1.6% RGC loss at day 3 to 54.8% ± 2.6% RGC loss by 8 weeks post-ischemic injury (Fig. 1D). Whereas, RGC counts in retinas contralateral to the injury remained constant through the period and were comparable to sham-operated controls (Supplementary Fig. 1). Thus, acute retinal ischemia triggers a prolonged phase of progressive RGC degeneration in the absence of a sustained insult.

T cells infiltrate into the retina after ischemic injury

Local inflammation represented by microglia/macrophage activation is a common event occurring after retinal injury. We thus performed CD11b immunolabeling to detect activated microglia/macrophage in retinal sections. At as early as 3 days post injury, significantly increased numbers of CD11b+ microglia/macrophages were noted in the ischemic retina compared to sham-operated controls (Fig. 2). Moreover activated microglia showed shortened dendritic processes and enlarged round cell bodies (Fig. 2A). Next, we asked if the adaptive immune system or T cells participate in ischemia-induced responses in the retina by double-immunostaining of CD4$^+$ T cell and RGC marker, anti-CD4 and β-III-tubulin (Tuj1) (Fig. 3A). While no T cells were detected in the uninjured contralateral retinas throughout the period (data not shown), infiltrated CD4$^+$ T cells were found in a close proximity of RGCs in the retinas subjected to reperfusion injury. The number of T cells counted in the flat-mounted retinas of sham-operated mice was minimal when examined at 2 weeks post operation (0.2 ± 0.2 cells/retina); whereas, a significant influx of T cells into the ischemic retina was detected from 1 to 4 weeks, reaching the peak at 2 weeks, after acute reperfusion injury (Fig. 3B). To exploit the subsets of T cells that infiltrated the ischemic retina, we assessed with qPCR the levels of cytokines, which

are hallmarks of different subsets of T_H cells: IFN-γ (T_H1), IL-4 (T_H2), IL-17 (T_H17), and TGF-β (T_{reg}),[14] in the retinas subjected to ischemic injury. Significant increases of T_H1 cytokine marker IFN-γ, but not other cytokines (TGF-β, IL-17, and IL-4), were detected in the ischemic retina at 2 and 4 weeks post injury (Fig. 3C). The subsets of infiltrated T cells were further verified by flow cytometry. The data support induction of local inflammation and CD4+ T_H1 cell infiltration following retinal ischemic/reperfusion injury.

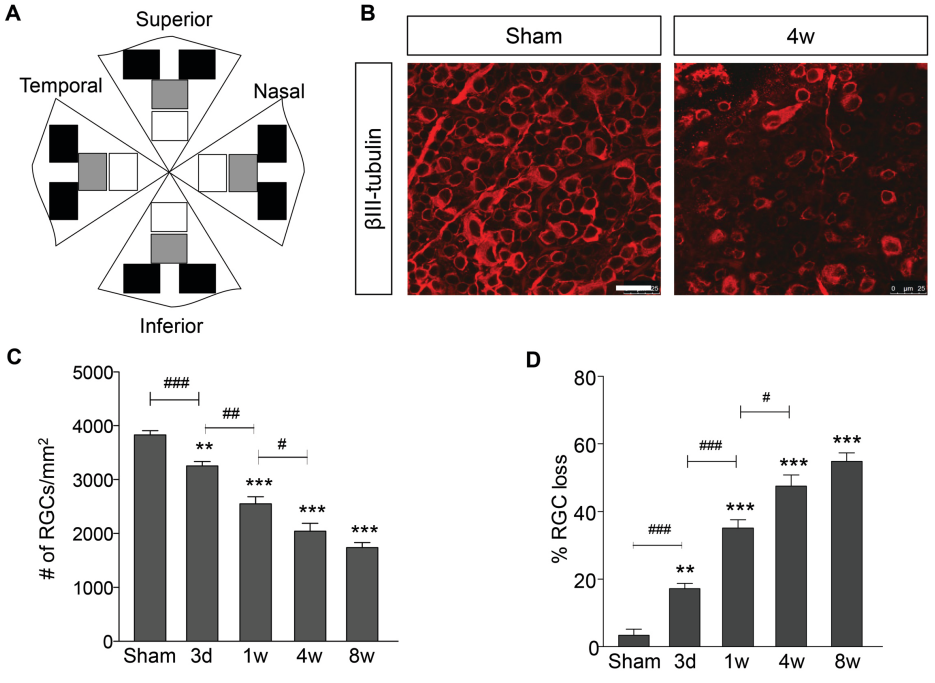
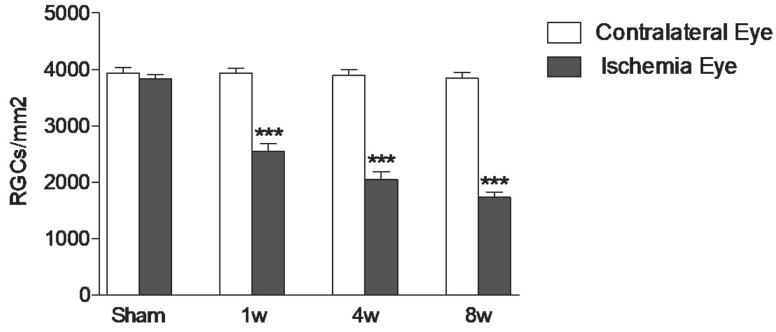


Figure 1. Acute retinal ischemia induces progressive neurodegeneration. A. Schematic illustration of retina sampling for RGC counts in a retinal flat-mount. **B.** Representative epifluorescent photomicrographs of β-III-tubulin (red) immunolabeled retinal flat-mounts taken from a sham-operated B6 mouse (Sham) and a mouse at 4 weeks after retinal ischemic injury (4w). Scale bar: 25 μm. **C.** Quantification of RGC densities in retinal flat-mounts at various time points after ischemic injury or at 4 weeks after sham operation (n = 6/group). **D.** Percentage of RGC loss over that of the uninjured contralateral eye in mice at various time points after retinal ischemia or sham operation. Value = mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the sham group or $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ as indicated, by one-way ANOVA.



Supplementary Figure 1. Acute retinal ischemia induces progressive neurodegeneration in the ischemic eye. Quantification of RGC densities in retinal flat-mounts taken from the eyes ipsilateral and contralateral to the injury at 1w, 4w, and 8w – post procedure or from sham-operated mice (n = 6/group). Value = mean ± S.E.M. ***P* < 0.01, ****P* < 0.001 compared to the sham group.

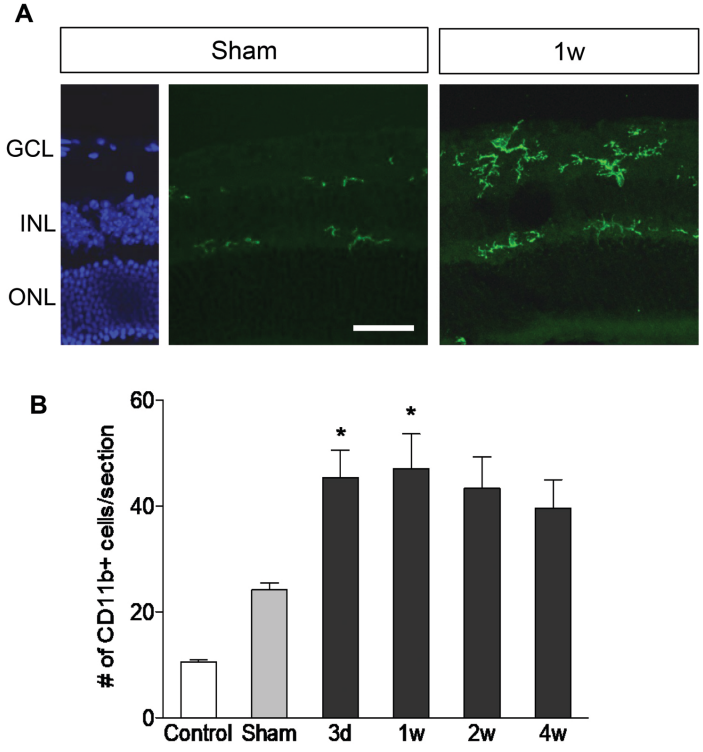


Figure 2: Acute retinal ischemia induces microglia/macrophage activation. A. Epifluorescent photomicrographs of CD11b immunolabeling (green) retinal sections taken from a sham-operated B6 mouse or at 1 (1w) and 4 weeks (4w) after ischemic injury. Scale bar: 10 µm. **B.** Quantification of $CD11b^+$ cells in retinal sections taken from uninjured eyes (control), mice at 4 weeks after sham-operation (Sham), or at 3 days (3d), 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury (n = 6/group). Value = mean ± S.E.M. **P* < 0.05 compared to sham-operated mice by one-way ANOVA.

Priming of T cell responses usually occurs first in the secondary lymphoid tissues, such as LNs, where naïve T cells become activated and respond to pathogenic antigens presented by their antigen presenting cells.[32] To assess whether transient ischemia leads to activation of immune responses and T cell activations in the eye-draining LNs, functional subsets of CD4+ T cells in the superior cervical LNs were analyzed with flow cytometry. T cell subsets, T_H1, T_H2, T_H17, and Treg cells, again were divided based on the expression profile of hallmark cytokines: IFN-γ, IL-4, IL-17, and TGF-β.[14] Superior cervical LNs were dissected from mice with retinal ischemia at day 3 and 1 – 4 weeks post injury, sham-operated mice at 2 weeks. Correlating with T cell infiltration into the ischemic retina, the frequencies of 3 subsets of $CD4^+$ T cells expressing IFN-γ (T_H1), IL-17 (T_H17) and TGF-β (T_{reg}) were significantly increased at as early as 1 week after ischemic injury (Fig. 3D). The increases of T_H1 and T_{reg} cell frequencies peaked at 2 weeks post injury and remained elevated by 4 weeks after retinal ischemia (Fig. 3D). Thus, acute retinal ischemia induced CD4+ T cell responses, particularly, that involved IFN-γ expressing T_H1 cells, and likely also Treg cells, in the retina and their draining LNs.

T cells mediate the prolonged phase of RGC degeneration following retinal ischemia

To determine if the T cell responses participate in ischemia-induced retinal neurodegeneration, we examined mice deficient in both T and B cells ($Rag1^{-/-}$ mice) or only T cells ($TCR\beta^{-/-}$).[33] While acute ischemic injury to the retina induced sustained RGC degeneration that progressed over 8 weeks, RGC loss in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice was significantly attenuated compared to B6 mice (Fig. 4A, B). At 1 week post injury, $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice showed a similar rate of RGC loss at 23.9% ± 2.8% and 24.0% ± 2.7%, respectively, as compared to 35.2% ± 2.4% RGC loss in B6 mice. No significant further loss of RGCs was detected in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice after week 1; by 4 weeks post injury, loss of RGCs counted from $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice was not significantly increased and remained at 28.2% ± 1.9% and 30.2% ± 2.9%, respectively. In contrast, loss of RGCs in B6 mice had significantly progressed to 47.6% ± 3.3% by week 4 post injury. These results indicate that T cells are essentially involved in perpetuating progressive neurodegeneration in retinal ischemia. The similar extents of RGC loss in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice suggest a primary role for T cells, but not B cells, in mediating neural damage following transient reperfusion injury.

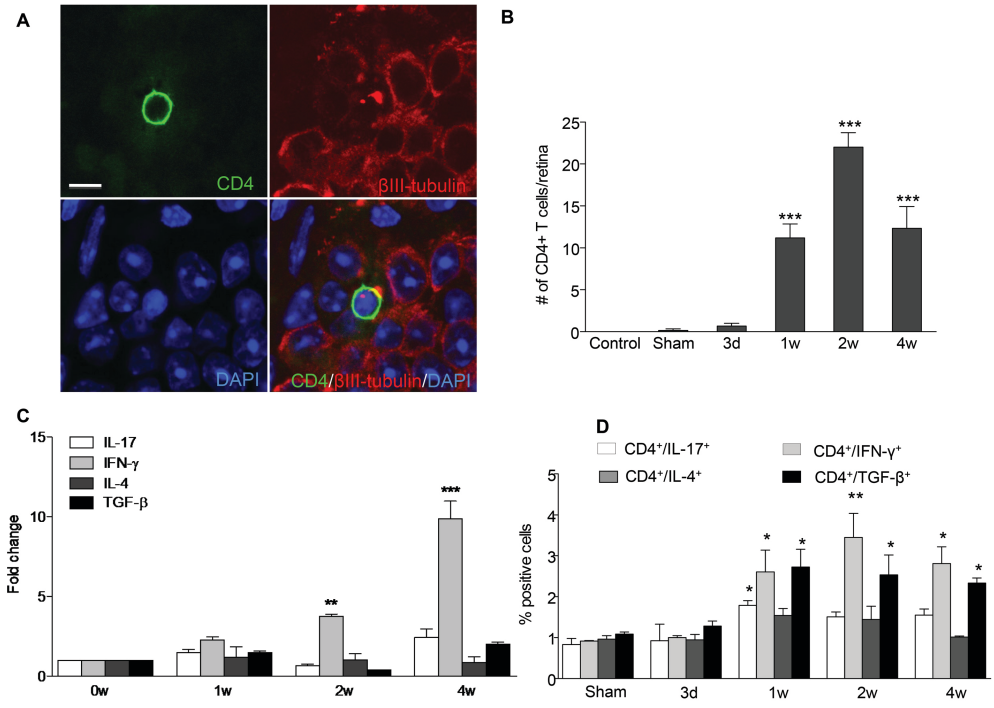


Figure 3. Acute retinal ischemic injury induces T cell infiltration into the retina. A. Epifluorescent photomicrographs of a retina flat-mount image taken from a mouse at 2 weeks after ischemic injury that was double-immunolabeled for CD4 (green) and β-III-tubulin (red) and counter-stained by a nuclear marker DAPI (blue). Scale bar: 10 μm. **B.** $CD4^+$ T cell counts in retina flat-mounts of an uninjured eye (control) and mice at 4 weeks after sham-operation (Sham) or at 3 days (3d) and 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury (n = 6/group). **C.** Results of qPCR revealing the fold changes in expression of hallmark cytokines of T_H cells in the retina of mice at day 0 (0w as a baseline), 1, 2, and 4 weeks after acute ischemia (n = 6/group). **D.** Flow cytometry quantification of frequencies of subsets of CD4+ T cells in the draining LNs of the eye taken from mice at 2 weeks after sham-operation (Sham) or after 3 days (3d), 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury. Shown were percentages of $CD4^+$ T cells that expressed IL-17, IFN-γ, Il-4, or TGF-β among freshly-isolated total LN lymphocytes (n = 6/group). *P < 0.05, **P < 0.01, ***P<0.001 compared to sham-operated mice by one-way ANOVA.

To investigate if T cells play a causative role in inducing RGC damage, $CD4^+$ T cells were isolated from the splenocytes of ischemia- or sham-operated B6 mice 2 weeks post procedure and adoptively transferred into $Rag1^{-/-}$ mice which had been subjected to retinal reperfusion injury 2 weeks earlier. Recipient $Rag1^{-/-}$ mice were sacrificed 2 weeks after adoptive T cell transfer. $Rag1^{-/-}$ recipient mice that were subjected to T cell injection from sham-operated mice showed a RGC loss (29.9% ± 1.5%) similar to that was seen in $Rag1^{-/-}$ mice without receiving a T cell transfer (28.2% ± 1.9%) (Fig. 4C, D). In contrast, $Rag1^{-/-}$ mice

that received T cell transfer from ischemic B6 mice showed a significantly increased loss of RGCs (44.4% ± 4.0%) compared to Rag1$^{-/-}$ mice without receiving T cell transfer or those who received T cells from sham-operated B6 mice. Together, these findings demonstrate that diseased CD4^{+} T cells from mice with retinal ischemic injury are sufficient to induce RGC damage.

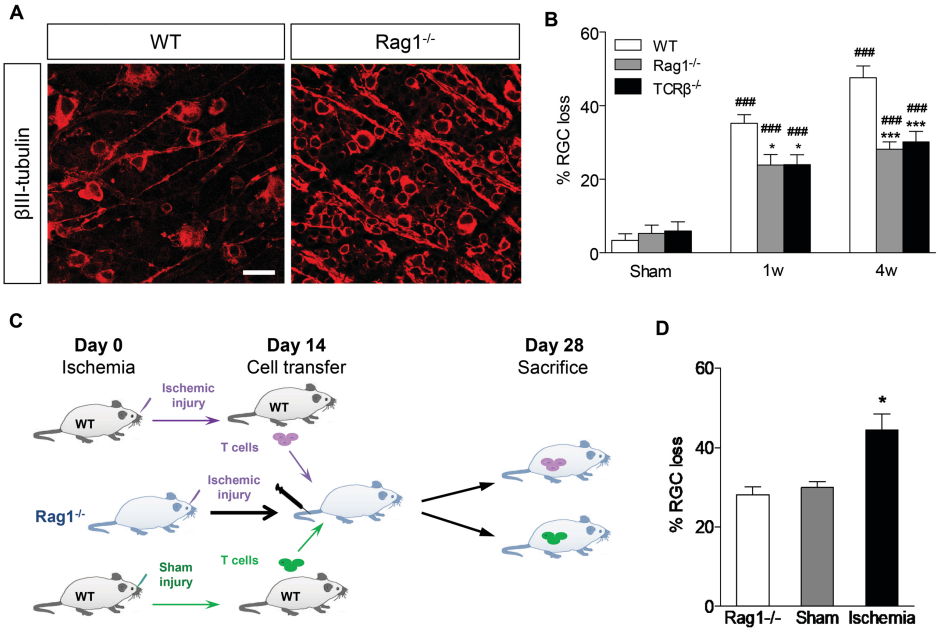


Figure 4. CD4+ T cell-mediated responses are essential and causative contributing factors to the progressive RGC loss after retinal ischemic injury. A. Representative epifluorescent photomicrographs of retinal flat-mounts of B6 and Rag1$^{-/-}$ mice at 4 weeks after retinal ischemia. Scale bar: 25 μm. **B.** Quantification of RGC loss in B6, Rag1$^{-/-}$ and TCRβ$^{-/-}$ mice at 1 and 4 weeks post-ischemic injury or at 4 weeks after sham operation (n = 6/group). Value = mean ± S.E.M.. *P < 0.05, ***P < 0.001 compared to B6 mice taken at the same time point; ###P < 0.001 compared to sham-operated group of mice within the same genotype group, by one-way ANOVA. **C.** Schematic illustration of adoptive T cell transfer: CD4+ T cells were isolated from the spleens of B6 mice 2 weeks after ischemic injury or sham-operation; the donor T cells were injected into the tail veil of recipient Rag1$^{-/-}$ mice that also received retinal ischemic injury 2 weeks prior to the cell transfer. Recipient mice were sacrificed 2 weeks after cell transfer. **D.** Percentage of RGC loss presented as RGC counts relative to that of the unoperated contralateral eyes (n = 6/group). Value = mean ± S.E.M.. *P < 0.05 by two-tailed student t test.

Anti-CD4 and IFN-γ antibodies attenuate retinal ischemia-induced RGC loss and improve retinal function

The findings prompted us to investigate the neuroprotective effect and therapeutic potential for retinal ischemic injury by local administration of blocking antibodies against

CD4+ T cells or IFN-γ. It has been shown that Ultra-LEAF anti-mouse CD4 blocked CD4-mediated cell adhesion and CD4+ T cell activation, causing in vivo depletion of CD4+ T cells.[24-29] Ultra-LEAF anti-mouse IFN-γ neutralizes the bioactivity of natural or recombinant IFN-γ.[30, 31] We found that intravitreal injections of anti-CD4 or anti-IFN-γ neutralizing antibodies significantly attenuated RGC loss compared to injections with IgG Isotype antibodies or sterile saline (Fig. 5A, B); whereas, the extent of RGC loss was comparable between saline- and IgG Isotype antibody-treated groups. Acute retinal ischemic injury is reported to induce ERG changes that are associated with functional impairment,[34] so we also evaluated ERG responses at 4 weeks post injury as a readout for their retinal functions. Vehicle treated ischemic retina showed significantly decreased a- and b-wave amplitudes in ERG scotopic-200 or flicker responses compared to sham-operated mice. In contrast, administration of anti-CD4 blocking antibody, but not anti-IFN-γ, prevented the reduction of a- and b-wave amplitudes, under both dark- and light-adapted conditions, following retinal ischemic injury in mice (Fig. 6A-C). These results strongly suggest that local administration of CD4+ T cell blocking antibody in the eye protects against secondary retinal neuron and function loss following reperfusion injury.

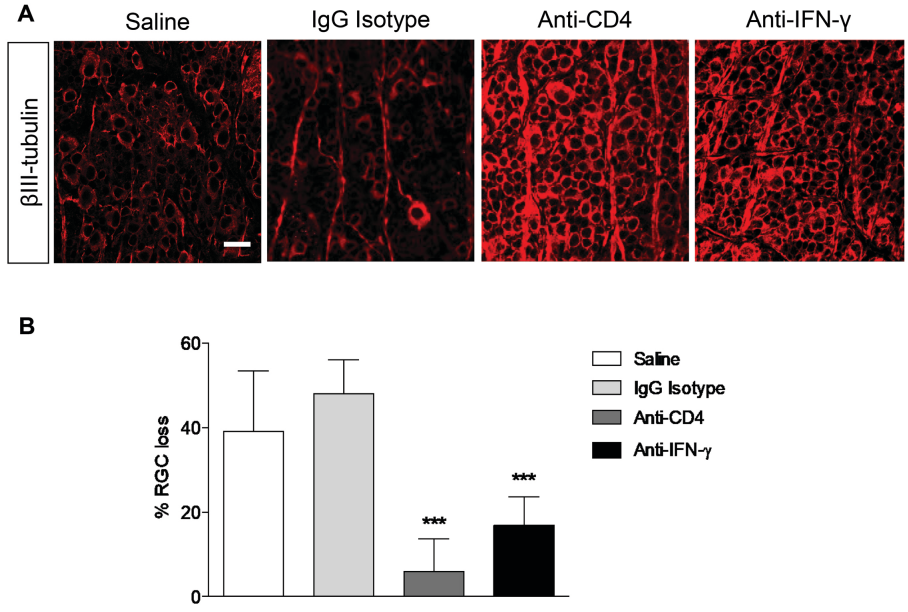


Figure 5. Local administration of anti-CD4+ or anti-IFN-γ antibody attenuates RGC loss after acute retinal ischemia. A. Epifluorescent images of βIII-tubulin-immunolabeled retinal flat-mounts taken from ischemic eye that received an intravitreal injection of saline-, IgG Isotype-, anti-CD4, or anti-IFN-γ. Scale bar: 25 μm. **B.** Percentage of RGC loss at 4 weeks after acute retinal ischemia. Value = mean ± S.E.M. (n > 6/group). ****P*<0.001 compared to the saline-injected group by one-way ANOVA.

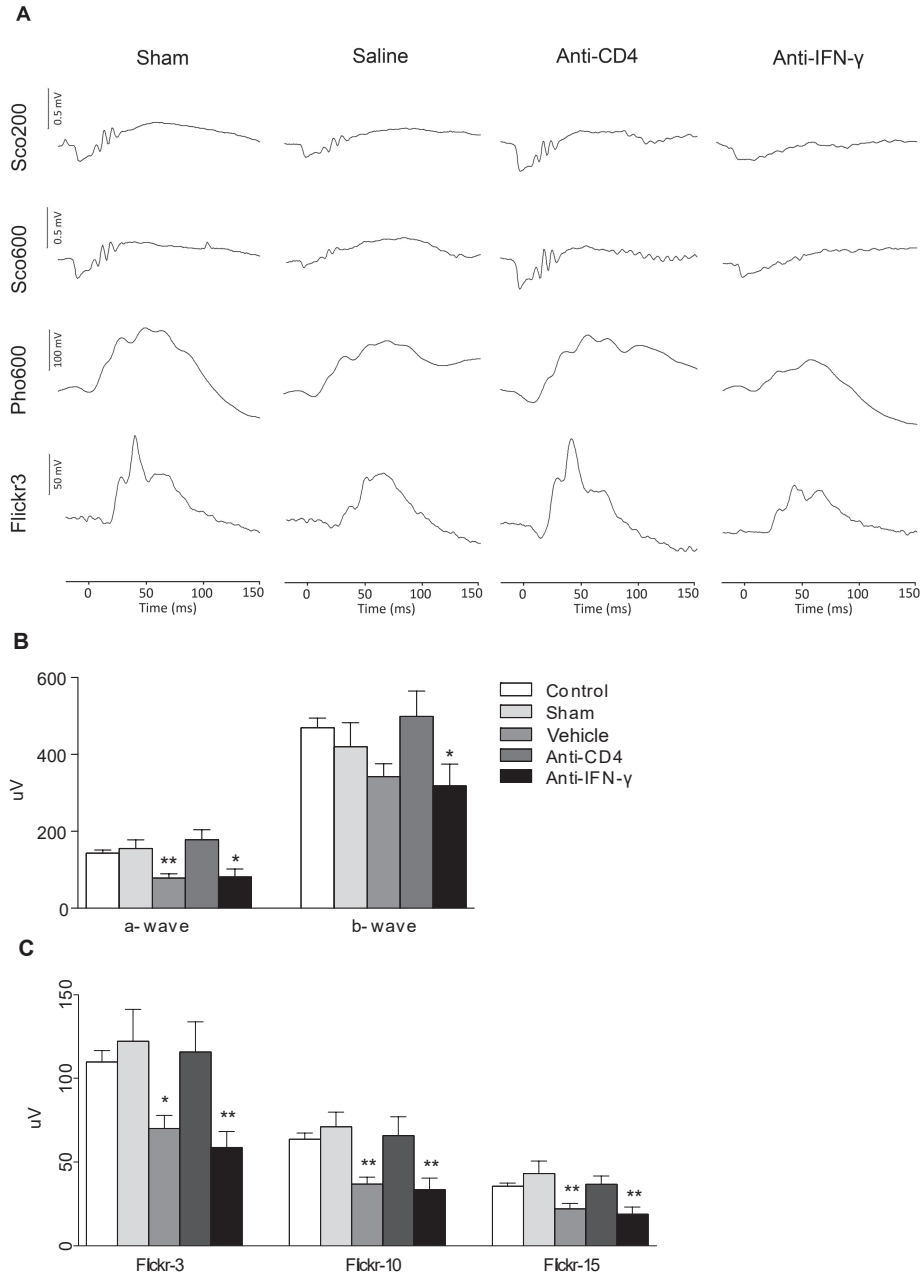


Figure 6. Local administration of anti-CD4 antibody preserves retinal function after acute retinal ischemia. A. Representative ERG waveforms from mice at 4 weeks after receiving sham operation or ischemic injury plus saline (vehicle)-, anti-CD4, or anti-IFN-γ intravitreal injection. **B, C.** Amplitudes of scotopic-200 a- and b-waves (B) or 3, 10, 15 Hz Flickr b-waves assessed in mice at 4 weeks after receiving no injury (control), sham operation (Sham), or ischemic injury plus intravitreal injections of saline- (vehicle), anti-CD4 (anti-CD4), or anti-IFN-γ (anti-IFN-γ). Value = mean ± S.E.M. (n > 6/group). *P<0.05, **P<0.01 compared to control eyes by one-way ANOVA.

DISCUSSION

In this study, we reported a prolonged phase of progressive RGC loss following acute retinal reperfusion injury and a role for CD4+ T cell-mediated responses in the etiology of neurodegeneration in ischemic retinopathy. We showed that transient retinal reperfusion injury led to microglia/macrophage activation and T cell infiltration. Moreover, T cell-mediated responses are responsible for progressive degeneration of RGCs despite of the absence of sustained insults. Adoptive transfer of T cells isolated from mice subjected to acute retinal ischemia was sufficient to drive progressive RGC damage, while ectopic suppression of $CD4^+$ T cell responses protected RGCs against ischemic insult-induced damage and preserved retinal function after injury. These results reveal a therapeutic window as well as a potential novel therapeutic strategy for limiting retinal neuron and function loss in the currently untreatable conditions of ischemic retinopathy or optic neuropathy.

Acute ischemic injury to the retina is accompanied by early activation of innate immune cascades, disruption of the blood retinal barrier, and leukocyte infiltration.[35, 36] However, little is known about the long-term impact of these immune responses on the retina. Here we discovered CD4+ T cell infiltration that was peaked around day 14 and persisted over 4 weeks after reperfusion injury. In addition, we observed the increase in $CD11b^+$ cells in the retina, followed by $CD4^+$ T cell infiltration and activation in the eye's draining LNs. In agreement with that was seen in ischemic stroke model of the brain, CD4+ T cells involved in retinal ischemia were primarily composed of INF-γ+ T_H1 and Treg cells.[37] Antigen-presenting cells in the retina and LNs, including microglia and macrophages, likely present retinal antigens from stressed or damaged RGCs to naïve T cells which further recruit T cells into the retina under a compromised blood retinal barrier.[9, 32]

Previous studies have shown that adaptive immune responses play an essential role in the pathogenesis of many neurodegenerative processes, such as ischemic stroke and traumatic brain injury.[13, 35, 38-40] Induction of ischemic stroke or traumatic brain injury in immunodeficient mice, including SCID, $Rag1^{-/-}$ mice, and IFN-$\gamma^{-/-}$ mice, resulted in attenuated CNS injury and reduced infarct size relative to immunologically-intact mice.[38-40] Adoptive transfer of activated/effector $CD4^+$T cells from ischemic or traumatic injured mice into immunodeficient mice significantly increased the size of traumatic brain injury and the number of apoptotic cells in the CNS.[39, 40] These data are in agreement with ours seeing

in the retinal ischemic model and support that activated CD4+ T cells are highly injurious. Specifically, we demonstrated a role for $CD4^+$ T cells in perpetuating a progressive loss of RGCs after ischemic injury, which is significantly attenuated in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice that lack T and B cells or only T cells. In addition, adoptive transfer of $CD4^+$ T cells from ischemia-induced B6 mice to $Rag1^{-/-}$ mice compromised the resistance of RGCs to ischemic injury and resumed the secondary damage in these mice. The fact that RGC loss was not detected in the contralateral eye of mice with ischemic injury suggests the prerequisite for local injury/inflammation or compromised blood-retina barrier to enable T cell-mediated neural damage. A similar observation was found in the mouse model of glaucoma, in which adoptive transfer of diseased $CD4^+$ T cells exacerbated RGC loss in $Rag1^{-/-}$ mice with elevated IOP, but not in naïve mice.[14] Correspondingly, pro-inflammatory T cells, such as IFN-γ- and IL-17-secreting $CD4^+$ T cells, were found to be the primary subsets that infiltrated the retina after ischemic-injury, as that was seen in glaucomatous mice.[14] Collectively, our data support that acute ischemic injury led to $CD4^+$T cell-mediated responses that involve particularly T_H1 type cells in the retina and eye-draining LNs, which contribute to a prolonged phase of RGC degeneration and/or retinal neural damage.

Our results indicate a therapeutic window and opportunity for currently untreatable conditions of ischemic retinopathy, such as that caused by central retinal artery occlusion and non-arteritic anterior ischemic optic neuropathy. We showed that local inhibition of $CD4^+$ T cell activities by intravitreal administration of CD4-blocking antibody protected RGCs against ischemic damage and preserved retinal function as assessed by ERG compared to the non-treated control group. As compared with anti-CD4 antibodies, IFN-γ antibodies were less effective, suggesting the involvement of not only T_H1 cell subset in the pathogenesis of ischemic injury. Future characterization of effector T cells that enter the retina and mediate retinal neuron damage will be necessary. In line with our finding, a study in a traumatic brain injury model showed similar benefit in attenuating acute injury-induced neuron tissue damage when mice were intravenously treated with immunosuppressants and T cell-inhibitory agents, such as cyclosporine A or FK506.[39] Antibodies against α4 integrin that prevent lymphocyte infiltration into post-ischemia brain injury, and methylprednisolone—an agent with inflammation-inhibitory effects and T cell suppressant, also reduced neural damage[41, 42] and promoted tissue healing.[43] In these studies, broader spectrum immunosuppressants and systematic administration via intravenous or intraperitoneal injection were employed. As the eye is more accessible

than the brain, it enables local administration of antibodies, thereby prevents systematic adverse effects. Our study demonstrates that intravitreal injection of antibodies specifically targeting CD4+ T cells is sufficient to prevent RGC and retinal function loss after ischemic optic neuropathy.

In summary, our study has provided novel evidence showing previously unappreciated roles for CD4+ T cells in post-ischemic retinal injury. Local administration of $CD4^+$ T cell blocking antibodies may present an effective therapeutic strategy for preventing RGC death and preserving retinal functions. This finding is in line with our previous report that CD4+ T cell responses are critically involved in propagating progressive neurodegeneration after retinal neuron insults, such as in glaucoma mouse models.[14] These findings point to novel therapeutic strategies of limiting or preventing neuron loss and preserving retinal function for currently untreatable conditions of ischemic retinopathy or optic neuropathy, which may be extended to treat CNS ischemic stroke.

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