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NEUROBIOLOGY

CD4⁺ T-Cell Responses Mediate Progressive Neurodegeneration in Experimental Ischemic Retinopathy



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Retinal ischemic events, which result from occlusion of the ocular vasculature share similar causes as those for central nervous system 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 thought to be intricately involved, but the exact roles remain elusive. We found that acute ischemia-reperfusion injury to the retina induced a prolonged phase of retinal ganglion cell loss that continued to progress during 8 weeks after the procedure. This phase was accompanied by microglial activation and CD4⁺ T-cell infiltration into the retina. Adoptive transfer of CD4⁺ T cells isolated from diseased mice exacerbated retinal ganglion cell loss in mice with retinal reperfusion damage. On the other hand, T-cell deficiency or administration of T-cell or interferon- γ -neutralizing antibody attenuated retinal ganglion cell degeneration and retinal function loss after injury. These findings demonstrate a crucial role for T-cell-mediated responses in the pathogenesis of neural ischemia. These findings point to novel therapeutic targets of limiting or preventing neuron and function loss for currently untreatable conditions of optic neuropathy and/or central nervous system ischemic stroke. (*Am J Pathol* 2020, 190: 1723–1734; <https://doi.org/10.1016/j.ajpath.2020.04.011>)

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 the American Stroke Association, as well as the American Academy of Ophthalmology, have recognized acute retinal ischemia as a stroke equivalent and recommend urgent etiologic workup, 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.

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Recent investigations reveal that the eye, which has been known for a long time to be an immune-privileged site,^{8,9} elicits immunologic responses under pathophysiologic 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 found that CD4⁺ T-helper (Th) cells participate in ischemic neurodegeneration and that mice with severe combined immunodeficiency that lacked T and B lymphocytes died less frequently of RGC causes after retinal ischemic injury than wild-type mice.¹³ In line with these observations, it was recently found that pathologic 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 (HSPs) were identified as pathogenic antigens of these T cells. Importantly, these T cells attacked RGCs by recognizing the surface HSPs that were induced after 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 after neural stress or injury.^{15,16}

As ischemic insult is reported to recruit T cells into the retina and up-regulate HSPs in RGCs, we hypothesized that CD4⁺ T-cell-mediated responses also play an important role in perpetuating retinal neurodegeneration in ischemic or reperfusion injury. In the present study, we sought to test this hypothesis by using T-cell-deficient mice and adoptive T-cell transfer and assessing T-cell responses. This study provided compelling evidence indicating that an acute ischemic event in the retina induced interferon (IFN)- γ -secreting CD4⁺ Th cell infiltration and a prolonged phase of neurodegeneration during 8 weeks, whereas administration of T-cell blocking antibodies attenuated RGC and retinal function loss in an experimental model of retinal reperfusion injury. These 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 central nervous system.

Materials and Methods

Mice

C57BL/6J wild-type (B6) mice, *CX3CR1-GFP*^{+/-} mice (in which the *GFP* gene has been inserted into the *CX3CR1* gene loci to allow direct visualization of microglia), and mice deficient for *Rag1* (*Rag1*^{-/-}) or T-cell receptor β (*TCR β* ^{-/-}) between 12 and 16 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed under a 12-hour 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 NIH and the Association for Research in Vision and Ophthalmology.

Acute Retinal Reperfusion Injury

Retinal ischemia was induced in B6, *Rag1*^{-/-}, *TCR β* ^{-/-}, or *CX3CR1-GFP*^{+/-} mice as previously described.^{17,18} Mice were anesthetized with a mixture of 120 mg/kg of ketamine and 20 mg/kg of xylazine in sterile saline (1:1:6). Retinal ischemia was induced unilaterally in the right eye, whereas the contralateral eye served as a nonischemic control. The pupil was dilated with 1% tropicamide (Bausch & Lomb Inc., Tampa, FL), and 0.5% proparacaine hydrochloride (Bausch & Lomb Inc.) was applied topically onto the cornea. The cornea was gently punctured near the center using a 30-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 physiologic (0.9% sodium chloride) bag (Hospira Inc., Lake Forest, IL). With elevation of the saline bag to 120 cm above the eye level, the IOP was increased to 90 mm Hg. 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 mice that underwent sham operation, 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 no IOP elevation was generated in these mice. Mice were sacrificed at day 3 on 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 1640 media (Sigma-Aldrich, St. Louis, MO) that contained 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. Red blood cells were lysed with red blood cell lysis buffer (Sigma-Aldrich). 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 mice undergoing sham operation at 2 weeks after the 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 >90% CD4⁺ T cells, as assessed by flow cytometry. The donor cells (2×10^8 cells in a volume of 200 μ L of sterile saline) were adoptively transferred into recipient *Rag1*^{-/-} mice 2 weeks after the induction of

retinal ischemia via tail vein injection. The same numbers of CD4⁺ T cells isolated from mice undergoing sham operation 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 overnight and transferred to 20% sucrose for 2 hours before being 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, MA), followed by reaction with an Alexa Fluor 488–conjugated secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA), and counterstained with the nuclear marker DAPI (Vector Laboratories, Burlingame, CA). The numbers of CD11b⁺ cells and CD4⁺ T cells were counted under direct fluorescence microscopy (Olympus IX51, Olympus, Tokyo, Japan). RGC loss was assessed quantitatively in retinal flat mounts using a standard protocol as previously described²⁰ with minor modifications. In brief, eyeballs were fixed in 4% paraformaldehyde for 3 hours at room temperature. Retinal flat mounts were incubated with a primary antibody against the 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. With use of the optic nerve head as the origin, four standard regions were selected from each quadrant: one peripheral, two intermediate, and one central (Figure 1A). In total, 16 rectangular regions (each 193 × 193 µm) of each retinal flat mount were photographed at ×400 magnification with a confocal microscope (Leica TCS-SP5, Leica, Wetzlar, Germany). 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 two investigators (T.H.K.V. and H.C.) under a blinded fashion.

Real-Time Quantitative PCR to Detect Cytokine Expression in Ischemic Retinas

Total RNA was extracted from mouse retina using RNeasy Plus Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA using Superscript III First Strand Kit (Invitrogen, Carlsbad, CA). Reaction mixture of real-time quantitative PCR contained cDNA, 2× Master Mix from a KAPA

SYBR Fast qPCR kit (Kapa Biosystems Inc., Wilmington, DE), and 10 mmol/L of specific primers. Quantitative detection of specific mRNA transcript was performed 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 *GAPDH* housekeeping gene.

ELISPOT Assays

Mouse IFN-γ Enzyme-linked ImmunoSpot (ELISPOT) assay (eBioscience, San Diego, CA) was performed as previously described¹⁴ for the determination of the frequency of IFN-γ–producing T cells in response to HSP27 or HSP60. Briefly, mouse splenocytes (1 × 10⁶ cells per well) were added to an ELISPOT plate (Miltiscreen-MAIPS4510) and incubated with antigens HSP27 or HSP60 (Sigma-Aldrich) at a final concentration of 10 µg/mL for 48 hours. Cell cultures incubated alone were used as controls. Results are given as the mean number of antigen-specific spot-forming cells after background subtraction from the control wells that contained no antigen.

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 at the 10, 12, or 2 o'clock positions of the eye to generate access for the glass micropipette. Using this entry wound, 2 µL of 1.0 mg/mL of Ultra-LEAF (low endotoxin and azide-free) purified anti-mouse CD4 (IgG2b, clone GK1.5; Biolegend, San Diego, CA), Ultra-LEAF purified anti-mouse IFN-γ (IgG1, clone XMG1.2; Biolegend), isotype IgG (Biolegend), or sterile saline was given intravitreally using a glass micropipette. The same entry site was used each time during the repeated injections in the 2-week period unless vessel growth was noted around the previous entry site in which case a new entry site in the adjacent area would be generated. More than 90% of the injected eyes survived and had no apparent signs of inflammation or degeneration throughout the period after multiple intravitreal injections. Reports have found 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}

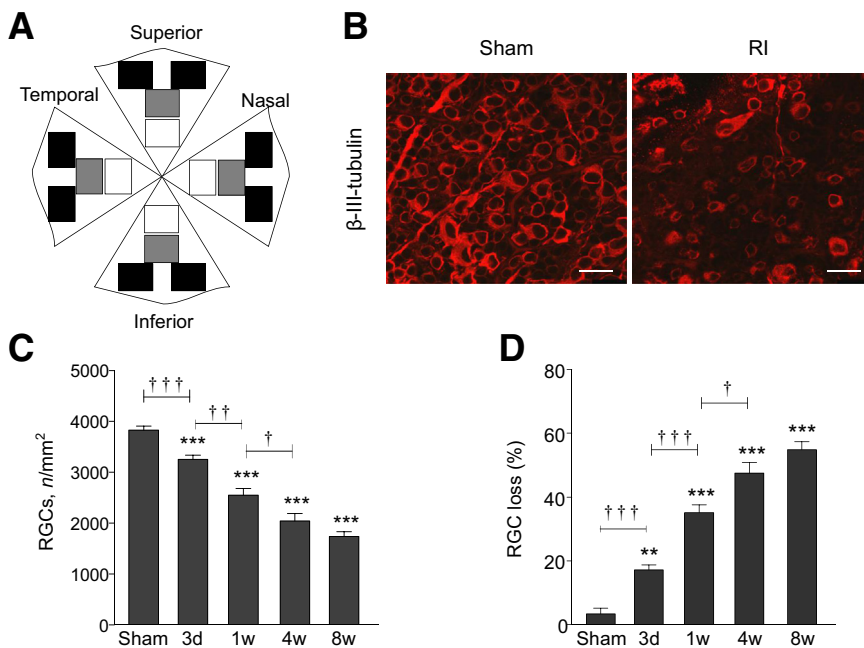


Figure 1 Acute retinal ischemia induces progressive neurodegeneration. **A:** Schematic illustration of retina sampling for retinal ganglion cell (RGC) counts in a retinal flat mount. **B:** Representative epifluorescent photomicrographs of β -III-tubulin (red) immunolabeled retinal flat mounts taken from a B6 mouse undergoing sham operation (sham) and a mouse at 4 weeks after retinal ischemic injury (RI). **C:** Quantification of RGC densities in retinal flat mounts at various time points after ischemic injury or at 4 weeks after sham operation. **D:** Percentage of RGC loss over that of the uninjured contralateral eye in mice at various time points after retinal ischemia or sham operation. Data are expressed as means $\pm$ SEM. $n = 6$ per group (**C**). $^{**}P < 0.01$, $^{***}P < 0.001$ versus the sham group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ (one-way analysis of variance). Scale bars = 25 μ m (**B**).

Flow Cytometry

To define the subsets of T cells involved in the pathologic process after acute retinal ischemia, cytokine expression by T cells in the eye's draining (superior cervical) lymph nodes (LNs) and the retina was analyzed. Superior cervical LNs were dissected, and cells were mechanically dissociated using two forceps. To analyze retinal T-cell infiltration, mice were transcardially perfused with saline. The retinas were dissected and dissociated with papain for 15 minutes at 37°C, which was stopped by the addition of ovomucoid protease inhibitor. Cell aggregates were separated by filtration through a 70- μ m nylon cell strainer (BD Falcon, San Jose, CA). Frequencies of CD4⁺ T-cell subsets as determined by their cytokine profiles, including IFN- γ (Th1), IL-17 (Th17), IL-4 (Th2), or transforming growth factor (TGF)- β [T-regulatory (Treg) cells], were determined after stimulation with phorbol 12-myristate 13-acetate (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) in the presence of monensin (Biolegend) for 4 hours.

Cells were then washed in IsoFlow (Beckman Coulter Inc., Brea, CA) and stained with surface fluorescein isothiocyanate-conjugated anti-mouse CD4 antibody (IgG2b, clone GK1.5; Biolegend). Thereafter, cells were permeabilized with Perm/Wash buffer (BD Biosciences, Franklin Lakes, NJ) and stained with phosphatidylethanolamine (PE)-labeled anti-mouse IFN- γ antibody (IgG1, clone XMG1.2; Biolegend), 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- β 1 antibody (IgG1, clone TW7-20B9) for detection of Th1, Th2, Th17, and Treg cells, respectively. For the identification of other immune cells, antibodies including Percp/cy5.5 anti-mouse CD19 (1D3/CD19) (B cells), allophycocyanin anti-mouse T-cell receptor γ/δ (γ/δ T cells), Alexa Fluor 488 anti-mouse CD49b (natural killer T cells), allophycocyanin anti-mouse CD8a (CD8 T cells), fluorescein isothiocyanate anti-mouse CD8b.2, and allophycocyanin anti-mouse Ly-6G (neutrophils) were used. The antibody-stained cells were analyzed

Table 1 List of Primer Sequences Used in Real-Time PCR

Gene	Forward	Reverse
<i>Ifng</i>	5'-ATGAACGCTACACACTGCATC-3'	5'-CCATCCTTTTGGCAGTTTCCTC-3'
<i>Il4</i>	5'-GGTCTCAACCCCCAGCTAGT-3'	5'-GCCGATGATCTCTCTCAAGTGAT-3'
<i>Il17a</i>	5'-TCAGCGTGTCCAAACACTGAG-3'	5'-TCTCGACCCGTGAAAGTGAAGG-3'
<i>Tgfb1</i>	5'-CTCCCGTGGCTTCTAGTGC-3'	5'-GCCTTAGTTTGGACAGGATCTG-3'
<i>GAPDH</i>	5'-AACTTTGGCATTGTGGAAGG-3'	5'-ACACATTGGGGGTAGGAACA-3'
<i>Ccl2</i>	5'-CAACTCTCACTGAAGCCAG-3'	5'-TTAACTGCATCTGGCTGAG-3'
<i>Il1b</i>	5'-AACCTGCTGGTGTGTGACGTTTC-3'	5'-CAGCACGAGGCTTTTGTGTGT-3'
<i>Tnf</i>	5'-TTCTCATTCCTGCTTGTG-3'	5'-TTGGGAACCTCTCATCCCT-3'

with a BD LSR II Flow Cytometer (BD Biosciences), and data were analyzed using Summit Software version 4.3 (Beckman Coulter Inc.).

Electroretinography

Animals were dark adapted for 5 hours before electroretinogram (ERG) recordings. All procedures were performed in a dark room under the dim red safety light. Mice were anesthetized with 120 mg/kg of ketamine and 20 mg/kg of xylazine, and the pupils were dilated with 1% tropicamide. Mice were placed in the sternal-abdominal position within the Ganzfeld 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 midfrontal head area and caudal area near the tail, respectively. Light stimulations were delivered with a Xenon lamp at 0.0002, 0.02, 2, 200, and 600 cd·s/m² for dark-adapted tests. Thereafter, animals were subjected to 7-minute light adaptation with a light intensity of 50 cd·s/m² before initiating the light-adapted tests. The light-adapted tests were conducted by Xenon light at 600 cd·s/m², green light at 13 cd·s/m², and blue light at 1 cd·s/m² sequentially. Flicker tests were executed with 6500 K of white light at 15 cd·s/m² 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 GraphPad Prism for Windows version 5.0 (GraphPad Software Inc., La Jolla, CA). The performed tests were two-sided, and $P < 0.05$ was considered as statistically significant. At least six animals were used for each experimental or control group. For the comparison between two groups, the *U*-test was performed, and for three or more groups, the nonparametric Kruskal-Wallis test was used.

Results

Acute Retinal Ischemia Induces a Prolonged Phase of Progressive Neurodegeneration

To investigate whether 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 mm Hg for 60 minutes. RGC loss was quantified at day 3 and weeks 1, 4, and 8 after ischemic injury or at 4 weeks after the procedure in mice undergoing sham operation, using Tuj1 labeling in retinal flat mounts as

previously described (Figure 1B).²⁰ As expected, no significant difference of RGC counts was noted in uninjured contralateral eyes at all time points after the procedure or in control eyes (sham operation) (Supplemental Figure S1). In contrast, starting from 3 days after injury, retinas subjected to reperfusion injury displayed progressive RGC loss (Figure 1, C and D and Supplemental Figure S1). Significantly fewer RGCs in the ischemic retinas (3250 ± 87 cells/mm²) were counted than that in control retina (3831 ± 78 cells/mm²) at as early as 3 days after the procedure ($P < 0.001$) (Figure 1, C and D). Although the ischemic injury lasted for only 60 minutes, in the absence of any sustained injury, progressive RGC loss continued to occur and 1737 ± 94 cells/mm² were counted by 8 weeks after injury. This amount was equivalent to a $17.2\% \pm 1.6\%$ RGC loss at day 3 to a $54.8\% \pm 2.6\%$ RGC loss by 8 weeks after ischemic injury (Figure 1D), whereas RGC counts in retinas contralateral to the injured eye remained constant through the period and were comparable to the controls (Supplemental Figure S1). Thus, acute retinal ischemia triggers a prolonged phase of progressive RGC degeneration in the absence of a sustained insult.

T-Cell Infiltration into the Retina and Induction of HSP-Specific T-Cell Responses after Ischemic Injury

Local inflammation represented by microglia or macrophage activation is a common event after retinal injury. This finding was verified using *CX3CR1-GFP*^{+/-} mice, in which a copy of *GFP* was inserted into the *CX3CR1* gene to allow direct visualization of microglia. It was noted that at 2 weeks after reperfusion injury in *CX3CR1-GFP*^{+/-} mice, microglia became activated, which revealed shortened dendritic processes and enlarged round cell bodies compared with those in the control eyes (Figure 2A). CD11b immunolabeling was also performed to quantify activated microglia and macrophage in retinal sections. As early as 3 days after injury, significantly increased numbers of CD11b⁺ microglia and macrophages were noted in the ischemic retina compared with controls ($P < 0.05$) (Figure 2B). Next, this study investigated 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 Tuj1 (Figure 3A). Although few T cells were detected in the uninjured contralateral retinas throughout the period (Supplemental Figure S2), infiltrated CD4⁺ T cells were found near RGCs in the retinas subjected to reperfusion injury. The number of T cells counted in the flat-mounted retinas of mice undergoing sham operation was minimal when examined at 2 weeks after operation (0.2 ± 0.2 cells per 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 ($P < 0.001$) (Figure 3B). The subsets of infiltrated CD4⁺ T cells were further verified by flow cytometry, and a significant increase

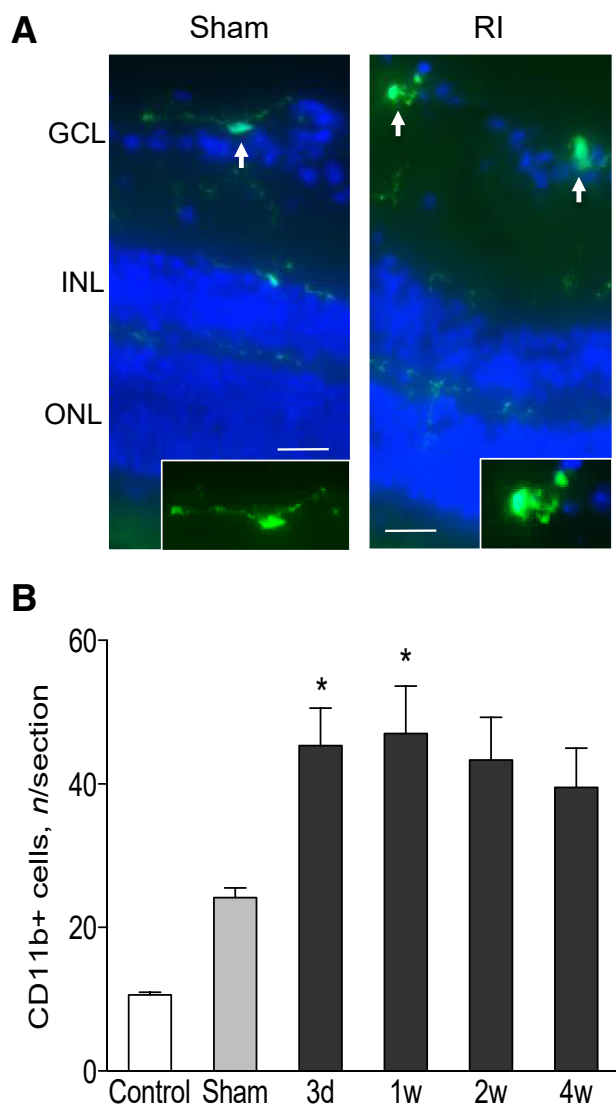


Figure 2 Acute retinal ischemia induces microglia-macrophage activation. **A:** Epifluorescent photomicrographs of green fluorescent protein (GFP) and microglia (green) on retinal sections taken from a *CX3CR1-GFP^{+/+}* mouse undergoing sham operation or at 2 weeks after retinal ischemic injury (RI). **Inset** showing a magnified image of the GFP and microglia (arrows). **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, 1 week, 2 weeks, and 4 weeks after acute ischemic injury. Data are expressed as means ± SEM. *n* = 6 per group (**B**). **P* < 0.05 versus sham mice (one-way analysis of variance). Scale bars = 25 μm (**A**). Original magnification, ×80 (**insets**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

of CD4⁺IFNγ⁺ T cells was noted (*P* < 0.05) (Supplemental Figure S3A) in ischemic eyes compared with controls. No significant increases of other adaptive immune cells, such as B cells, CD8⁺ T cells, γ/δ T cells, natural killer cells, or neutrophils, were detected in the retinas subjected to ischemic injury compared with controls at 2 weeks after the procedure (Supplemental Figure S3B). In addition the expression of *Ifng* was significantly increased 2 and 4 weeks after ischemic injury (*P* < 0.01 and *P* < 0.001, respectively)

(Figure 3C) but not in the uninjected contralateral or control eyes (Supplemental Figure S4). The data support the induction of local inflammation and CD4⁺ T-cell infiltration after retinal ischemic and reperfusion injury.

Priming of T-cell responses usually occurs first in the secondary lymphoid tissues, such as LNs, where naive 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 activation in the eyes draining LNs, functional subsets of CD4⁺ T cells in the superior cervical LNs were analyzed with flow cytometry. The T-cell subsets Th1, Th2, Th17, and Treg cells were divided based on the expression profile of hallmark cytokines: IFN-γ, IL-4, IL-17, and TGF-β, respectively.¹⁴ Superior cervical LNs were dissected from mice with retinal ischemia at days 3 and 1 to 4 weeks after injury and at 2 weeks in mice undergoing sham operation. Correlating with T-cell infiltration into the ischemic retina, the frequencies of 3 subsets of CD4⁺ T cells expressing IFN-γ (Th1), IL-17 (Th17), and TGF-β (Treg) were significantly increased as early as 1 week after ischemic injury (*P* < 0.05) (Figure 3D). The increases of Th1 and Treg cell frequencies peaked at 2 weeks after injury and remained elevated by 4 weeks after retinal ischemia (Figure 3D). Thus, acute retinal ischemia induced CD4⁺ T-cell responses, particularly responses that involved IFN-γ-expressing Th1 cells, and likely also Treg cells, in the retina and their draining LNs.

Previous studies found that retinal stress or injury induces HSP-specific (particularly HSP27 and HSP60) T-cell infiltration into the retina and contributes to progressive neurodegeneration.¹⁴ Because reperfusion injury is also reported to induce HSP27 and HSP60 expression in the retina, this study investigated whether the T-cell responses observed above were HSP27 and HSP60 specific. ELISPOT assay of mouse splenocytes is a standardized assay to measure T-cell responses. With ELISPOT, significantly higher frequencies of HSP27 -and HSP60- specific IFN-γ-secreting Th1 cells were detected at both 1 and 4 weeks after ischemic or reperfusion injury than that of controls (*P* < 0.05) (Figure 3E). The data strongly support that retinal ischemic-reperfusion injury induces HSP-specific T-cell responses, which may participate in the pathogenesis of progressive neurodegeneration.

T Cells Mediate the Prolonged Phase of RGC Degeneration after Retinal Ischemia

To further verify that the T-cell responses participate in ischemia-induced retinal neurodegeneration, this study examined mice deficient in both T and B cells (*Rag1*^{-/-} mice) or only T cells (*TCRβ*^{-/-}).³³ Although acute ischemic injury to the retina induced sustained RGC degeneration that progressed during 8 weeks, RGC loss in *Rag1*^{-/-} and *TCRβ*^{-/-} mice was significantly attenuated compared with B6 mice (*P* < 0.001) (Figure 4, A and B). At 1 week after

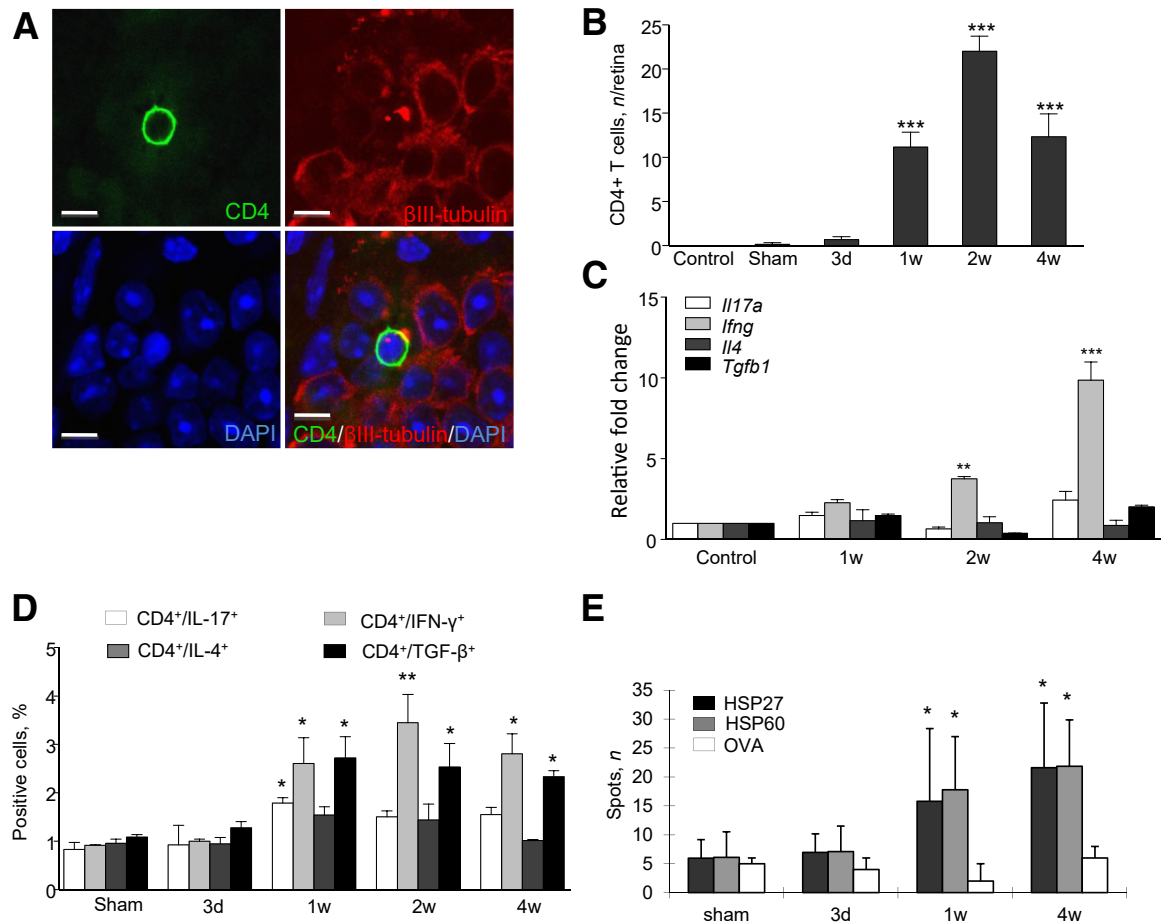


Figure 3 Acute retinal ischemic injury induces T-cell infiltration into the retina. **A:** Epifluorescent photomicrographs taken from the ganglion cell layer of the retinal flat mount of a mouse at 2 weeks after ischemic injury. The retina was double-immunolabeled for CD4 (green) and β -III-tubulin (red) and counterstained by a nuclear marker DAPI (blue). **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, 1 week, 2 weeks, and 4 weeks (4w) after acute ischemic injury. **C:** Results of real-time quantitative PCR showing the fold changes in expression of hallmark cytokines of T helper cells in the retina of mice at day 0 (0 weeks as a baseline), 1 week, 2 weeks, and 4 weeks after acute ischemia. **D:** Flow cytometry quantification of frequencies of CD4⁺ T-cell subsets in the draining lymph node of the ischemic eye taken at 2 weeks after sham operation (sham) or at 3 days, 1 week, 2 weeks, and 4 weeks after acute ischemic injury. Shown are percentages of CD4⁺ T-cell subsets that expressed IL-17, interferon (IFN)- γ , IL-4, or transforming growth factor (TGF)- β among freshly isolated total lymph node lymphocytes. **E:** Frequencies of heat shock protein (HSP) 27- and HSP60-specific T-cell responses. Splenocytes of mice with sham operation or ischemic injury were stimulated with HSP27 or HSP60 in culture, and the frequencies of IFN- γ -secreting cells were assessed by ELISPOT. $n = 6$ per group (**B–D**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus sham mice (one-way analysis of variance). Scale bars = 10 μ m (**A**).

injury, *Rag1*^{−/−} and *TCR β* ^{−/−} mice had a similar rate of RGC loss at 23.9% $\pm$ 2.8% and 24.0% $\pm$ 2.7%, respectively, compared with 35.2% $\pm$ 2.4% RGC loss in B6 mice. No significant further loss of RGCs was detected in *Rag1*^{−/−} and *TCR β* ^{−/−} mice after week 1; by week 4 after injury, loss of RGCs counted from *Rag1*^{−/−} and *TCR β* ^{−/−} mice was 28.2% $\pm$ 1.9% and 30.2% $\pm$ 2.9%, respectively. In contrast, loss of RGCs in B6 mice had progressed to 47.6% $\pm$ 3.3% by week 4 after 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 β* ^{−/−} mice suggest a primary role for T cells, but not B cells, in mediating neural damage after transient reperfusion injury.

To investigate whether T cells play a causative role in inducing RGC damage, CD4⁺ T cells were isolated from the

splenocytes of B6 mice undergoing ischemia-induced or sham operation 2 weeks after the procedure and adoptively transferred into *Rag1*^{−/−} mice that had been subjected to retinal reperfusion injury 2 weeks earlier. *Rag1*^{−/−} mice received no cells or adoptive T-cell transfer from mice undergoing sham or ischemia-induced operations; these recipient *Rag1*^{−/−} mice were then sacrificed after another 2 weeks (Supplemental Figure S5). *Rag1*^{−/−} mice that received T-cell transfer from ischemic B6 mice had a significantly increased loss of RGCs compared with *Rag1*^{−/−} mice that did not receive T-cell transfer or those who received T cells from B6 mice in the sham operation group ($P < 0.05$) (Figure 4, C and D). No significant difference in RGC loss was noted between *Rag1*^{−/−} recipient mice that received T-cell injection from mice in the sham group and *Rag1*^{−/−} mice that did not receive a T-cell transfer

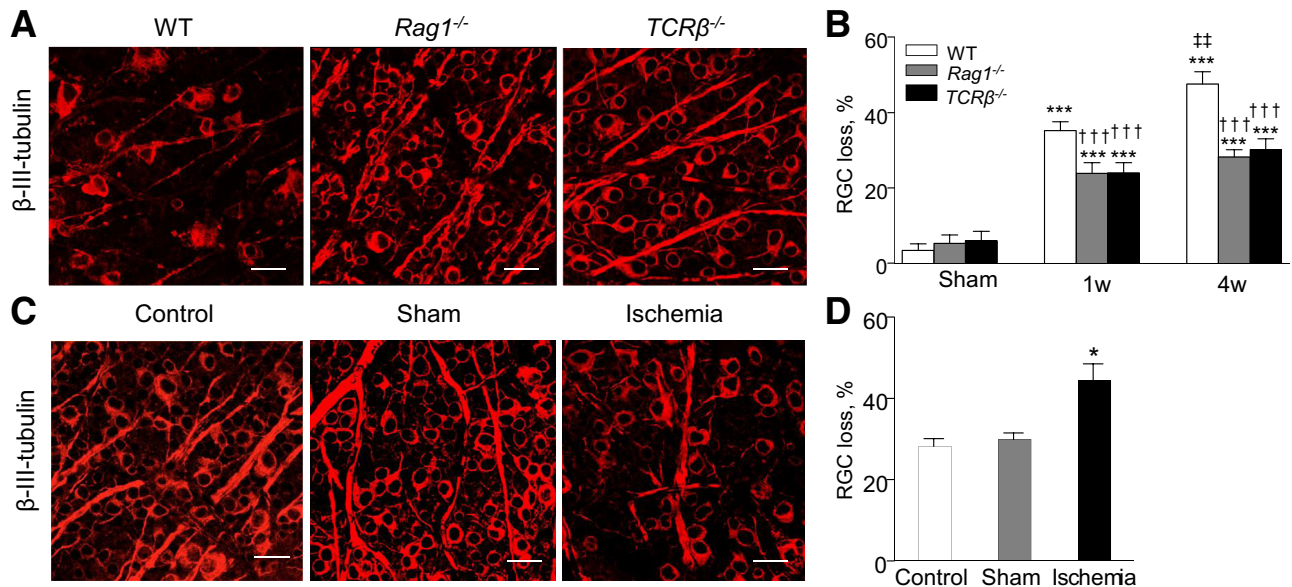


Figure 4 CD4⁺ T cell-mediated responses are essential and causative to the progressive retinal ganglion cell (RGC) loss after retinal ischemic injury. **A:** Representative epifluorescent photomicrographs of retinal flat mounts of B6, *Rag1*-deficient (*Rag1*^{-/-}), and TCRβ-deficient (*TCRβ*^{-/-}) mice at 4 weeks after retinal ischemia. **B:** Quantification of RGC loss in B6, *Rag1*^{-/-}, and *TCRβ*^{-/-} mice at 1 week and 4 weeks after ischemic injury or at 4 weeks after sham operation. **C:** Representative epifluorescence photomicrographs of retinal flat mounts of *Rag1*^{-/-} mice at 4 weeks after retinal ischemia after receiving no cell transfer (control) or adoptive transfer of CD4⁺ T cells isolated from mice undergoing sham operation (sham) or ischemia mice (ischemia). **D:** Percentages of RGC loss presented as RGC counts relative to that of the unoperated and sham-operated controls on contralateral eyes. Data are expressed as means ± SEM. *n* = 6 per group (**B** and **D**). **P* < 0.05, ****P* < 0.001 versus sham mice of the same genotype (two-tailed *t*-test); †††*P* < 0.001 versus B6 wild-type (WT) mice at the same time point; ‡‡*P* < 0.01 versus mice of the same genotype at the 1-week time point (one-way analysis of variance). Scale bars = 25 μm (**A** and **C**).

(Figure 4, C and D). These findings indicate that diseased CD4⁺ T cells from mice with retinal ischemic injury are sufficient to induce RGC damage.

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 after the onset of the disease. Because retinal infiltration of T cells peaked at approximately 2 weeks after ischemia, antibodies were administered intravitreally twice a week during the first 2 weeks after injury to maximize the chance of attenuating T-cell-mediated immune attack and retinal neurodegeneration. Anti-mouse CD4 has been reported to block CD4-mediated cell adhesion and CD4⁺ T-cell activation, causing *in vivo* depletion of CD4⁺ T cells.^{24–29} Anti-mouse IFN-γ neutralizes the bioactivity of natural or recombinant IFN-γ.^{30,31} Local administration of anti-CD4 or anti-IFN-γ neutralizing antibodies via intravitreal injections significantly attenuated RGC loss compared with injections with IgG isotype antibodies or sterile saline (*P* < 0.001) (Figure 5). In contrast, the extent of RGC loss was comparable between saline- and IgG isotype antibody-treated groups

(Figure 5). No apparent inflammatory responses or changes in expression of inflammatory cytokines were observed after multiple intravitreal injections of saline or isotype control antibody when compared with uninjected ischemic retina (Supplemental Figure S6). None of the injected antibodies altered the level of *Ifng* expression (Supplemental Figure S7).

Acute retinal ischemic injury is reported to cause damage to the neuroretina, including photoreceptors, whose function can be assessed through whole-field ERG.³⁴ To evaluate the functional effect of anti-CD4 or anti-IFN-γ administration, we performed ERG measurements were performed at 4 weeks after injury. Vehicle-treated ischemic retina had significantly decreased a- and b-wave amplitudes in ERG scotopic-200 or Flicker responses compared with mice in the sham group. 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 after retinal ischemic injury in mice (*P* < 0.01) (Figure 6). No significant differences were observed in the ratios of the b-wave amplitude to the a-wave amplitude among groups, suggesting that the decrease in b-wave amplitude was the result of photoreceptor loss or dysfunction. These results strongly suggest that local administration of CD4⁺ T-cell blocking antibody in the eye protects against secondary retinal neuron and function loss after reperfusion injury.

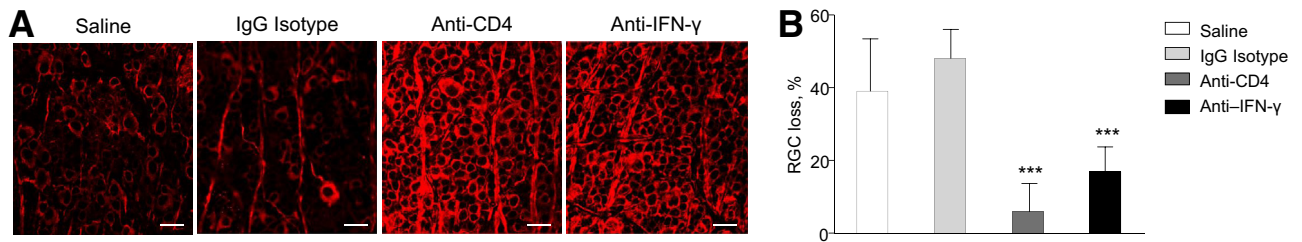


Figure 5 Local administration of anti-CD4⁺ or anti-interferon (IFN)- γ antibody attenuates retinal ganglion cell (RGC) loss after acute retinal ischemia. **A:** Epifluorescent images of β -III-tubulin-immunolabeled retinal flat mounts taken from an ischemic eye that received an intravitreal injection of saline, IgG isotype, anti-CD4, or anti-IFN- γ . **B:** Percentage of RGC loss at 4 weeks after acute retinal ischemia. Data are expressed as means $\pm$ SEM. $n > 6$ per group). *** $P < 0.001$ versus the saline-injected group (one-way analysis of variance). Scale bars = 25 μ m.

Discussion

This study reported a prolonged phase of progressive RGC loss after acute retinal reperfusion injury and a role for CD4⁺ T-cell-mediated responses in the origin of neurodegeneration in ischemic retinopathy. Transient retinal reperfusion injury was followed by microglia or

macrophage activation, T-cell infiltration, and HSP-specific T-cell responses. In the absence of sustained insults, T cells are involved in propagating a prolonged phase of RGC degeneration after acute injury. Adoptive transfer of T cells isolated from mice subjected to acute retinal ischemia was sufficient to drive progressive RGC damage, whereas ectopic suppression of CD4⁺ T-cell responses protected

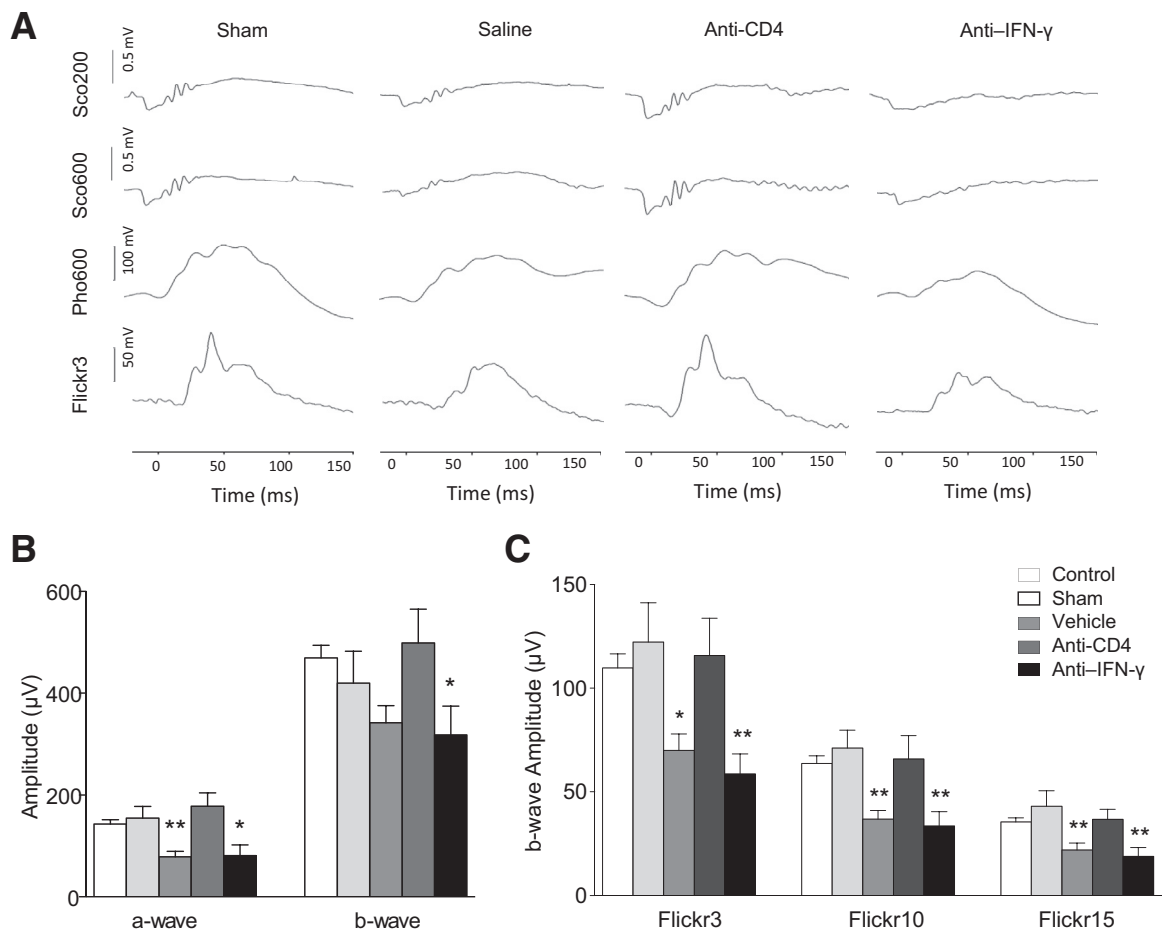


Figure 6 Local administration of anti-CD4 antibody preserves retinal function after acute retinal ischemia. **A:** Representative electroretinogram waveforms from mice at 4 weeks after undergoing the sham operation or ischemic injury plus saline (vehicle), anti-CD4, or anti-interferon (IFN)- γ intravitreal injection. **B** and **C:** Amplitudes of scotopic-200 a- and b-waves (**B**) or 3-, 10-, and 15-Hz Flicker b-waves (**C**) 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, or anti-IFN- γ . Data are expressed as means $\pm$ SEM. $n > 6$ per group). * $P < 0.05$, ** $P < 0.01$ versus control eyes (one-way analysis of variance).

RGCs against ischemic insult–induced damage and preserved retinal function after injury. These results implicate a therapeutic window and potential novel therapeutic targets for limiting retinal neuron and function loss in the currently untreatable conditions of ischemic retinopathy or optic neuropathy.

CD4⁺ T cells propagate a prolonged phase of neurodegeneration in glaucoma.¹⁴ A previous report describes sequential events initiated by retinal neuron stress or insults that up-regulate HSPs and activate HSP-specific T-cell responses, contributing to the pathogenesis of progressive RGC loss in glaucoma.¹⁴ Retinal reperfusion injury also induces HSP up-regulation, followed by microglia and T-cell activation and a prolonged phase of RGC loss similar to that seen in the glaucomatous retina. Consistently, T-cell deficiency attenuated reperfusion injury–induced RGC loss, whereas adoptive transfer of T cells from diseased mice into *Rag1*^{−/−} mice exacerbated RGC loss. Together, these findings suggest a similar mechanism in operation underlying the causes of ischemic injury–induced neural damage.

Acute ischemic injury to the retina is known to be accompanied by early activation of innate immune cascades, disruption of the blood retinal barrier, and leukocyte infiltration.^{35,36} This study discovered that these immune responses led to CD4⁺ T-cell infiltration, which peaked at approximately 2 weeks and persisted for 4 weeks after reperfusion injury. In agreement with findings in an ischemic stroke model of the brain, CD4⁺ T cells that are involved in retinal ischemia primarily consist of IFN-γ⁺ Th1 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 naive T cells, which further recruit T cells into the retina under a compromised blood retinal barrier.^{9,32} Studies have found that adaptive immune responses play an essential role in the pathogenesis of many neurodegenerative processes, including ischemic stroke and traumatic brain injury.^{13,35,38–40} Immunodeficiency observed in severe combined immunodeficiency, *Rag1*^{−/−} mice, and *IFN-γ*^{−/−} mice led to attenuated central nervous system injury and reduced infarct size relative to immunologically intact mice in ischemic stroke and traumatic brain injury models.^{38–40} Adoptive transfer of activated or 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 central nervous system.^{39,40} These data are in line with the current study data in the retinal ischemic model and support that activated CD4⁺ T cells are highly injurious. This study found in the glaucomatous mouse model that donor CD4⁺ T cells isolated from the diseased mice, but not control mice, infiltrated the retina of *Rag1*^{−/−} mice after adoptive transfer and participated in the process of neural damage. The observation that local administration of anti-CD4 antibody via intravitreal injections attenuated retinal ischemic injury–induced RGC loss further supports that infiltrated T

cells are critically involved in the propagation of neural damage after retinal reperfusion injury.

The finding that RGC loss was not detected in the contralateral eye of mice with ischemic injury suggests the prerequisite for local injury or inflammation and/or compromised blood-retina barrier to enable T-cell–mediated neural damage. A similar observation was noted 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 naive mice.¹⁴ Correspondingly, proinflammatory 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 seen in glaucomatous mice.¹⁴ Collectively, these data support that acute ischemic injury led to CD4⁺ T-cell–mediated responses that involve particularly Th1-type cells in the retina and eye-draining LNs, which contribute to a prolonged phase of RGC degeneration and/or retinal neural damage.

Importantly, the present study revealed a two-phase neurodegeneration process after acute ischemic neural injury: an initial phase that occurs immediately after acute retinal ischemia, which is not affected by the presence of T cells (a T-cell–independent phase) and a prolonged T-cell–dependent phase of neuron loss that lasts >2 months and is abolished in *Rag1*^{−/−} and *TCRβ*^{−/−} mice. In line with this observation, CD4⁺ T-cell–mediated neurodegeneration is also involved in ischemic stroke or traumatic brain injury as well as in the experimental models of glaucoma.¹⁴ Current treatment for neural damage caused by ischemic stroke has a short therapeutic window, normally within 4 hours after injury. T-cell deficiency (*Rag1*^{−/−} or *TCRβ*^{−/−}) limited the RGC loss to approximately 25% as measured at 4 weeks after ischemia because it eliminated the prolonged T-cell–dependent phase of neural damage, whereas wild-type mice exhibited >50% RGC loss by 4 weeks and continued to progress thereafter. Local administration of antibodies that targeted CD4⁺ T cells starting 3 days after injury effectively attenuated neural damage and rescue retinal function. In the present study, this attenuation was achieved through weekly eye injections for 3 weeks. Although the current study (Supplemental Figure S6) and others found little effect of weekly eye injections on the levels of proinflammatory cytokines in mice, their effects on retinal microglia and Müller cells should be carefully considered and further evaluated.⁴¹ In any case, these results point to a much wider therapeutic window for ischemic stroke than the currently available treatment and the exciting possibility of targeting CD4⁺ T cells to prevent neuron loss.

These results suggest an opportunity for currently untreatable conditions of ischemic retinopathy, such as that caused by central retinal artery occlusion and nonarteritic anterior ischemic optic neuropathy. Administration of anti-CD4 antibodies was more effective than administration of IFN-γ antibodies, implicating the involvement of not only a IFN-γ–secreting Th1 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. A study in a traumatic brain injury model found a 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 the $\alpha 4$ integrin that prevent lymphocyte infiltration into postischemia brain injury and methylprednisolone (an agent with inflammation-inhibitory effects and T-cell suppressant) also reduced neural damage^{42,43} and promoted tissue healing.⁴⁴ In these studies, broader-spectrum immunosuppressants and systematic administration via intravenous or intraperitoneal injection were used. Because the eye is more accessible than the brain, it enables local administration of antibodies, thereby preventing systemic adverse effects. This study found that intravitreal injection of antibodies that specifically target CD4⁺ T cells is sufficient to prevent RGC and retinal function loss after ischemic optic neuropathy.

In summary, this study has provided novel evidence showing previously unappreciated roles for CD4⁺ T cells in postischemic 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 a previous report showing 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 central nervous system ischemic stroke.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.04.011>.

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