

Disrupting the CD47/SIRP α Axis Protects Against Traumatic Optic Neuropathy

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Abstract

Traumatic optic neuropathy (TON) is a serious injury to the optic nerve that can lead to vision impairment or blindness. Current treatment options include corticosteroids or surgical decompression. There is no consensus on the treatment of TON due to insufficient evidence supporting the effectiveness of current options. Cluster of differentiation 47 (CD47) is a transmembrane protein that can be upregulated on apoptotic cells. It prevents their phagocytic clearance or efferocytosis by communicating a “don’t eat me” signal when it binds to signal regulatory protein α (SIRP α) on myeloid cells. We found that CD47 is upregulated in the retina after the optic nerve crush (ONC) injury model of TON, along with the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α). Antibody neutralization of TNF- α after ONC resulted in blunted CD47 levels whereas treating ex-vivo retinal explants with recombinant TNF- α resulted in increased levels of CD47, indicating that TNF- α stimulates CD47 expression. To investigate the therapeutic potential of blocking the CD47/SIRP α axis after ONC, we intravitreally treated WT mice with anti-CD47, anti-SIRP α , or anti-IgG (control) post-ONC injury. Anti-CD47 treatments resulted in improved efferocytosis, attenuated neurodegeneration, improved retinal thickness, and enhanced retinal function as compared to anti-IgG control treatment post-ONC. Conversely, anti-SIRP α did not show statistically significant neuronal preservation after ONC. Myeloid-specific SIRP α deletion reduced retinal thinning and only showed a neuroprotective trend for retinal ganglion cell count and function after ONC. Collectively, our data indicate that the CD47-SIRP α axis is a promising therapeutic target to improve retinal outcomes following ONC injury, with CD47 neutralization proving more effective than targeting SIRP α .

Introduction

Traumatic optic neuropathy (TON) is a condition where the optic nerve is damaged due to direct or indirect trauma. The optic nerve is a bundle of nerve fibers that transmits visual information from the eye to the brain. TON can occur due to a variety of causes, including blunt or penetrating trauma, compression, or stretching of the optic nerve. The symptoms of TON can include sudden loss of vision, pain around the eye, and changes in color perception. TON is often irreversible, and vision loss is permanent.¹ TON is characterized by inflammation, immune response and neuronal degeneration following injury. The lack of understanding of the mechanisms involved in tissue injury and repair after TON is a significant barrier to developing novel clinically effective treatments.

Efferocytosis is the phagocytosis of apoptotic cells (AC). It is a highly evolutionarily conserved process that maintains tissue homeostasis.² Myeloid cells, including microglia and macrophages, are known as professional phagocytes and are integral for the clearance of AC. Under healthy conditions, “eat me” signals, such as phosphatidylserine (PtdSer) displayed on AC, encourage the engulfment of the dying cell by myeloid cells.³ Efferocytosis is crucial for resolving the inflammatory response and facilitating tissue repair after injury. Since efferocytosis relies on these “eat me” signals to clear AC, blockade of anti-efferocytotic “don’t eat me” signaling on AC, such as cluster of differentiation 47 (CD47) - signal regulatory protein α (SIRP α) axis, has the potential to increase the efficacy of efferocytosis after injury.⁴

The rapid clearance of dying cells reduces the deleterious effect of inflammation caused by lingering AC.⁵

CD47 is an integrin-associated protein ubiquitously expressed on neurons and other cell types in the human body. It is a commonly known “don’t eat me” signal.² CD47 binds to the ligand SIRPα, a regulatory membrane glycoprotein expressed on the surface of microglia and macrophages. CD47-SIRPα signaling works as an immune checkpoint by which myeloid cells can recognize cells displaying CD47 as “self”.⁶ When CD47 binds to SIRPα, the tyrosine residues of SIRPα are phosphorylated, activating Src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2, impeding phagocytosis potentially via deactivation of non-muscle myosin-IIA.¹ Upregulation of CD47 has been implicated in dysfunctional efferocytosis in cardiovascular diseases and cancer.^{1,3} The inflammatory cytokine, tumor necrosis factor-α (TNF-α) has been implicated in CD47 upregulation in these conditions.⁷ Yet, the regulation of CD47 in retinal injury remains poorly defined. Recent studies explored the role of CD47 in mouse models of retinitis pigmentosa and autoimmune uveitis.^{8,9} Interestingly, CD47 augmentation protects cone photoreceptors from genetic retinal degeneration, whereas its deficiency ameliorates ocular autoimmune inflammation, emphasizing that CD47 may play diverse and disease-specific roles in ocular conditions that we cannot fully untangle at this time. However, the contribution of the CD47-SIRPα axis to injury progression in TON is unknown.

In this study, we demonstrate that TNF-α mediates CD47 upregulation in the inner retina following optic nerve crush (ONC) model of TON, which inhibits efferocytosis and retinal tissue repair. By disrupting the CD47-SIRPα axis through the use of neutralizing antibodies or by specifically deleting SIRPα in myeloid cells, we are able to protect against retinal neurodegeneration and improve retinal function after ONC. These finding provides proof of concept that targeting the CD47-SIRPα pathway could be a promising therapeutic approach for treating TON.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences approved all animal experiments. Mice were housed at 25°C with a light/dark cycle and fed ad libitum maintenance chow. Wild-type (WT) C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

Generation of myeloid-specific SIRPα KO mice

SIRPα^{fl/fl} on a C57BL/6J background (obtained from Dr. Hisashi Umemori at Harvard Medical School) were used to generate myeloid-specific SIRPα KO mice (M-SIRPα KO).¹⁰ SIRPα^{fl/fl} mice were bred with mice expressing tamoxifen-inducible Cre recombinase under the direction of the Cx3cr1 promoter [Jackson Laboratory Stock No: 020940 (*Cx3cr1*^{CreERT2})] as described above to generate microglia and

MΦ cell-specific SIRPα knockout (*Cx3cr1^{CreERT2}*; SIRPα^{fl/fl} or M-SIRPα KO) mice.¹¹ Littermate SIRPα^{fl/fl} mice were used as controls. Mice genotyping is shown in **(Fig. S1A, B)**. We have characterized these mice and confirmed that they display normal retina morphology and function **(Fig. S1C-E)**. To account for any off-target effects of tamoxifen, both SIRPα KO and SIRPα^{fl/fl} mice were treated with tamoxifen (75mg/kg) (Sigma Aldrich) as an intraperitoneal injection daily for 5 consecutive days using a 20 mg/mL stock of tamoxifen in corn oil. This causes deletion of SIRPα in microglia and infiltrating monocytes/MΦ since both cell types express *Cx3cr1^{CreERT2}*.^{12–14} Experiments were conducted 1 week after the last dose of tamoxifen, when myeloid Cre activity is high and tamoxifen is eliminated from the circulation, since it is a neuroprotective agent.^{15,16}

Optic nerve crush model

Mice were anesthetized with isoflurane and treated with a single subcutaneous injection of buprenorphine extended-release (3.25 mg/kg) for analgesia. To induce optic nerve crush (ONC) injury, the left optic nerve was exposed and crushed for 3 seconds using a self-closing forceps (Dumont, #N7 forceps) as we previously described.^{17,18} The contralateral eye was utilized as a non-injury sham.

Antibody Treatment

To investigate the relationship between TNF-α and CD47 expression, WT mice received a 1 μL intravitreal injection of anti-TNFα (conc. 9.81 mg/ml, BioXCell) on day 3 post-ONC injury. For CD47/SIRPα antibody treatment experiments, WT mice received 1 μL intravitreal injection of CD47 (conc. 8.31 mg/ml, BioXCell), SIRPα (conc. 5.47 mg/ml, BioXCell), or IgG (conc. 10.22 mg/ml, BioXCell) antibodies on day 4 post-ONC. Injections were given via a microinjection syringe pump (World Precision Instruments). Antibody treatment did not affect normal retina morphology and function in sham eyes **(Fig. S1F-H)**.

Antibody labeling

For antibody penetration studies, SIRPα antibodies were labeled with rhodamine (Thermo Scientific) and injected on day 4 post-ONC, whereas saline injection served as the control. Mice were sacrificed at days 4 through 7 post-ONC. Eyeballs were collected and fixed in 4% paraformaldehyde, and retinas were dissected into flatmounts on slides. Zeiss LSM 880 with Airyscan confocal microscope was used for fluorescence imaging.

We conducted the anti-CD47 and anti-SIRPα neutralization in two separate experiments with each set having their anti-IgG control group. Since there were no differences between the OCT and PERG for the anti-IgG group from the two experiments, we decided to pool this data in our final analysis to increase the N value for our studies. This was not done for the NeuN labeling data since fluorescence intensity can differ from one immunolabeling/imaging condition to another.

Western Blot

For vivo experiments, retinas were collected, snap-frozen, and stored at -80°C for later analysis. Retinas were then homogenized and suspended in RIPA lysis buffer (Thermo Scientific) and centrifuged at 20,000 × *g*. The protein content was then estimated using Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Scientific). The samples' lysates were run on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies used were anti-TNF-α (Abcam, cat #41-0MT0-25CF) and anti-CD47 (R&D Systems, cat #PIPA5109497).

Immunofluorescent labeling

Eyeballs were collected and fixed in 4% paraformaldehyde overnight at 4°C. After fixation, they were transferred to phosphate-buffered saline (PBS) and stored at 4°C until further analysis.

Neurodegeneration was assessed at 14 days post-ONC. Retinas were dissected from eyeballs, and neurons were immunolabeled using the neuronal marker anti-NeuN (MilliporeSigma, cat # ABN78MI) and flatmounted. Four images per retina were taken from the retinal mid-periphery, and neurodegeneration was analyzed as the NeuN⁺ area in the ganglion cell layer (GCL) using ImageJ software. CD45⁺ (Fisher, AF114) cells and CD68⁺ (Cell Signaling, #97778) cells were manually counted. In a different cohort of mice, eyeballs were collected on day 4 post-ONC for the anti-TNFα treatment groups. CD47 levels were analyzed using anti-CD47 (R&D Systems). Images were taken using a Zeiss LSM 880 with an Airyscan confocal microscope. Lastly, for SIRPα antibody rhodamine labeling as aforementioned, CD45 (Fisher, AF114) was used for colocalization of the two.

Efferocytosis Index

For the efferocytosis indexes, eyeballs were collected and fixed in 4% paraformaldehyde overnight at 4°C on day 5 post-ONC. The efferocytosis index was assessed using Click-iT™ Plus TUNEL Assay (Invitrogen) and anti-IBA-1 (FUJIFILM Wako, cat #011-27991). The co-localization of TUNEL⁺ and IBA-1⁺ cells was manually counted. The efferocytosis index was calculated as the ratio of TUNEL⁺ IBA-1⁺ cells to TUNEL⁺ cells.

Retinal Optical Coherence Tomography (OCT)

Pupils were dilated using 1% tropicamide (Akron Pharmaceuticals, IL) and mice were anesthetized with ketamine/xylazine prior to optical coherence tomography (OCT, Bioptigen Inc., Durham, NC) imaging to assess retinal thickness between groups (rectangular volume mode – 3 frames/scan, 1000 A scan/B scan × 100 B scan, 1.4 mm × 1.4 mm). Images were analyzed using InVivoVue™ Diver 2.4 software (Bioptigen Inc., Durham, NC).

Pattern Electroretinography (PERG)

Celeris electroretinogram system (Diagnosys LLC, Cambridge, UK) was used to assess retinal PERG functional differences between groups. Mice were dark-adapted overnight before PERG assessment at 14 days post-ONC. Pupils were dilated using 1% tropicamide (Akron Pharmaceuticals, IL) and mice were

anesthetized with ketamine/xylazine prior to testing. PERG was performed under dim red lighting. Eyes were assessed with one pattern stimulator used for the test eye and a full field stimulator used as a reference electrode for the fellow eye. The amplitudes of P1, N2, and |P1-N2| peaks were used to assess retinal functionality.

Retinal Explants

WT mice were anesthetized and sacrificed via cervical dislocation. Eyecups were harvested after sacrifice, and retinas were immediately harvested from eyecups in ice-cold HBSS containing 1% penicillin-streptomycin. Retina was placed face up in transwell inserts (Thermo Scientific) in a 24-well plate supplied with Neurobasal-A medium (Invitrogen) with 2% B27 (Invitrogen), 1% N2 (Invitrogen), and 2 mM GlutaMAX (Invitrogen) as we described previously.¹⁷ Retina explants were kept in a humidified incubator at 37°C with 5% CO₂ and treated with 0, 20, or 50 ng/ml of recombinant mouse TNF- α (R&D Systems) for 24 hours. Then, they were snap-frozen for Western blotting or fixed in 4% paraformaldehyde for fluorescent immunolabeling.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 10 software, and data were presented as mean $\pm$ standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant. A student's t-test was used for two-group comparisons, with Welch's correction if unequal variances. One or two-way analysis of variance (ANOVA) with Tukey's post hoc test was used for comparison of multiple groups.

Results

CD47 and TNF- α are upregulated in the retina after ONC injury

ONC is a widely used model to study TON. After ONC injury, cells in the ganglion cell layer (GCL) become apoptotic, and immune cells are recruited to the retina. We first conducted a time course of AC induction after crush using TUNEL labeling of WT retina cross-sections, which showed peak accumulation of AC at 5 days after ONC (Fig. 1A). Flatmount immunolabeling showed proliferation/infiltration of IBA-1 + myeloid cells starting at day 2 and persisting till day 7. Immune cells' recruitment to AC facilitates their clearance through a reparative process called efferocytosis, which is tightly regulated by 'eat me' and 'don't eat me' signals. The 'don't eat me' signal, CD47, has been found to be upregulated in certain pathological conditions, including cancers and atherosclerosis.^{3,19} Here, we investigated the role of CD47 and its ligand SIRP α in TON. To do this, we first performed ONC on WT mice with the contralateral eye used as a non-injured sham. Western blot of whole retinal lysates revealed an upregulatory trend of CD47 on days 4 and 5 post-ONC that reached statistical significance at day 10 post-ONC (Fig. 1C, D). Immunolabeling of retina sections revealed an increase in CD47 levels in the retinal GCL, which is the

layer most susceptible to injury in the ONC model (Fig. 1E), as well as in the crushed area of the optic nerve (Fig. 1F). Investigation of publicly available single-cell RNA-seq data revealed upregulation of CD47 mRNA in the mouse retinal ganglion cells within the first 48 hours post-ONC injury (Fig. S2).

Previous research has identified the cytokine tumor necrosis factor- α (TNF- α) as an inducer of CD47.¹⁹ Interestingly, we found a sustained upregulation of TNF- α (both 26 and 52 kDa bands) in whole retinal lysates, starting at day 3 post-ONC injury and continuing through day 14 (Fig. 1G-I). Publicly available data revealed that TNF- α is mainly expressed by retinal microglia and vitreous macrophages (hyalocytes) in the human eye (Fig. S3A), with microglia being its main source in the mouse retina (Fig. S3C). Interestingly, SIRP α exhibited a similar expression trend to TNF α , but it was not as exclusive to microglia as TNF α (Fig. S3B, D). After ONC, both TNF α and SIRP α showed sustained expression in mouse retinal myeloid cell populations, and their expression increased over time (Fig. S4, 5).

TNF- α mediates CD47 upregulation after ONC injury.

Since the upregulation of TNF- α protein levels precedes CD47 upregulation, we conducted a series of experiments to determine whether TNF- α plays a role in the upregulation of CD47 in retinal ganglion cells. To do this, we incubated WT retinal explants with increasing concentrations of recombinant TNF- α (0, 20, and 50 ng/ml) for 24 hours. Explants were then processed for Western blot and immunolabeling. Western blotting of retinal lysates revealed a significant upregulation of CD47 following treatment with TNF- α at concentrations of 20 and 50 ng/ml (Fig. 2A, B). To determine if TNF-mediated CD47 upregulation is associated with retinal ganglion cell death, we immunolabeled retinal explant flatmount with NeuN (neuronal marker), which showed a neurodegeneration trend at 20 ng/ml and significant degeneration with 50 ng/ml recombinant TNF- α treatment, * $p < 0.05$ (Fig. 2C, D). This indicated to us that TNF-mediated CD47 upregulation precedes TNF-induced neuronal death.

To further determine the contribution of TNF- α to CD47 upregulation in vivo, we administered anti-TNF- α antibodies intravitreally to WT mice after ONC, using the contralateral eyes as sham controls. Since we first saw the upregulation of TNF- α on day 3 post-ONC, we chose day 3 to treat the mice with anti-TNF- α , 1 μ L intravitreal injection (conc. 9.81 mg/ml) administered to the injured and contralateral sham eyes. Retinas were collected at day 4 post-ONC for Western blot and immunolabeling. Western blot analysis of whole retinal lysates revealed no significant difference in CD47 expression between groups. However, treatment with anti-TNF- α resulted in downregulation of TNF- α , suggesting successful neutralization (Fig. 2E-G). Immunolabeling revealed upregulation of CD47 in the retinal GCL after ONC, whereas treatment with anti-TNF- α abrogated this increase (Fig. 2H-I). This suggested a localized TNF-mediated upregulation of CD47 after ONC that was not detectable in the Western blot conducted on whole retinas.

CD47 neutralization improves efferocytosis after ONC

The upregulation of the 'don't eat me' signal CD47 after ONC suggested to us impaired phagocytic clearance of efferocytosis of AC after crush. To investigate the role of CD47/SIRP α in efferocytosis and

injury resolution after ONC, we tested the therapeutic potential of CD47 and SIRPα neutralizing antibodies, using anti-IgG as a control. Since CD47 shows delayed protein upregulation, we employed a delayed intravitreal administration of the anti-CD47 neutralizing antibodies at day 4.

On day 5 post-ONC, retinas were collected for efferocytosis index which was determined based on the colocalization of TUNEL (an apoptosis marker) and IBA-1 to mark microglia and macrophages (Fig. 3A). Retinas from mice treated with anti-CD47 showed decreased AC number after ONC as compared to the anti-IgG-treated group (Fig. 3B). Furthermore, anti-CD47 treatment resulted in an increase in the efferocytosis index measured as the ratio of myeloid cell-associated AC to free AC (Fig. 3C), suggesting that CD47 neutralization improves microglia-mediated clearance of dead cells in the retina following ONC injury. We then aimed to evaluate how early enhanced efferocytosis influences the number of phagocytic cells at a later time point following ONC. Employing the same treatment paradigm with anti-CD47 on a different cohort of WT mice and conducting retinal immunolabeling at day 14 showed a strong induction of CD45+/CD68+ phagocytic cells after crush as compared to shams. Retinas from mice treated with anti-CD47 revealed a downward trend in the count of CD45+/CD68+, although this trend was not statistically significant (Fig. 3D-F).

CD47 neutralization attenuates neurodegeneration and improves retinal function

To determine whether the increased efferocytosis with CD47 neutralizing resulted in a neuroprotective effect, we treated WT mice with anti-CD47 or anti-IgG as described above on day 4 and retinal thickness, function and neuron count were assessed at day 14 after ONC. NeuN labeling revealed attenuated neurodegeneration with anti-CD47 treatment as compared to anti-IgG (Fig. 4A, B). Retinal thickness assessed using OCT revealed rescue of retinal ganglion cell complex thickness with anti-CD47 (Fig. 4C, D). Retinal ganglion cell function evaluated with PERG showed improved P1-N2 amplitude difference with anti-CD47 treatment (Fig. 4E-H). Collectively, CD47 neutralization resulted in neuroprotection and improved retinal thickness and function after ONC.

Anti-SIRPα neutralization attenuates neurodegeneration and improves retinal function

We next aimed to determine if neutralization of SIRPα, the receptor for CD47 on myeloid cells will result in similar neuroprotection to CD47 neutralization. WT mice were treated with anti-SIRPα or anti-IgG after ONC using the same experimental protocol employed for CD47. Mice were subjected to OCT and PERG to assess retinal thickness and function, then retinas were collected for flatmount immunolabeling at day 14 after ONC.

Anti-SIRPα treatment showed a slight neuroprotective trend assessed with NeuN flatmount immunolabeling, although it was not statistically significant (Fig. 5A, B). Similarly, retinal thickness

assessed by OCT imaging did not show a difference between the anti-SIRPα and anti-IgG groups after ONC (Fig. 5C-D). Retinal functionality assessed using PERG was also not different between groups with only a slight protective trend with anti-SIRPα treatment (Fig. 5E-H). Since we did not detect robust differences between the anti-SIRPα and anti-IgG treated groups, we sought to confirm that antibodies were retained in the retina after treatment. We injected rhodamine-labeled anti-SIRPα on day 4 and collected retinas from day 4 through day 7 post-ONC. Antibody signal was retained in the retina through day 7 and colocalized with the leukocyte marker CD45, suggesting that antibody treatment was successful in binding to SIRPα expressed on myeloid cells (Fig. 5I).

Myeloid-specific SIRPα deletion preserves retinal thickness after ONC

We then used M-SIRPα KO mice and SIRPα^{f/f} mice to further investigate the role of the CD47/SIRPα axis in ONC and to confirm the anti-SIRPα results shown above. For all experiments, SIRPα KO mice and their SIRPα^{f/f} littermates received tamoxifen injections for a consecutive 5-day period. Since tamoxifen has been shown to be neuroprotective, experiments began one week after tamoxifen injections.²⁰ On day 14 post-ONC injury, myeloid deletion of SIRPα resulted in a neuroprotective trend for NeuN immunolabeling. However, the results were not significant (Fig. 6A, B). Retina thickness assessed using OCT showed improved GCC thickness in the M-SIRPα KO retinas as compared to SIRPα^{f/f} littermates after ONC (Fig. 6C, D). Functionality, measured via PERG, showed an improvement trend in RGC function for M-SIRPα KO but was not significant (Fig. 6E-H). Collectively, the deletion of myeloid SIRPα demonstrated less effective protection compared to the blockade of CD47.

Discussion

In this study, we reported four major findings: First, we found upregulation of the 'don't eat me' signal CD47 in the inner retina during the subacute phase of the ONC injury. Second, we have identified TNF-α as the mediator of CD47 upregulation. Third, our study provides the first evidence of neuronal protection and improved retinal function with blockade of the CD47/SIRPα axis after ONC injury. Fourth, better outcomes were achieved with CD47 neutralization over SIRPα blockade or deletion; and this protection was mediated by enhanced efferocytosis of AC by myeloid cells.

CD47 is a myeloid cell immune checkpoint that inhibits efferocytosis (the removal of AC) by macrophages and other phagocytic cells. It achieves this by interacting with the SIRPα receptor on these cells, thus preventing them from engulfing and removing cells that express CD47. While CD47 is expressed on almost all healthy cells to help them be recognized as "self" by the immune system, its upregulation has been linked to pathological conditions. CD47 upregulation on AC has been linked to disease progression in cancer and atherosclerosis through impaired efferocytosis. Our data is the first to implicate CD47 upregulation in the pathogenesis of TON. Since TNF-α upregulates CD47 in many cell types, we investigated its regulation of CD47 after ONC.²¹ We provided compelling evidence, in vivo and

ex vivo, implicating TNF- α as a regulator of CD47 after ONC. TNF- α upregulates CD47 primarily in the retinal GCL after ONC.

Publicly available data included in this paper show that TNF- α is mainly produced by myeloid cells in the retina. TNF- α plays a complex role in RGC injury and survival, which could be explained by the opposing effects of its receptors, TNFR1 and TNFR2. TNFR1 is known to induce apoptosis, and an earlier study showed that deletion of TNFR1 decreased neurodegeneration after ONC; however, this effect was only prominent after 2 weeks from injury.²² TNFR1 appears to mediate similar RGC neurodegeneration following retinal ischemia, while TNFR2 plays a protective role.²³ Inhibition of TNF with etanercept, which blocks both soluble and membrane-bound TNF- α , increased RGC survival after ONC.²⁴ Interestingly, a study using TNF- α KO mice showed enhanced neurodegeneration in these mice after crush, suggesting a protective role of TNF- α . The authors confirmed this protective role of TNF- α using intravitreal pretreatment with exogenous TNF- α . However, TNF- α treatment was protective only when given 5 days prior to ONC, and TNF- α treatment at 7 days after crush did not affect neuronal survival.²⁵ However, we have found that recombinant TNF- α led to worsened RGC neurodegeneration in a dose-dependent manner in retinal explants. Interestingly, TNF- α upregulated CD47 in a sub-lethal dose, suggesting that the induction of CD47 by TNF- α is independent of its pro-apoptotic effects.

Improving efferocytosis after neuronal injury has been increasingly studied in recent years for its potential therapeutic effect.^{26,27} Enhancing efferocytosis has been shown to improve traumatic brain injury outcomes.²⁷ Here, we provide the first evidence that efferocytosis plays a reparative role in TON. We have found that blocking the “Don’t eat me” signal, CD47, with neutralizing antibodies results in increased efferocytic efficiency, as determined by the colocalization of TUNEL + cells with IBA-1 + cells. Furthermore, injured retinas treated with anti-CD47 had a decrease in AC, possibly due to the increased interaction and clearance of dying cells by myeloid cells. Neutralization of CD47 ameliorated neurodegeneration, preserved inner retina thickness, and rescued retinal ganglion cell function after ONC injury. These findings complement other studies, which have also found blockade of the CD47/SIRP α axis in stroke and uveitis improves neuronal cell survival and functional outcomes.^{8,28} Interestingly, despite a neuroprotective trend, we did not observe a significant preservation in NeuN + neurons or retinal thickness and function following antibody blockade of SIRP α . Our data show that CD47 may be a more effective therapeutic target than SIRP α in ONC, which can be due to the fact that blockade of CD47 can enhance efferocytosis independent of SIRP α . A recent study has shown CD47 neutralization unmasks pro-phagocytic ligands such as SLAMF7, which CD47 can bind to through cis-interaction.²⁹ Furthermore, CD47 functions involve more than just phagocytosis. For example, when thrombospondin-1 (TSP-1) binds to CD47, it blocks nitric oxide signaling, typically promoting vasodilation and cell survival. Therefore, blockade of CD47 would also block CD47/TSP-1 signaling, possibly leading to increased blood and oxygen supply to the site of injury, resulting in improved cellular metabolism, function, and survival.³⁰ Additional studies are needed to define these pathways in TON further. Another possible explanation for the discrepancy between CD47 and SIRP α neutralization is that the expression of SIRP α

on neurons has been shown to counterintuitively enhance microglial phagocytosis during retinal development by acting as a decoy receptor to prevent microglial SIRPα-CD47 interaction.³¹

To dissect the role of myeloid cell SIRPα, we generated and utilized a myeloid-specific knockout of SIRPα under the Cx3cr1 cre to further investigate the inhibition of myeloid SIRPα in ONC. We chose the microglia-specific Cx3cr1 cre since the ONC model is thought to involve a predominantly microglial response to injury.^{32–34} Surprisingly, we found that myeloid deletion of SIRPα only showed significant preservation of retinal thickness. The fact that antibody neutralization or myeloid deletion of SIRPα failed to confer significant neuroprotection or functional improvement suggests that SIRPα inhibition alone may be insufficient to halt ONC-induced neurodegeneration. Our data show that blockade of the ligand CD47 and the receptor SIRPα do not always have the same impact, with the two possibly having diverse signaling in ONC. More work is needed to dissect these mechanisms.

In summary, our studies highlight the therapeutic potential of enhancing efferocytosis by blocking the CD47-SIRPα axis in ONC, resulting in neuroprotection and improved retinal function. We show that CD47 neutralization is more effective in ONC. Since CD47 neutralizing antibodies and small molecule inhibitors are already in clinical testing for certain cancers, this will facilitate their translation to the clinic for TON.³⁵ Furthermore, the delayed treatment employed in this study suggests a wide window for intervention with these antibodies.

Declarations

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Figures

Figure 1.

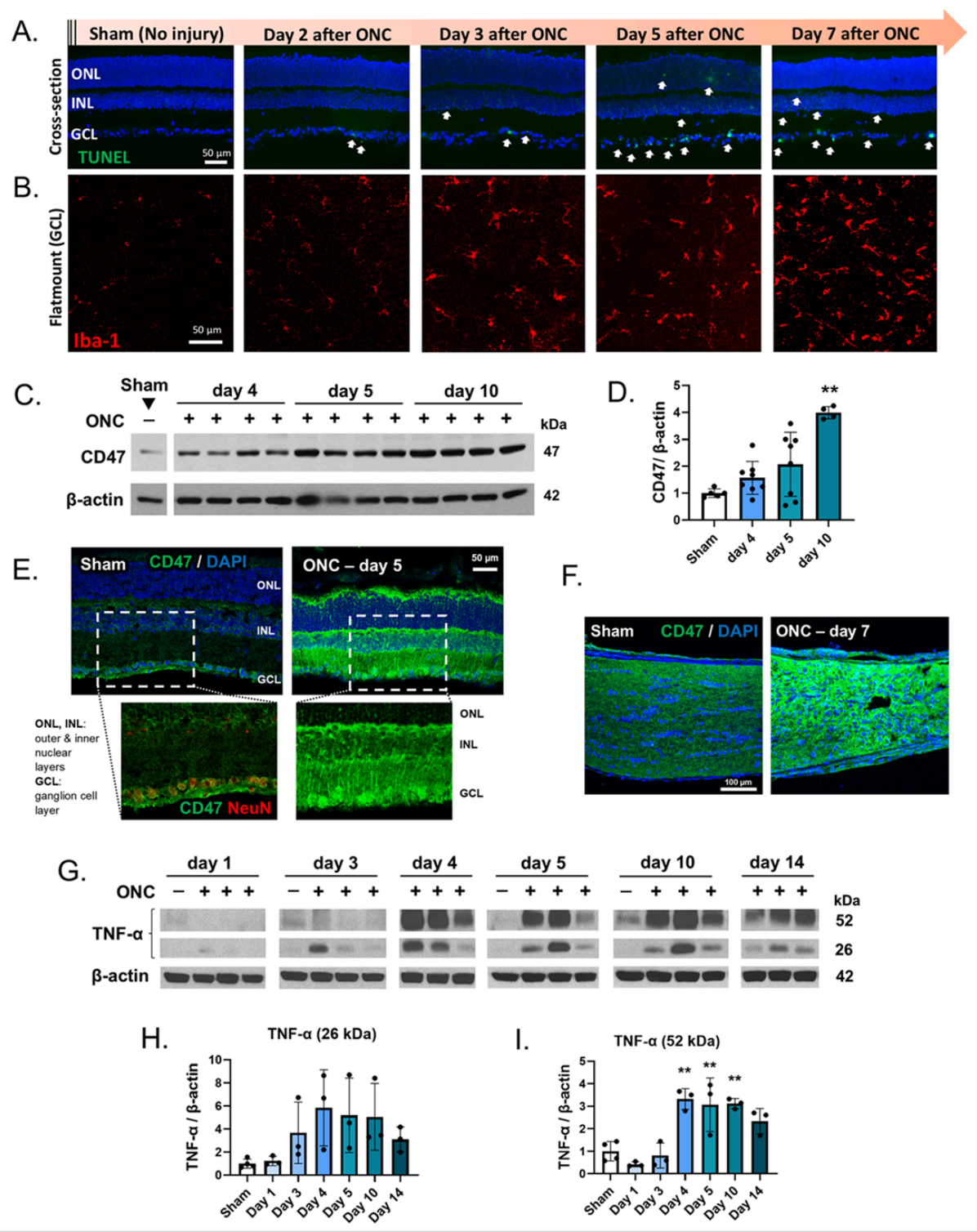


Figure 1

Temporal dynamics of apoptosis, myeloid cell infiltration, and CD47 and TNF- α expression following ONC injury. **A.** The time course of apoptosis, marked by TUNEL (green) labeling on WT retina cross-sections and counterstained with DAPI (blue), shows a progressive accumulation of AC (arrows) that peaks around day 5 after ONC. **B.** Myeloid cell response after ONC, as determined by flatmount immunolabeling at the same time points, shows activation (enlarged soma) and proliferation of IBA-1⁺ (red) myeloid cells

(microglia/macrophages). Images are representative of N=3 animals. GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. **C, D.** Western blot analysis of WT retina lysates reveals a CD47 upregulatory trend at days 4 and 5, with a statistically significant increase at day 10 after ONC. N=4-8 per group, ** $p < 0.01$ vs sham, one-way ANOVA. **E.** Immunolabeling of WT retina sections shows strong upregulation of CD47 in the inner retinal layer including the GCL at day 5 after ONC. **F.** Immunolabeling of WT optic nerve longitudinal sections shows upregulation of CD47 at the crush site 7 days after ONC as compared to sham. **G-I.** Time course of TNF- α protein (membrane-bound 26 kDa and trimer 52 kDa forms) levels, determined by Western blot on WT whole retinal lysates, shows upregulation of TNF- α after ONC. N=3-4 per group. Western blot quantification of retinal lysates reveals significant upregulation of soluble TNF- α (52 kDa) starting day 4 post-ONC. N=3-4 per group, ** $p < 0.01$ vs sham, one-way ANOVA.

Figure 2.

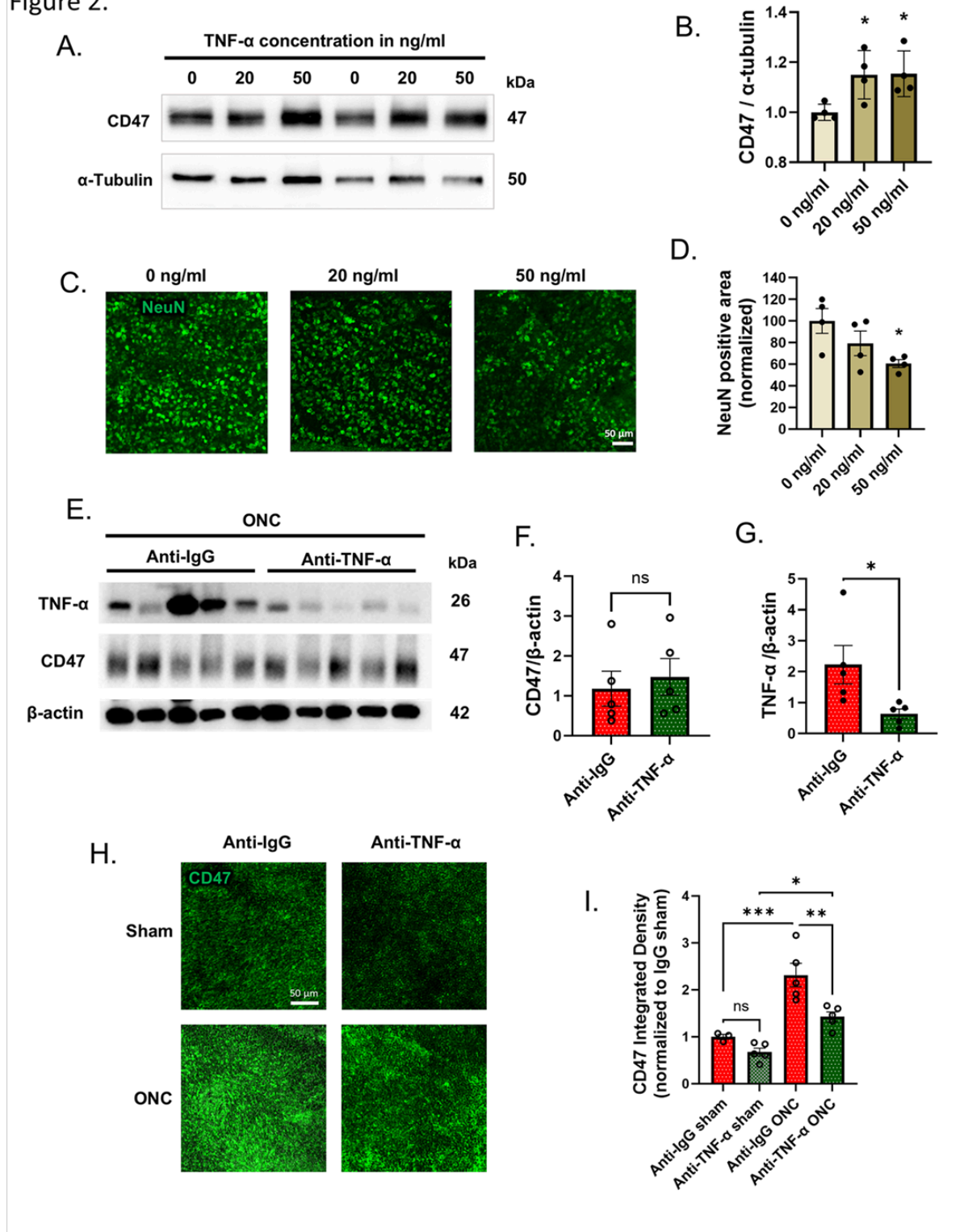


Figure 2

TNF- α upregulates CD47 in the retinal ganglion cell layer. **A, B.** Western blot on WT retinal explants treated with 20 or 50 ng/ml recombinant TNF- α shows significant upregulation of CD47. N=4, * p <0.05 one-way ANOVA. **C, D.** Flatmount immunolabeling of retina explants using the neuronal marker NeuN shows significant neurodegeneration with 50 ng/ml recombinant TNF- α treatment. N=4, * p <0.05 one-way ANOVA. **E-G.** Western blotting on retinas from WT mice treated intravitreally with TNF- α neutralizing

antibodies after ONC shows decreased TNF- α levels as compared to anti-IgG control. Whole retina lysates showed no difference in CD47 protein levels between the two groups. N=5, *p<0.05, unpaired t-test, ns=not significant. **H, I.** Immunolabeling of retina flatmounts from WT mice treated with TNF- α neutralizing antibodies after ONC shows downregulation of CD47 in the GCL as compared to anti-IgG-treated retinas. N=3-5, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA.

Figure 3.

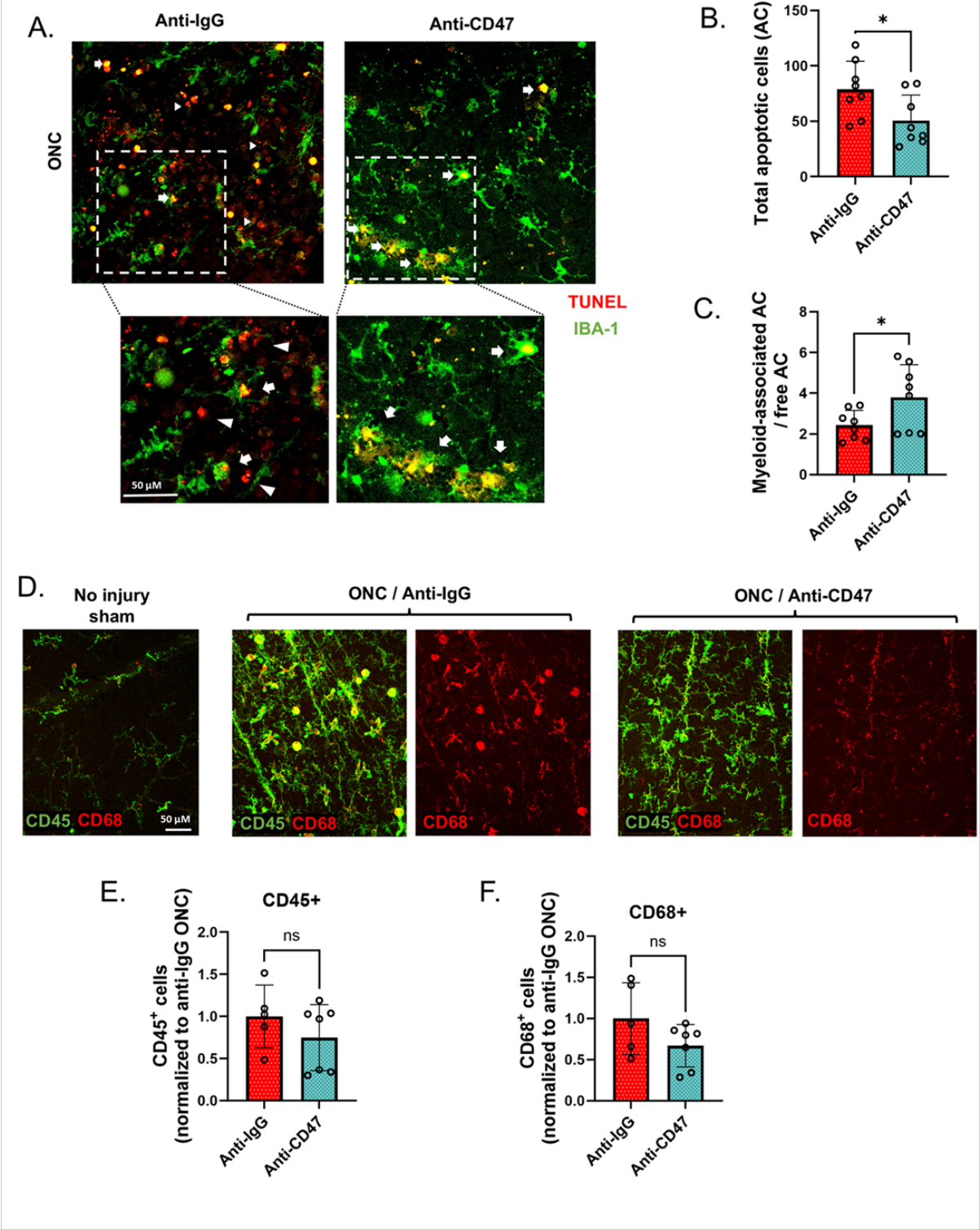


Figure 3

CD47 neutralization facilitates efferocytosis. **A-C.** Immunolabeling of retina flatmounts from WT mice treated with CD47 neutralizing antibodies shows decreased TUNEL⁺ AC and increased efferocytosis index determined by colocalization of AC with IBA-1⁺ myeloid cells 5 days post-ONC. The efferocytosis index was measured as the ratio of myeloid cell-associated AC to free AC. N=8-9, *p<0.05, unpaired t test. **D-F.** Treatment with anti-CD47 after ONC shows a decreasing trend but not a significant reduction in the number of CD45⁺ or CD68⁺ phagocytic cells at day 14 after ONC. N=5-7, unpaired t test, ns=not significant.

Figure 4.

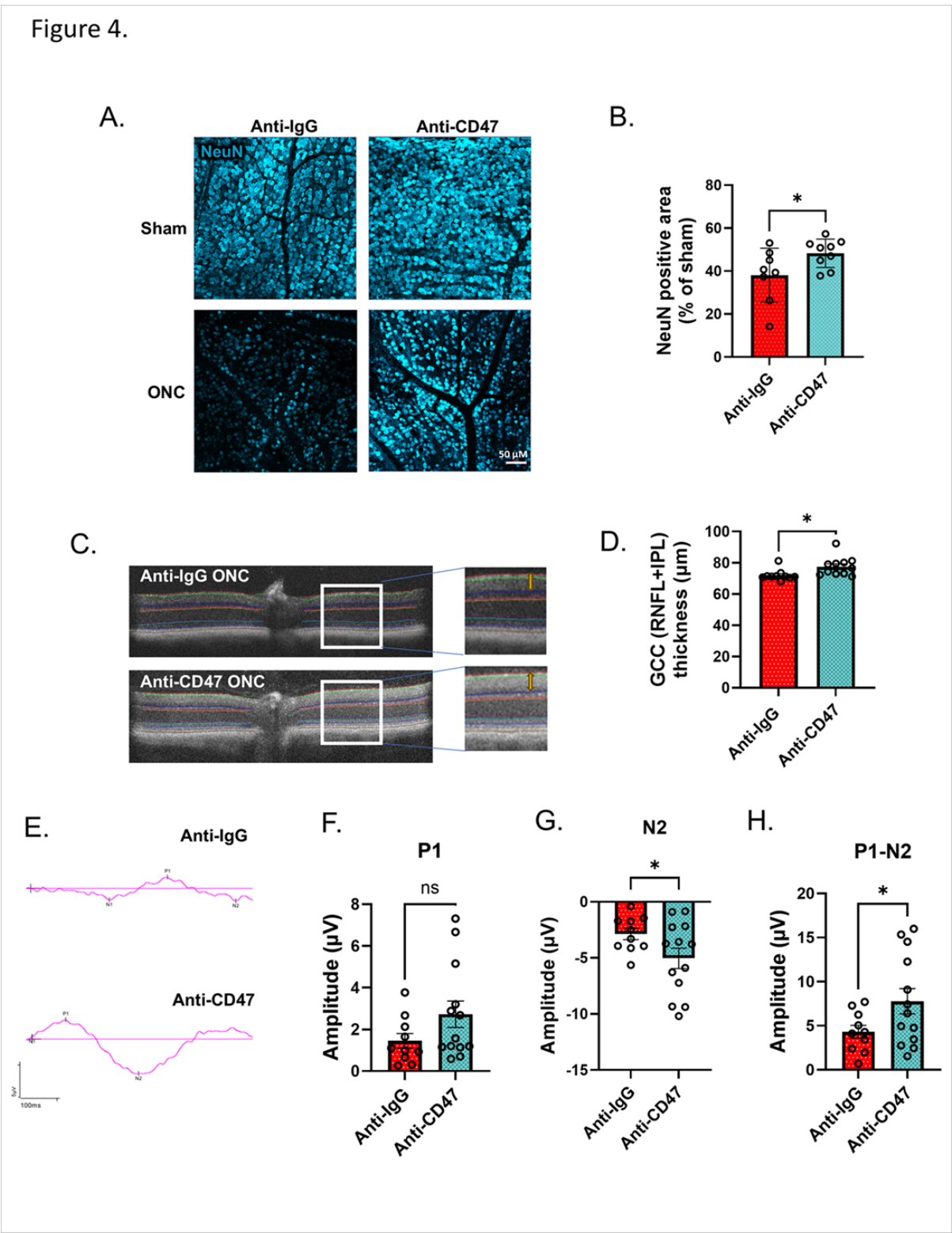


Figure 4

CD47 neutralization is neuroprotective after ONC. **A, B.** WT retina flatmount immunolabeling at day 14 post-ONC using the neuronal marker, NeuN, shows preservation of neurons at the GCL with anti-CD47 treatment as compared to anti-IgG control treatment. N=8-9 per group, * $p < 0.05$, unpaired t test. **C, D.** OCT imaging of ONC injured treatment groups shows rescue of the thickness of the ganglion cell complex (GCC) with anti-CD47 treatment as compared to anti-IgG group. N=10-11 per group, * $p < 0.05$, Welch's unpaired t test. GCC was calculated as retinal nerve fiber layer (RNFL) + inner plexiform layer (IPL). **E-H.** PERG representatives and quantification show improved retina function with anti-CD47 treatment after ONC, determined as the amplitude difference between the P1 and N2 peaks. N=10-13 per group, * $p < 0.05$, Welch's t test, ns=not significant.

Figure 5.

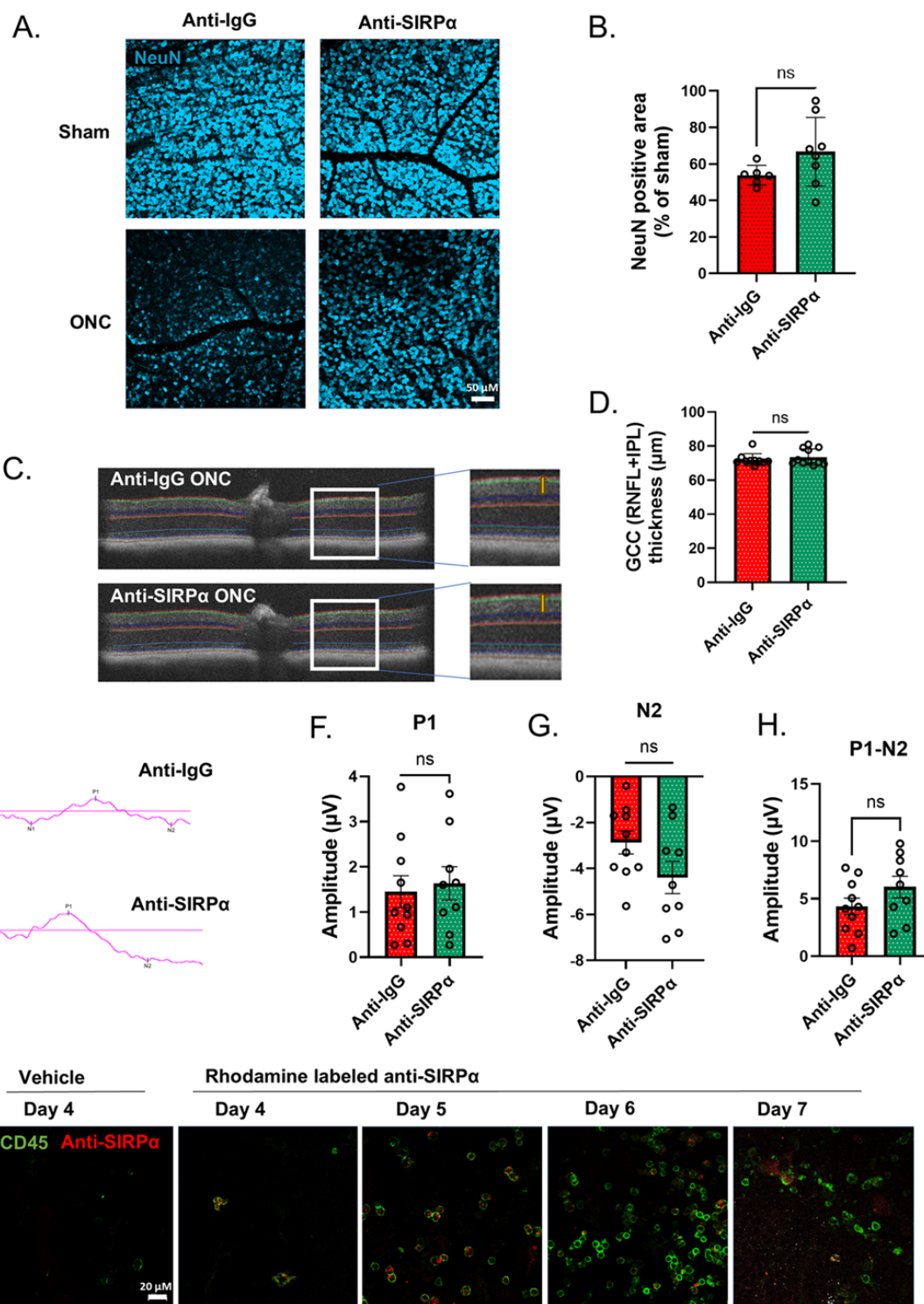


Figure 5

Anti-SIRPα treatment shows a slight neuroprotective trend, A, B. WT Retina flatmount immunolabeling at day 14 post-ONC using the neuronal marker, NeuN. Treatment with anti-SIRPα shows a neuroprotective trend but did not reach statistical significance. N=6-8, ns=not significant, unpaired t test. **C, D.** OCT imaging after ONC and treatment with anti-SIRPα or anti-IgG shows no difference in GCC thickness between the groups. N=10-12 per group, unpaired t test. **E-H.** PERG representatives and quantification for

treatment groups show a trend towards improved retina function with anti-SIRPα as compared to anti-IgG after ONC, but did not reach significance. N=9-10 per group, unpaired t test, *p<0.05. I. Retina flatmounts showing rhodamine-labeled anti-SIRPα antibodies intravitreally injected on day 4 are retained in the retina up to day 7 after ONC and colocalized with the leukocyte marker, CD45 (green).

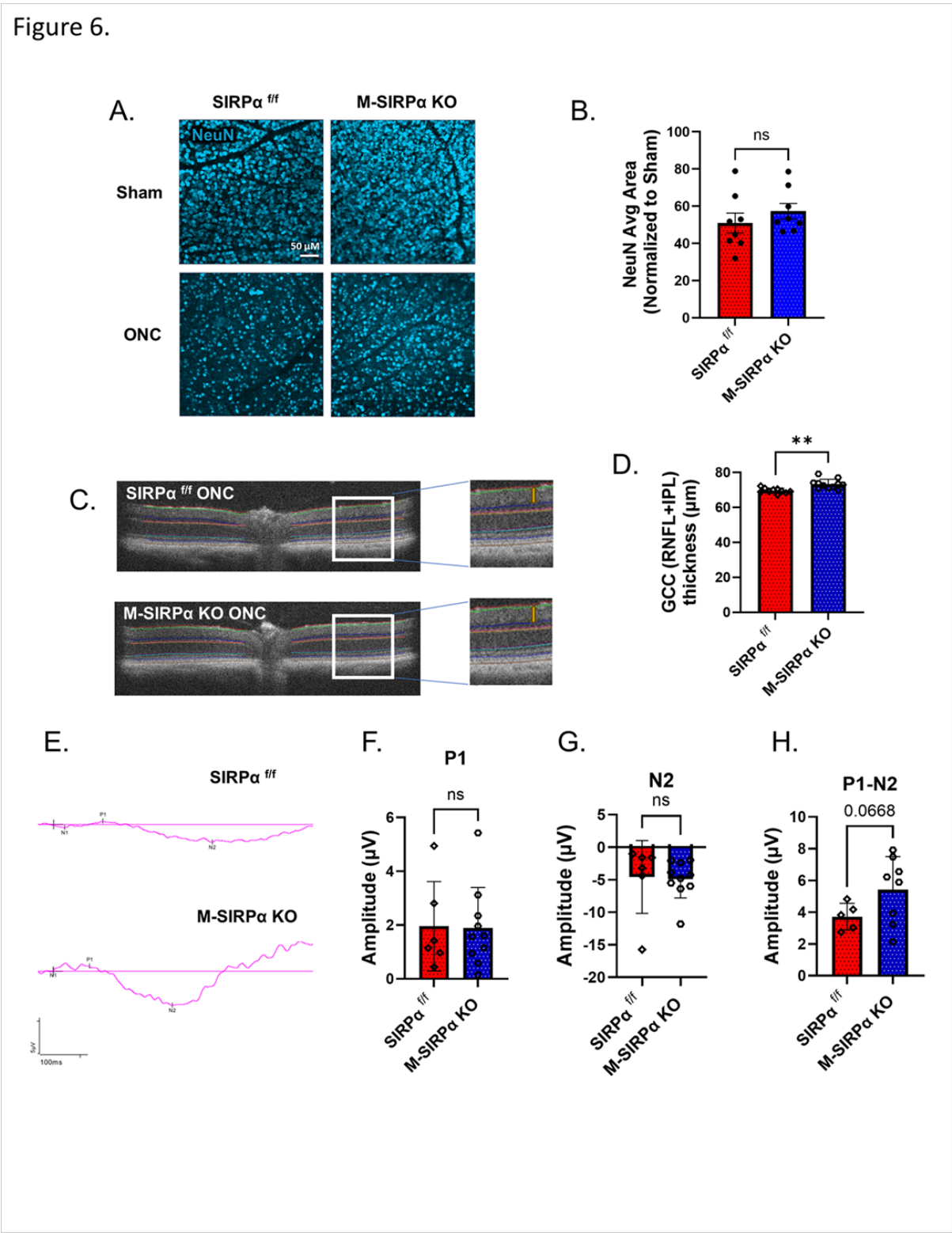


Figure 6

Myeloid-specific SIRP α deletion preserves retinal thickness after ONC. **A-B.** Immunolabeling of retina flatmounts using the neuronal marker, NeuN, shows a neuroprotection trend in the myeloid-specific SIRP α KO mice at day 14 post-ONC injury as compared to SIRP $\alpha^{f/f}$ littermates but did not reach statistical significance. N=8, unpaired t test, ns=not significant. **C-D.** OCT imaging shows a statistically significant preservation of GCC thickness in the myeloid-specific SIRP α KO mice vs SIRP $\alpha^{f/f}$ littermate controls. N=7 per group, unpaired t test. **E-H.** PERG representatives and analysis show a trend towards improved retinal function in M-SIRP α KO mice vs SIRP $\alpha^{f/f}$ littermate controls at day 14 after ONC, but this did not reach statistical significance (p=0.06). N=6-8 per group, unpaired t test.

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