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Damage-Induced Senescent Immune Cells Regulate Regeneration of the Zebrafish Retina

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Abstract

Zebrafish spontaneously regenerate their retinas in response to damage through the action of Müller glia (MG). Even though MG are conserved in higher vertebrates, the capacity to regenerate retinal damage is lost. Recent work has focused on the regulation of inflammation during tissue regeneration, with temporal roles for macrophages and microglia. Senescent cells that have withdrawn from the cell cycle have mostly been implicated in aging but are still metabolically active, releasing a variety of signaling molecules as part of the senescence-associated secretory phenotype. Here, we discover that in response to retinal damage, a subset of cells expressing markers of microglia/macrophages also express markers of senescence. These cells display a temporal pattern of appearance and clearance during retina regeneration. Premature removal of senescent cells by senolytic treatment led to a decrease in proliferation and incomplete repair of the ganglion cell layer after N-methyl-D-aspartate damage. Our results demonstrate a role for modulation of senescent cell responses to balance inflammation, regeneration, plasticity, and repair as opposed to fibrosis and scarring.

Graphical Abstract

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Gregory J. Konar and Zachary Flickinger have equally contributed to this research.

Author Contributions

G.J.K.: conceptualization, experimentation, data analysis, and writing the original draft. Z.F.: conceptualization, experimentation, writing, reviewing, and editing. S.S.: experimentation and data analysis. K.T.V.: experimentation. C.E.L.: experimentation. C.D.: experimentation. A.L.: experimentation. W.L.: experimentation. J.G.P.: conceptualization, funding acquisition, investigation, methodology, supervision, writing, review, and editing.

Conflict of Interest Statement

There are no conflicts of interest to be reported by any of the authors of this article.

Supplementary Materials

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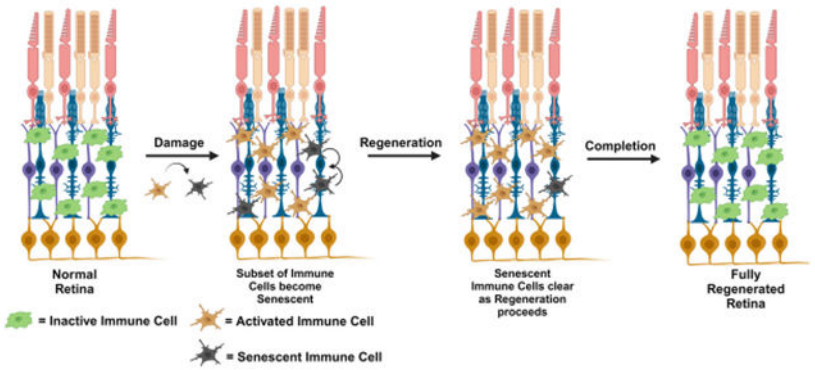

Introduction

Degenerative eye diseases such as age-related macular degeneration (AMD), retinitis pigmentosa, or Stargardt disease affect millions of people each year. Each of these conditions is marked by profound atrophy and the loss of one or more layers of the retina, which are unable to regenerate or recapitulate lost retinal neurons after damage. In contrast to humans and other mammals, zebrafish (*Danio rerio*) have the endogenous ability to regenerate their retina following damage[1,2]. Damage to the zebrafish retina triggers a conserved mechanism of cellular regeneration driven by Müller glia (MG), which serve as resident stem cells[3–7]. Post-injury, MG dedifferentiate and undergo asymmetric division for both self-replacement and the generation of progenitor cells that proliferate to replace any lost neurons[1,2]. Despite the conservation of MG, regeneration is blocked in higher vertebrates. However, recent work and comparative analyses of zebrafish, chick, and mouse retina regeneration suggest that activation or derepression of gene regulatory networks can enable limited reprogramming of mammalian MG[8–13]. Mechanistic understanding of how zebrafish regenerate is crucial to discover novel factors that promote complete regeneration of the mammalian retina.

Recent papers have demonstrated a role for the immune system and inflammation in retina and retinal pigment epithelium regeneration[14–20]. These effects appear to be mediated by resident and infiltrating macrophages and microglia that show dynamic transcriptional and morphological changes during regeneration[21–25]. There are diverse populations of retinal microglia/macrophages, but the precise identity of distinct subsets of these cells throughout regeneration remains to be determined, and it is not clear how changing patterns of secreted signaling molecules from these diverse subpopulations regulate the initiation and resolution of retina regeneration[10,25–29].

Senescence is a cellular process triggered by a myriad of molecular factors, including aging, oxidative stress, telomeric shortening, DNA damage, inflammation, or epigenetic dysfunction[30]. During senescence, cells exit the cell cycle in a programmed growth arrest event regulated by p53, p16 (Cdkn2a), and p21 (Cdkn1a)[31–33]. Despite withdrawing from the cell cycle, senescent cells remain metabolically active and adopt a senescence-associated secretory phenotype (SASP), which promotes the secretion of cytokines and growth factors that directly modulate the local cellular microenvironment[34,35]. While senescence has been

more well studied related to aging and cancer, it also plays a seemingly paradoxical role in development and regeneration[36]. Transient initial exposure to pro-inflammatory SASP signaling appears to be required for regeneration, whereas chronic SASP exposure restricts the regeneration and proliferation of somatic or stem-like cells[31,37]. Transient versus chronic senescent cell detection is apparent in models of spinal cord injury when comparing mammals and fish, where mammals show a chronic accumulation of senescent cells while fish show transient senescent cell expression[38]. Targeting senescent cells with senolytic drugs (senotherapy) has been shown to aid in regeneration in multiple mammalian tissues, including the liver, kidney, muscle, and spinal cord[39–43].

In the retina, senescent cells are often associated with age-related diseases such as AMD, and their targeting in retinal pigmented epithelial (RPE) cells has shown success in reducing AMD-like phenotypes in animal models of AMD[44–47]. However, outside of the RPE, senescence of the inner retinal layers has seldom been investigated for its utility in contributing to the regeneration of retinal neurons. Here, we provide the first evidence that in the adult zebrafish retina, there is a temporally conserved, transient senescent response to damage mediated by a subset of macrophages/microglia. Premature clearance of these senescent cells inhibits proliferation and leads to incomplete repair of the retinal ganglion cell (RGC) layer after N-methyl-D-aspartate (NMDA) damage.

Materials and Methods

Zebrafish husbandry and maintenance

Wild-type AB zebrafish were maintained at 28.0 °C on a 12:12 hour light–dark cycle. A random assortment of male and female zebrafish was used during the experimentation. All experiments were performed in accordance with Vanderbilt University Institutional Animal Care and Use Committee approval #M1800200.

NMDA damage model

Adult zebrafish aged 5–10 months were anesthetized using a 0.016% Tricaine solution (MESAB, Acros Organics). The sclera of the left eye was cut using a sapphire blade, and 0.5 uL of a 100 mM NMDA solution was intravitreally injected. An equal volume of 1X phosphate-buffered saline (PBS) was used as an injection control for the NMDA damage.

Pharmacologic treatments

For Metformin, a stock solution of 50 mM Metformin (PHR1084, Millipore Sigma, USA) was prepared in distilled water. Prior to use, the Metformin was diluted 1:500 in fish water to achieve a final working concentration of 100 uM in the tank. Metformin water was changed daily to ensure consistent drug levels throughout the experiment. For ABT-263 (Navitoclax), a 30 uM working solution of ABT-263 was made using 5% Tween-80, 20% PEG-300, and 2% DMSO in PBS, and 0.5 uL were injected intravitreally.

Immunohistochemistry (IHC), EdU labeling, and TUNEL assays

Fluorescent staining was performed as previously described[48]. Briefly, zebrafish eyes were collected and fixed in 4% paraformaldehyde overnight at 4 °C. Eyes were then moved to a

sucrose gradient and stored overnight in 30% sucrose at 4 °C. Following sucrose treatment, eyes were moved to a 2:1 Cryomatrix with 30% sucrose solution for 2 hours and embedded in Cryomatrix (Fisher Scientific). Sections were collected on a Leica cryostat using charged Histobond slides (VWR International, USA) at a thickness of 15 um, then dried on a heating block and stored at −80 °C. For IHC, slides were warmed and then incubated in 1X PBS for 30 min to rehydrate. Slides were then subjected to antigen retrieval as previously described[49], before cooling to room temperature (RT). Blocking solution (3% donkey serum, 0.1% Triton X-100 in 1X PBS) was applied to the slides and incubated for 2 hours at RT before incubation with primary antibodies overnight at 4 °C. The antibodies used for IHC are listed in Table 1.

After primary antibody incubation, slides were washed and stained with secondary antibodies in 1% donkey serum, 0.05% Tween-20 in PBS along with 1:1000 To-Pro-3 (Invitrogen) for 2 hours at RT. Secondary antibodies used include donkey anti-mouse 488 (1:500), donkey anti-rabbit 488 (1:500), donkey anti-mouse cy3 (1:500), and donkey anti-rabbit cy3 (1:500) (all Jackson Immuno). Slides were then washed, mounted, and coverslipped using VectaShield Antifade Mounting Medium (Vector Labs).

For EdU labeling, adult zebrafish received 0.5 uL intravitreal injections of 10 mM EdU at both 24 hours post-damage (hpi) and 48 hpi. Incorporation was detected using the Click-iT™ Plus EdU Cell Proliferation Kit (555 fluorophore, ThermoFisher, USA). For TUNEL staining, a Click-iT™ Plus TUNEL Assay Kit was used (488 fluorophore, ThermoFisher, USA).

Senescence-associated b-galactosidase (SA-βGal) staining

SA-βGal staining was performed on retina sections using a kit from Cell Signaling Technology. Briefly, slides were warmed on a heating block and incubated in PBS. SA-βGal staining solution was warmed to 37 °C with the pH adjusted to between 5.9 and 6.1 and then applied to retinal sections and incubated overnight at 37 °C. Samples were then washed and incubated with a 1:1000 dilution of To-Pro-3 (ThermoFisher) in PBS for nuclear staining, mounted using VectaShield antifade mounting medium (Vector Labs), and sealed using clear nail polish.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on retinas dissected from NMDA-treated eyes as described[50]. Briefly, retinas were collected into Trizol (Invitrogen) for total RNA purification. mRNA was converted to cDNA using an Accuscript High-Fidelity 1st Strand cDNA Synthesis Kit, and RT-qPCR of the cDNA samples was performed using SYBR Green (Bio-Rad Laboratories). Samples were run on a 96-well plate using a Bio-Rad CFX96 real-time system. RT-qPCR runs were normalized to 18S rRNA levels. Analysis was performed using the ΔΔCt method. The primer sets used are listed in Table 2.

Imaging and image processing

For imaging of IHC, EdU, or TUNEL staining, a META Zeiss LSM Meta 510 confocal microscope was used through the Vanderbilt Center for Imaging Shared Resource (CISR)

Core. Slides stained with SA-βGal and ToPro were imaged using a Nikon AZ100M Widefield microscope through the Vanderbilt CISR Core. Confocal images were processed using Zen Blue version 3.1, with follow-up analyses done using ImageJ. AZ100M images were first processed using NIS-Elements Viewer 5.21 and then subsequently analyzed using ImageJ.

Quantification and statistical analysis

Experiments involving immunostaining and cell number quantification were evaluated in an unbiased and blinded manner. Briefly, stained slides that comprised the optic nerve region were evaluated in the central retina. Both dorsal and ventral regions were counted to encompass the entire retinal region, spanning approximately ~300–400 mm from the optic nerve in either direction. Regions of extreme structural damage or nearer to the ciliary marginal zone were excluded from counts so as not to cause artificially elevated numbers. Cell counts from two independent central retina sections were counted and averaged for each injected eye. For gap counting, slides were stained with HuC/D primary antibodies and To-Pro-3 nuclei markers, and regions of missing RGCs were counted under a fluorescent microscope. Sphericity analysis was performed using Imaris Software version 10.1 with MatLab plug-ins. Significance was calculated using either a student's t-test or a two-way ANOVA with Tukey's post hoc test for intergroup comparisons in GraphPad Prism 10.0.1. All data represented in graphical form are the average ± standard error of the mean. The number of fish used in each experiment is described in the figure legends.

Results

Detection of senescent cells after NMDA damage

Traditionally, senescence has been viewed as largely associated with aging, but recent evidence supports a role for senescence in development and regeneration[36,51,52]. After spinal cord damage, senescent cells can be transiently detected in zebrafish, whereas in mice, senescent cells accumulate over time, and targeting senescent cells in mice with senolytic drugs can improve tissue repair[38]. We sought to determine if senescent cells are detectable in the regenerating retina, whether they follow a similar burst and clearance profile as in the zebrafish spinal cord damage model, and whether temporal treatment with senolytics might affect repair. To first determine whether we could detect senescent cells, we damaged zebrafish retinas by intravitreal injection of NMDA, which causes excitotoxic damage to RGCs (Fig. 1A)[8]. We then stained tissue sections for SA-βGal, a pH-dependent lysosomal marker of senescent cells[53–55]. In undamaged or PBS control retinas, we rarely, if ever, detected SA-βGal+ cells (Fig. 1B). In contrast, after NMDA damage, SA-βGal+ cells were detectable above background levels starting at 2 days post-injury (dpi) ($p < 0.05$). The levels of SA-βGal+ cells peaked around 12 dpi ($p < 0.0001$) and then began to decline at times that coincided with the end stages of regeneration (Fig. 1B). We used nuclear staining with To-Pro 3 to visualize the different layers of the retina, which showed that the SA-βGal+ cells were detected across multiple layers of the retina after NMDA damage (Fig. 1C).

We never observed SA-βGal staining in undamaged or PBS control retinas, but because tissue fixation and imaging for SA-βGal staining can potentially affect quantitative

analysis of senescent cells[56], we quantified the levels of p21 as a second marker of senescence[30,38,57]. For this, we isolated retina RNA at the same time points as in Figure 1A and conducted RT-qPCR for p21 because none of the available antibodies against p21 proved reliable for immunostaining and colocalization of zebrafish retinal sections. We found that expression of p21 peaked at 12 dpi and then began to return to near-baseline levels by 18 dpi in a pattern very similar to the detection pattern of SA-βGal (Fig. 1D). We also confirmed that the senescent cells we detected were not apoptotic by demonstrating an absence of colocalization between SA-βGal staining and TUNEL staining (Supplemental Fig. 1).

The expression pattern and timing of appearance and clearance of senescent cells in the inner retina are comparable to the temporal appearance and clearance of SA-βGal+ cells after spinal cord damage in zebrafish, albeit on a shorter time frame, and stand in contrast to senescent cell accumulation in the mammalian retina or RPE after damage[38,44,58,59]. Reduced detection or clearance of senescent cells as regeneration proceeds may constitute a key difference between regenerative responses in zebrafish compared to higher vertebrates.

Macrophages/microglia become senescent after retina damage

The location of SA-βGal+ cells within the damaged retina appeared similar to previous immunolocalization of microglia and macrophages[21]. Thus, we immunostained NMDA-injected retinal sections at 1–5, 8, and 15 days post-damage with 4c4 antibodies, which target the galectin-3 antigen in zebrafish microglia and macrophages[60]. We detected an increase in 4c4+ cells after damage with a peak of 4c4+ macrophages/microglia at early times post-damage[21–23,25,61] (Fig. 2A). Only a subset of 4c4+ macrophages/microglia were found to colocalize with SA-βGal+ cells, and colocalization was only observed after 1 dpi, coinciding with the induction of a regenerative response (Fig. 2A). Quantitation of the relative levels of colocalized 4c4+/SA-βGal+ cells showed that approximately 20%–45% of 4c4+ cells colocalize with SA-βGal+ at early times post-damage, peaking at 4 dpi ($n = 4–6$ retinas) ($p < 0.0001$) before decreasing to control levels by 15 dpi ($n = 3$ retinas) ($p > 0.05$) (Fig. 2B). Our data are consistent with an early role for macrophages and microglia in initiating retina regeneration[21,22,25,62], but additionally argue that only a subset of these cells adopt a senescent phenotype. Since senescent cells release a variety of pro-inflammatory cytokines and growth factors, our data also support experiments that have shown an essential early role for inflammation to induce regeneration[14–17,20,25,61,63–65].

To assess what percent of SA-βGal$^+$ cells colocalize with microglia/macrophages or whether other cell types express SA-βGal after damage, we quantified the number of SA-βGal+ cells in each retinal section, the total number of SA-βGal$^+$/4c4$^+$ cells, and then determined the fraction that colocalizes ($n = 3$ retinas) (Fig. 2C). We found on average that at 2 dpi, 7 of the 12 SA-βGal+ cells colocalized with 4c4+ cells, and this increased to 16 of the 20 SA-βGal+ cells colocalizing with 4c4+ cells at 5 dpi. These data suggest that the primary senescent cell type that is detectable in response to damage is derived from microglia/macrophages[16,17].

As a first test of what other cell types express SA-βGal in response to damage, we determined the fraction of SA-βGal+ cells that also immunostain for markers of photoreceptors (Zpr1), MG (glutamine synthase), and RGCs (HuC/D). We found that only

3 of the 93 RGCs expressed SA-βGal. While only 1 of 31 MG showed expression of SA-βGal+, often near their endfeet in the RGC layer (Supplemental Fig. 2). We did not detect colocalization of Zpr1+ photoreceptors with SA-βGal+ cells under conditions of NMDA damage. Together, the data show that there are other retinal cell types that become senescent after NMDA damage, but they become senescent at a far lesser frequency than microglia/macrophages.

Senotherapeutic drugs reduce proliferation after NMDA damage and inhibit regeneration

The link between senescence and aging has prompted the development of drugs to clear senescent cells. We tested the effects of two different senotherapeutic drugs on retina regeneration that clear senescent cells by different mechanisms: ABT-263 and Metformin. ABT-263 (Navitoclax) is a Bax-Bcl2 inhibitor that clears senescent cells through stimulation of apoptosis[66,67]. Metformin is a common antidiabetic drug that targets multiple aging and cancer pathways and has been shown to function as a senolytic agent through the stimulation of autophagy[68–71]. Interestingly, senotherapeutics, especially Metformin, are being used to treat AMD[58].

Treatment of NMDA-damaged retinas with Metformin (Fig. 3A) led to a significant decrease in SA-βGal$^+$ cells at 5 ($p < 0.05$) and 10 dpi ($p < 0.001$), confirming that Metformin functions as a senolytic ($n = 6–11$) (Fig. 3B,C). ABT-263 treatment also reduced the number of senescent cells in NMDA-treated retinas between 5 ($p < 0.005$) and 10 dpi ($p < 0.05$) ($n = 3–5$) (Fig. 3D,E). Finally, combination treatment of both Metformin and ABT-263 also reduced the number of senescent cells in NMDA-treated retinas between 5 ($p < 0.05$) and 10 dpi ($p < 0.005$) ($n = 5–8$) (Fig. 3F,G). These results confirm that senolytic treatment leads to a decrease in senescent cell numbers and that the damage-induced appearance of senescent cells after retina damage can be modulated by senolytic treatment.

Senolytic treatment limits cellular proliferation in NMDA damaged retinas

Following retinal or brain injury in zebrafish, an initial inflammatory response is required to initiate regeneration[14–16,20,25,63]. MG responds to inflammatory signals from dying neurons[72] and from resident and infiltrating macrophages and microglia[15,20–22,25]. Senescent cells release cytokines and other inflammatory signals that could increase the proliferation of immune cells (and potentially MG) during the initial stages of retina regeneration. To test whether senolytic clearance of SA-βGal$^+$ cells affects proliferation in the damaged retina, we injected EdU at various times after NMDA injury in the presence or absence of ABT-263, Metformin, and the combination thereof, followed by EdU staining of sections at 5 and 10 dpi. All three senoytic treatments showed modest but significant decreases in proliferation as measured by EdU incorporation (Metformin $p < 0.05$, ABT $p < 0.05$, Combo $p < 0.005$) (Fig. 4). These results support the hypothesis that senescent cells mediate signaling events, one of which induces proliferation after retina damage.

Previous work has indicated that microglia undergo morphological changes that accompany activation in response to damage[21]. We tested whether senolytic treatment might induce similar changes but did not observe significant differences in localization or polarization state (Supplemental Fig. 3).

Premature removal of senescent cells impairs RGC regeneration

We next sought to test whether senescent cells play a role in retina regeneration by determining whether clearance of senescent cells using senolytics affects regeneration after NMDA damage. For this, we stained sections for HuC/D, a marker of RGCs and amacrine cells[13], in retinas treated with either PBS, NMDA, or NMDA with a combination of Metformin and ABT-263. In PBS-treated eyes, we observed a continuous RGC monolayer with only occasional gaps or breaks across the entire time course (Fig. 5A,B). Without senolytic treatment, NMDA damage induced increased numbers of gaps and breaks in the RGC layer at 1 dpi ($p < 0.0001$) (Fig. 5A). As regeneration occurred over time, the number of gaps and breaks decreased to near-control levels by 18 dpi (Fig. 5B). In contrast, the combination of senolytic treatment in the presence of NMDA damage caused a significant increase ($p < 0.05$) in the number of gaps and breaks in the RGC that continued from 10 dpi ($p < 0.005$) out to 18 dpi ($p < 0.0001$) (Fig. 5A,B). Compared to the PBS control retinas, both NMDA-treated retinas showed a similar damage response at the onset of regeneration, but premature removal of senescent cells led to the downstream inability to regenerate the RGC monolayer. These data support a role for senescent cells to properly modulate regenerative responses in the retina.

To determine if the breaks in the RGC layer coincide with an increase in the number of apoptotic cells in damaged retinas, we used TUNEL assays to identify apoptotic cells in retinas treated with NMDA and the combination of both senolytic agents. With the addition of NMDA, we observed the expected increase in the number of $TUNEL^+$ cells throughout the retina. However, we observed a further 1.8-fold increase in the number of $TUNEL^+$ cells after the combination of Metformin and ABT-263 treatment ($p < 0.05$) (Supplemental Fig. 4A,B). This is consistent with decreased repair of the RGC layer in the presence of senolytics and the induction of apoptosis by ABT-263. Metformin has also been shown to induce apoptosis[73], but it can also induce autophagy (Supplemental Fig. 5)[74].

Discussion

Here, we show that following NMDA damage, senescent cells are detectable during zebrafish retina regeneration. Senescent cell detection after retina damage in zebrafish is transient, with a progressive accumulation of senescent cells beginning around 2–3 dpi, reaching a peak around 12 dpi, and then decreasing as regeneration proceeds. We also show that a subset of macrophages and microglia that are detectable after damage express SA-βGal and that expression of a second marker of senescence (p21) peaks at the same time that detection of SA-βGal peaks (12 dpi). Finally, we show that premature removal of senescent cells via senolytic treatment leads to decreased proliferation following NMDA damage with incomplete regeneration of the RGC layer.

Recent results support a modulatory role for inflammation during retina regeneration[14–20,25]. Senescent cells are metabolically active, releasing a variety of cytokines and growth factors, many of which are pro-inflammatory[75,76]. We propose that senescent cells play a key role in initial inflammatory responses during retina regeneration, consistent with roles for senescent cells in plasticity and regeneration[36,51,52]. The inflammatory contributions of senescent cells are part of the overall response to damage, working downstream of or in combination with

existing damage-associated molecular profiles that naturally arise after damage. We also propose that senescent signaling to MG is necessary to initiate regeneration, supporting a role for inflammatory signaling during retina regeneration. This is consistent with the observed decrease in repair of the RGC layer. Senolytic treatment limited RGC break repair after 5 dpi, indicating a defect in signals driving MG-derived regeneration of RGCs caused by the loss of senescent cells. Beyond the retina, senescent cells have been shown to play a role in fin and spinal cord regeneration in zebrafish[38,42]. The combined data support a conserved and important role for senescent cells during retina regeneration. We refer to the senescent cells that are detectable after NMDA treatment of the retina as damage-induced senescent immune (DISI) cells (Fig. 6).

Inflammation and regeneration

Zebrafish possess the innate ability to regenerate any part of their body[77]. Understanding the surrounding microenvironmental cues and cellular factors that facilitate this capability is important for a complete understanding of regeneration and the role that such signaling may play in different mechanisms of regeneration, such as blastema formation in fin regeneration and MG-derived retina regeneration. During MG-derived retina regeneration, the data support a model in which early pro-inflammatory responses give way to anti-inflammatory responses as regeneration proceeds[14–18,78].

A senescent cell burst during the early stages of regeneration followed by a later decline in senescent cells is consistent with a role in modulating the inflammatory response through the SASP. Secreted metabolites by senescent cells include cytokines, proteases, and extracellular matrix components[79]. One prominent cytokine associated with SASP signaling is interleukin-6 (IL-6), a cytokine that works to control localized inflammatory responses[80–82]. Interestingly, IL-6 displays both pro- and anti-inflammatory roles[83], and its release by senescent cells is critical for cellular reprogramming[84]. Given that a subset of immune cells become senescent and adopt the SASP, this can be viewed as an alternate source for cytokine release to maintain the transient inflammatory environment needed for proper regeneration in the zebrafish. While it remains to be seen what additional factors are being secreted by senescent cells in the retina and any temporal changes in such factors, the shift from a proinflammatory environment championed by microglia and macrophages into a more anti-inflammatory or pro-regenerative environment correlates with the temporal appearance and clearance of senescent cells that we observe during retina regeneration. This is also consistent with results from spinal cord repair experiments in mice and zebrafish, where senescent cells are cleared in regeneration-competent zebrafish but persist and even continue to increase post-spinal cord injury in mice[38].

Secondary to cytokine release from senescent cells is the release of various growth factors, including granulocyte macrophage colony-stimulating factor (gmCSF) and matrix metalloprotease 9 (mmp-9)[18,85]. GmCSF promotes retina regeneration[85], while mmp-9 is upregulated in both senescent cells and MG-derived progenitors and plays a role in modulating inflammation during the early and late stages of photoreceptor regeneration[18]. This highlights the ability of SASP factors to influence the microenvironment through paracrine and autocrine signaling that in turn modulates the responses of both immune

cells and MG. Initial upregulation of anti-inflammatory factors followed by later secretion of pro-regenerative factors appears to provide the precise microenvironmental context for promoting regeneration after damage in the adult zebrafish retina.

Senolytics and regeneration

Senolytic treatment has the potential to serve as a therapeutic mechanism for many different diseases affecting a variety of organs, including the eye[44], spinal cord[38], liver[57], as well as cancer and other age-related diseases[86–88]. Most experiments examining senescence and the use of senolytics have been performed in mammalian models where there is a distinct lack of senescent cell clearance over time[38]. Removal of senescent cells via senolytic treatment has the potential to alleviate degenerative diseases[89,90]. In the zebrafish retina, we show that senescent cell clearance occurs naturally and is temporally controlled. Zebrafish retina regeneration is generally completed within 3 weeks post-injury[1,7], and the clearance of the senescent cells we observed is consistent with this time frame. Premature clearance of senescent cells caused incomplete repair of the RGC layer supporting temporal mechanisms regulating the appearance and clearance of senescent cells, which we hypothesize coincides with the switch from pro-inflammatory to pro-regenerative responses.

Inflammation and regeneration in mice and zebrafish

Work in zebrafish retina regeneration has shown a critical role for initial inflammatory responses and dynamic changes in microglia and macrophages[15–17,21,22,25]. In contrast, work in mice indicates that microglia suppress retina regeneration[10]. This could indicate that mechanistic differences in inflammatory signaling underlie the ability to regenerate when comparing fish and mice. Alternatively, it is possible that temporal and context-specific differences can explain apparent contradictions between fish and mice regarding a role for immune cells and inflammation[20]. From spinal cord regeneration experiments[38] and our work here, it seems that the ability to clear senescent cells is crucial for regeneration.

In summary, our results demonstrate that in response to NMDA-induced ganglion cell damage, there is a population of senescent cells that display a temporal pattern of appearance and clearance as regeneration proceeds. These cells appear to be a subset of macrophages and microglia consistent with precise control of inflammation during regeneration. Inducing premature clearance of these senescent cells through either autophagic or apoptotic mechanisms showed a surprising decrease in proliferation and truncation of RGC repair following damage. Taken together, these data highlight a temporally conserved population of DISI cells that contribute to a complete and robust regenerative response in the zebrafish and provide new potential therapeutic targets to induce retina regeneration in mammals and humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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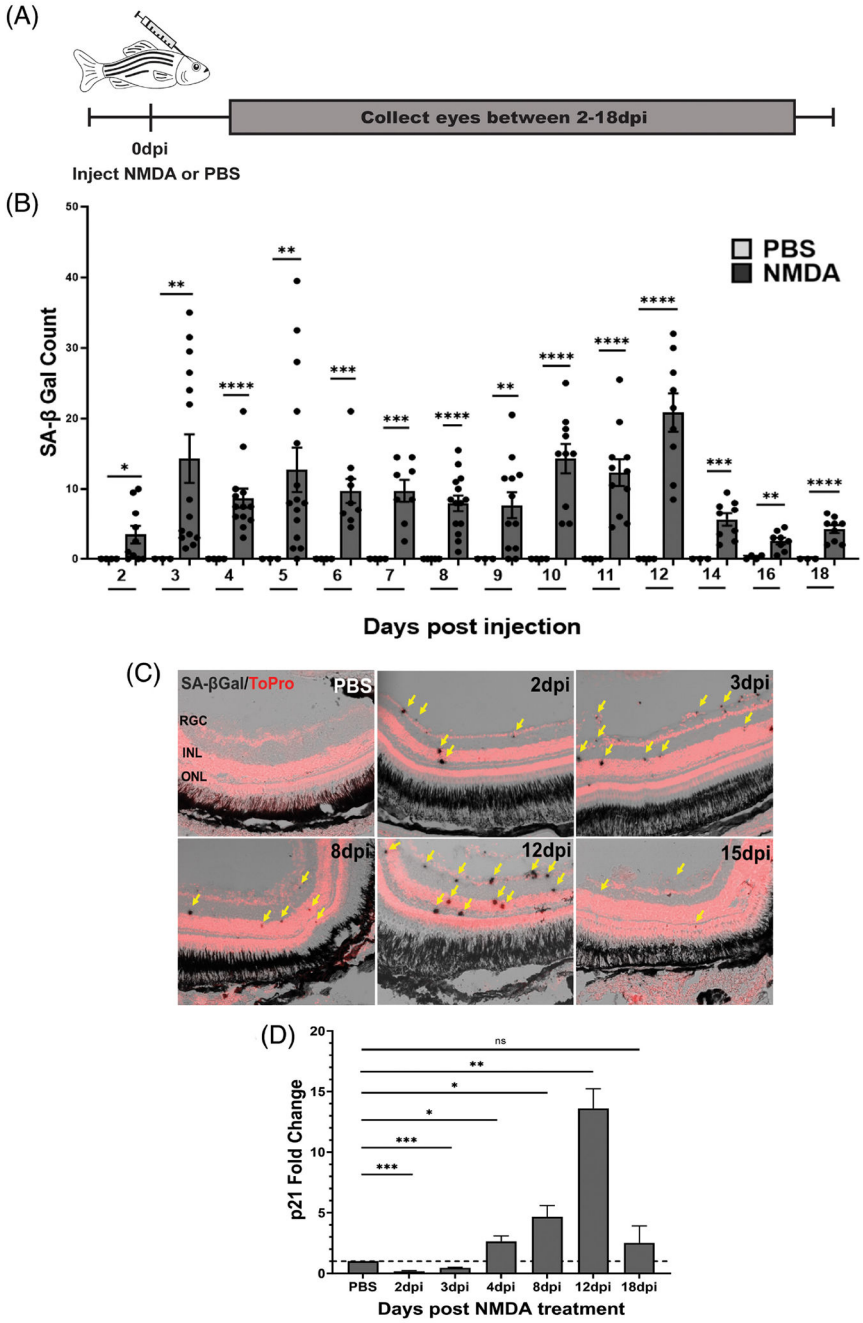

Figure 1. Detection of senescent cells in the zebrafish retina after N-methyl-D-aspartate (NMDA) damage.

(A) Schematic highlighting the experimental timeline. Wild-type AB adult zebrafish aged 5–10 months were intravitreally injected with either 0.5 uL of 100 mM NMDA or phosphate-buffered saline (PBS), and then eyes were collected between 2 and 18 days post-injury (dpi) for analysis. **(B)** Eyes injected with NMDA or PBS were sectioned and stained for senescence-associated b-galactosidase (SA-βgal) expression. Senescent cells were detected beginning at 2 dpi (n = 10), followed by declining numbers after 12 dpi (n = 9). Undamaged

or PBS control eyes were largely devoid of senescent cells. **(C)** Representative images of SA-βgal-stained retina sections. Nuclear staining was performed using To-Pro-3 in red, and SA-βgal cells are in dark gray. **(D)** RT-qPCR for p21 showed expression peaking around 12 dpi and decreasing to near basal levels by 18 dpi (n = 3). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. All graphs show individual eyes with mean ± standard error of the mean (s.e.m.). All statistics were done using the Student's t-test, where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

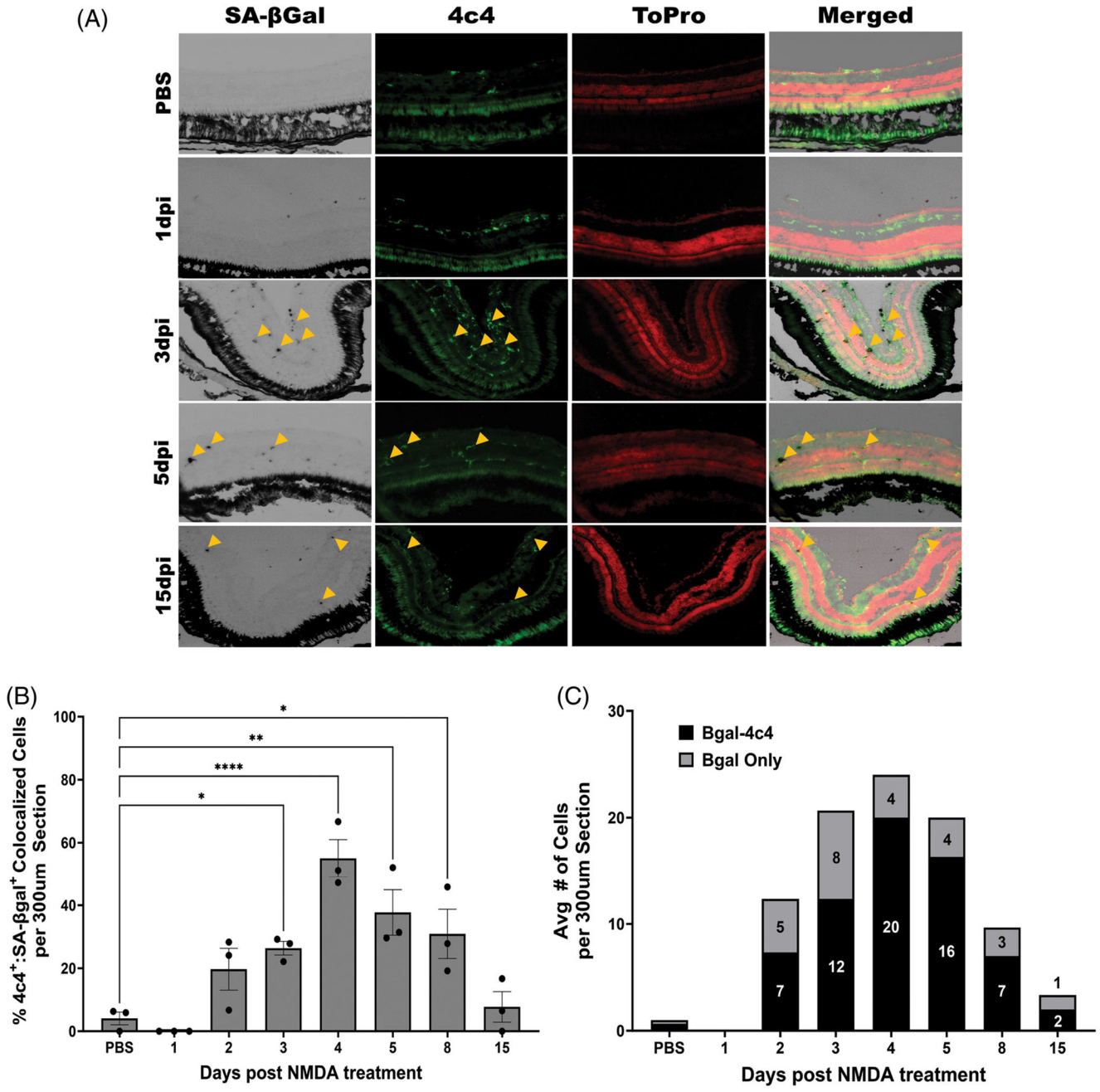

Figure 2. Colocalization of 4c4 and SA-βgal after NMDA damage.

(A) NMDA-treated retina sections from 1–5, 8, and 15 dpi were immunostained for 4c4, a marker of macrophages/microglia, and for SA-βgal. Damaged retinas showed colocalization of 4c4 (green) and SA-βgal (dark gray) starting at 2 dpi and persisting throughout later time points. Arrows show a select population of cells that were both SA-βgal+ and 4c4+. **(B)** Graph showing the percentage of 4c4+ microglia/macrophages that were also SA-βgal+ in a 300-um section of the retina across the time course. A subset of 4c4+ cells showed SA-βgal+ staining, ranging from ~20% to 45% of total 4c4+ cells showing a senescent

phenotype (n = 3). **(C)** Average number of SA-βgal+/4c4+ cells at each time point evaluated across 300 um sections compared to the number of SA-βgal+-only cells in the retina at the same time point. The majority of SA-βgal+ colocalized with 4c4+ cells across all time points starting at 2 dpi and constituted a majority of the total SA-βgal+ cells at all time points with senescent cells detected (n = 3 retina). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. All graphs show individual eyes with mean ± standard error of the mean (s.e.m.). All statistics were done using the Student's t-test, where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

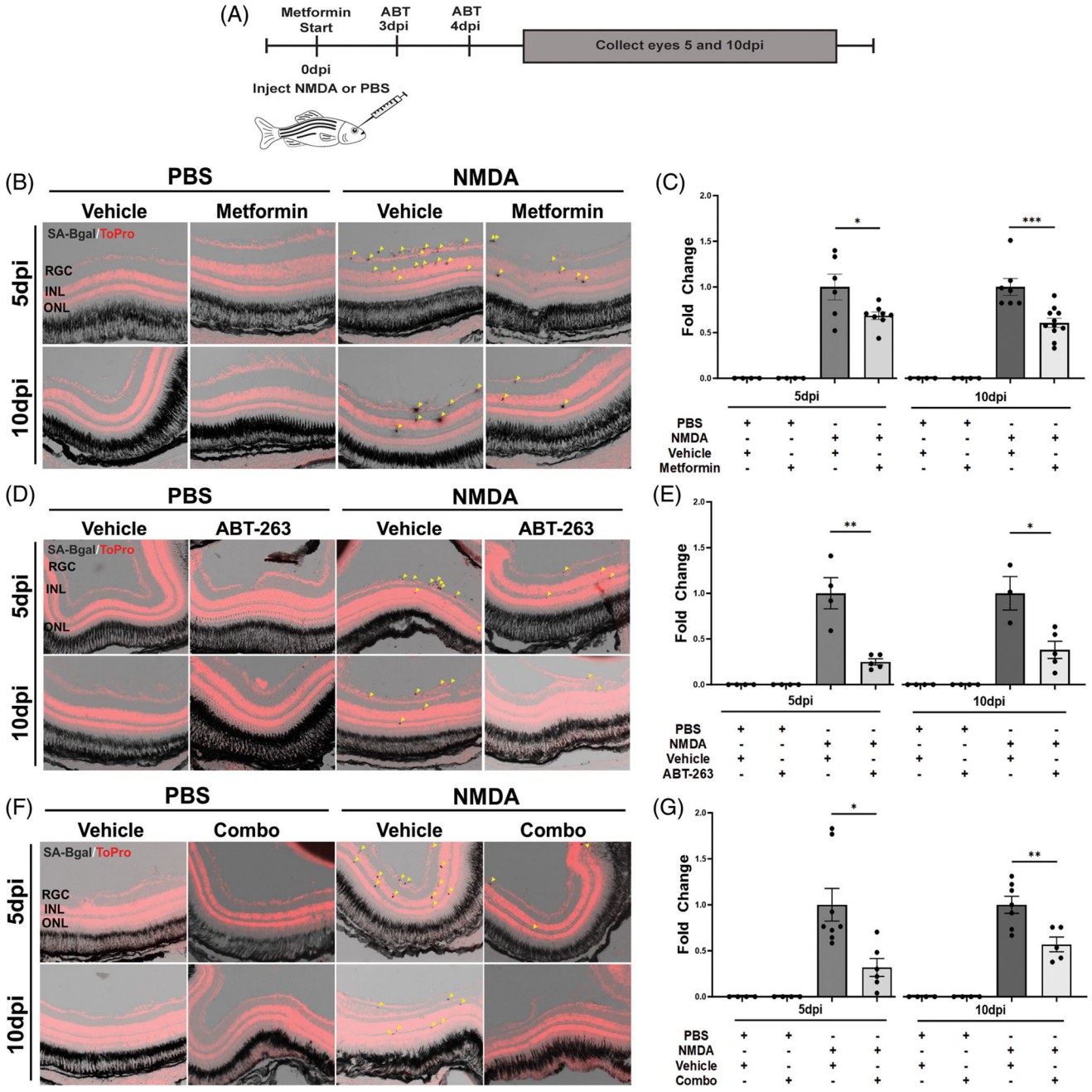

Figure 3. Senolytic treatment decreases SA-βgal+ cells in NMDA-damaged retinas.

(A) Schematic depicting the experimental timeline of senolytic dosing in conjunction with NMDA damage. Adult wild-type (AB) fish were intravitreally injected with 0.5 uL of 100 mM NMDA or PBS and then placed in tanks containing either 100 uM Metformin or standard tank water. Fish receiving ABT-263 injections received 0.5 uL intravitreal injections of 30 uM ABT-263 on days 3 and 4 after the initial NMDA injection. **(B,C)** Metformin treatment reduced the number of senescent cells in NMDA-treated retinas between 5 and 10 dpi (n = 5–11). **(D,E)** ABT-263 treatment reduced the number of

senescent cells in NMDA-treated retinas between 5 and 10 dpi (n = 3–5). **(F,G)** Combination treatment of Metformin and ABT-263 additionally reduced the number of senescent cells in NMDA-treated retinas between 5 and 10 dpi (n = 4–8). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. All graphs show individual eyes and mean ± s.e.m. All statistics were performed using the Student's t-test, where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

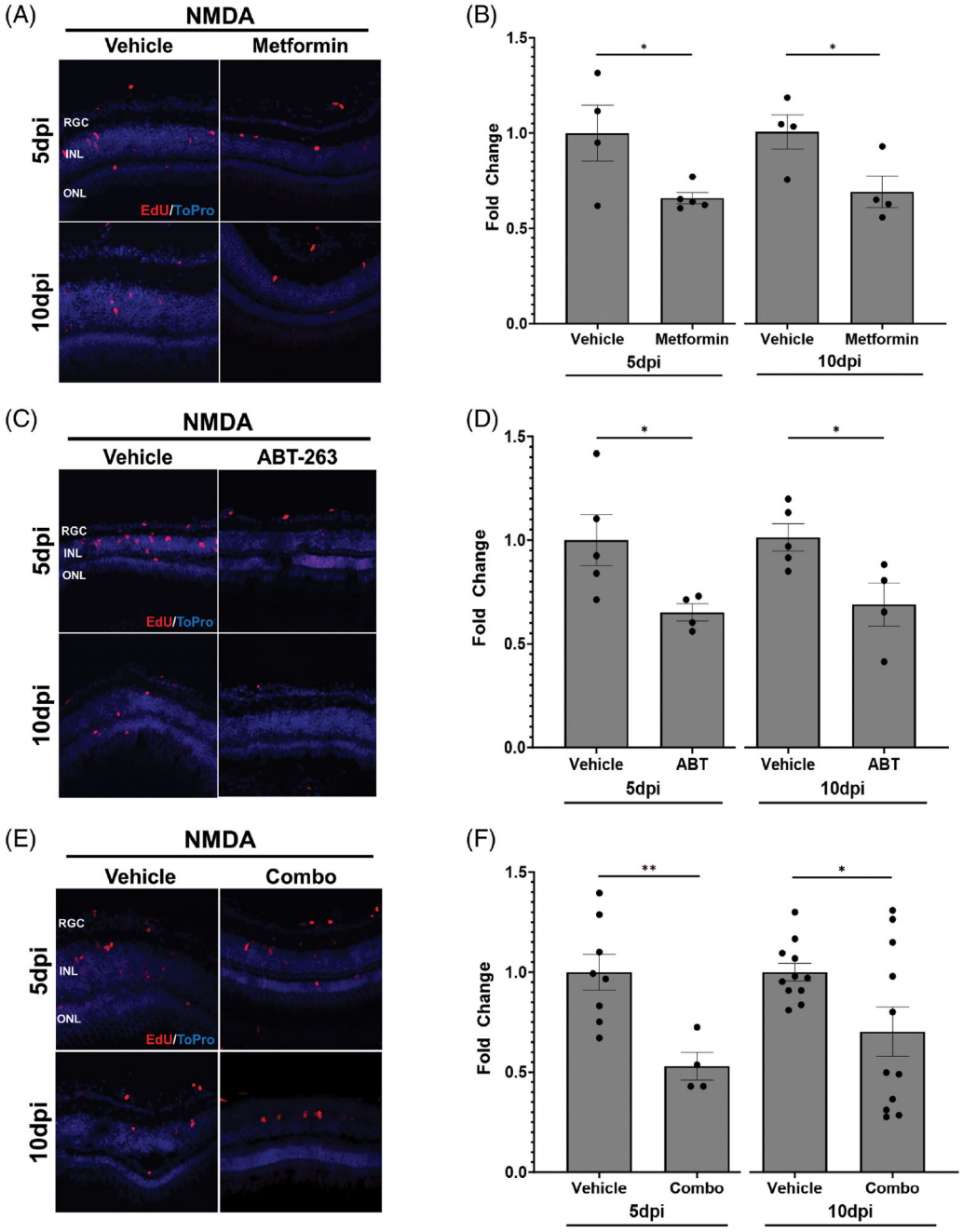

Figure 4. Senolytic treatment limits proliferation in NMDA-damaged retinas.

(A,B) Wild-type AB zebrafish received 0.5 uL intravitreal injections of 100 mM NMDA, were placed in Metformin-treated or regular tank water (vehicle), and were then intravitreally injected with EdU at the indicated time points. Metformin treatment showed a significant decrease in the number of EdU+ cells in NMDA-treated retains (n = 4–5). **(C,D)** Wild-type zebrafish were intravitreally injected with ABT-263 or a PBS vehicle three and four days after NMDA injection. ABT-263 treatment produced a significant decrease in EdU+ cells in NMDA-treated retinas at both 5 and 10 dpi (n = 3–5). **(E,F)** Combination

treatment with Metformin and ABT-263 showed a decrease in EdU+ cells in NMDA-treated retinas at 5 and 10 dpi (n = 4–12). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. All graphs show individual eyes and mean ± s.e.m. All statistics were performed using the Student's t-test, where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

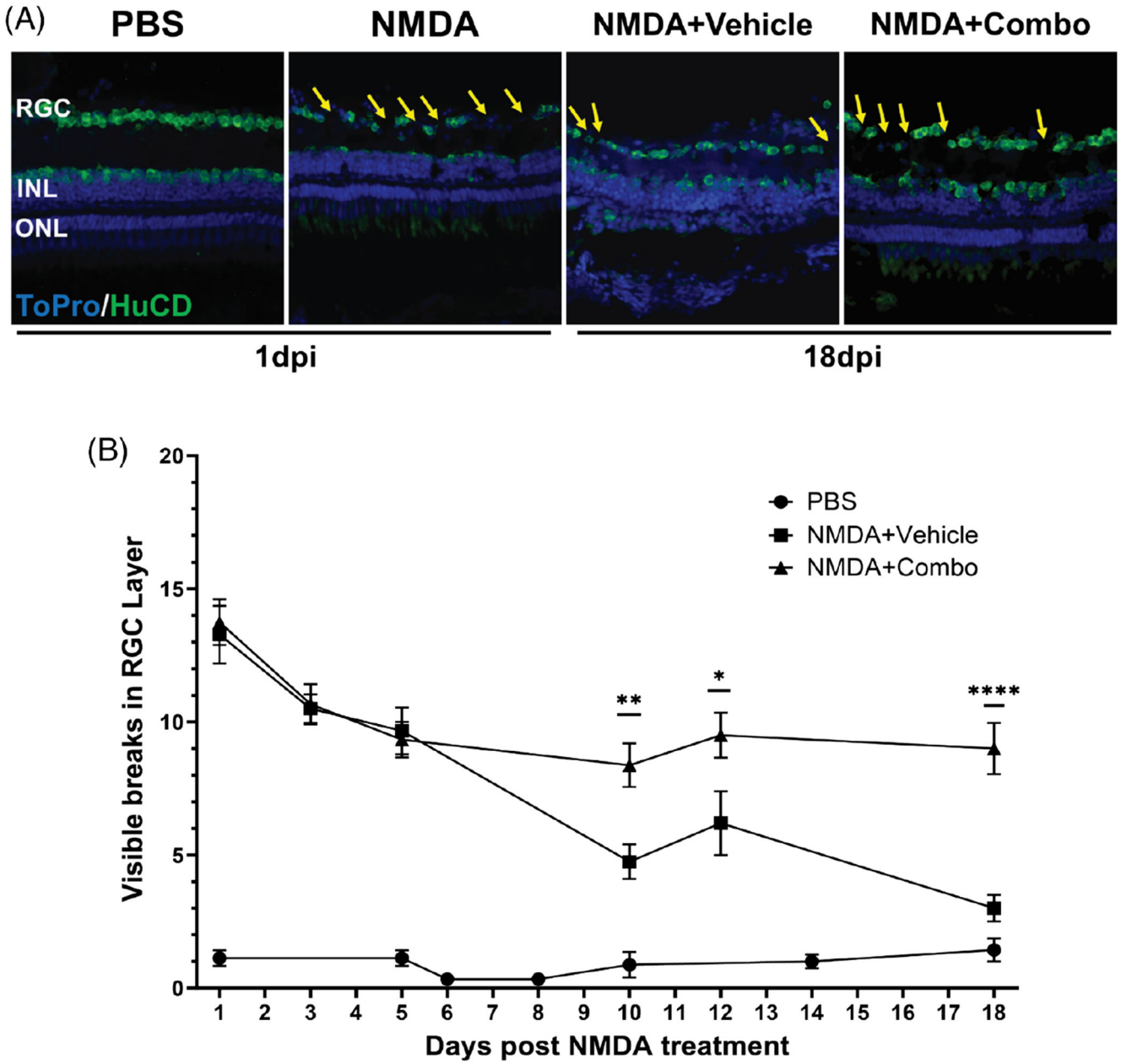

Figure 5. Premature removal of senescent cells impairs RGC regeneration in NMDA-damaged retinas at 10 dpi.

(A) Wild-type AB zebrafish were intravitreally injected with 0.5 uL of 100 mM NMDA or PBS and then subjected to a combination of the senolytic drugs Metformin and ABT-263 for 18 days. Representative images of PBS and NMDA-treated retinas at 1 dpi and of NMDA-treated retinas with or without the combo therapy of senolytics at 18 dpi show a complete RGC monolayer (PBS) or gaps in the RGC layer. **(B)** Longitudinal staining of all treatment conditions showed a low basal level of RGC gaps in the PBS condition, with a significantly higher disruption in NMDA-treated retinas. Over time, the NMDA + Vehicle retinas started to regenerate the RGC monolayer while the retinas treated with NMDA +

Combo failed to regenerate the lost RGCs, showing a statistical difference starting at 10 dpi ($p < 0.01$) and persisting to 18 dpi ($p < 0.0001$). All data are the product of between $n = 5$ retinas. INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. All graphs show individual eyes and mean ± s.e.m. All statistics were performed using the Student's t-test, where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

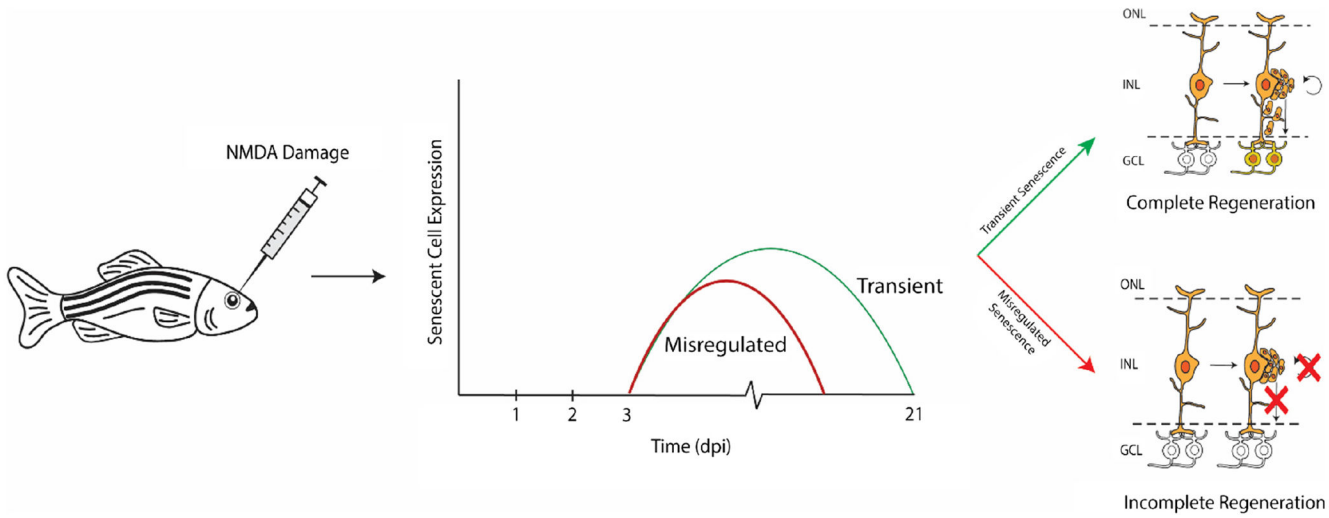

Figure 6. Model of senescence and zebrafish retina regeneration.

After damage to the retina, the appearance and clearance of senescent cells are proposed to precisely modulate paracrine and autocrine signaling to allow proper regeneration. The hypothesis is that senescent cells secrete pro-inflammatory molecules that are essential for initiating retina regeneration but that as regeneration proceeds, signaling shifts toward a pro-regenerative state, followed by the eventual clearance of senescent cells. Altered or prolonged pro-inflammatory signaling is proposed to block regeneration.

Table 1.

List of antibodies used in immunohistochemistry staining protocol.

Name	Target	Concentration	Source
Proliferating cell nuclear antigen	Actively proliferating cells	1:500	Abcam
HuC/D	Retinal ganglion cells	1:500	Invitrogen
4c4	Macrophages/microglia	1:1000	Hitchcock Lab (University of Michigan)
Glutamine synthase	Müller glia	1:500	Abcam
Zpr1	Photoreceptors	1:500	Patton Lab

Table 2.

Primer sequences used for reverse transcription quantitative polymerase chain reaction of N-methyl-D-aspartate-treated retinas.

Gene Name	Forward Sequence 5′-3′	Reverse Sequence 5′-3′
p21	CAGCGGGTTTACAGTTTCAGC	TGAACGTAGGATCCGCTTGT
18S rRNA	ACGCGAGATGGAGCAATAAC	CCTCGTTGATGGGAAACAGT