Context-Dependent Effects of Inflammation On Retina Regeneration

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Abstract

Inflammation is required for the proliferation of Müller glia (MG) into multipotent progenitors (MGPCs) in the injured fish and avian retinas. However, its function in retina regeneration has not been fully understood. Here we investigated the role of inflammation in three different retinal regeneration paradigms in zebrafish (stab-injury, NMDA-injury and insulin treatment). We first show that different types of immune cells and levels of inflammatory cytokines were found in the retinas of these paradigms. Though zymosan injection alone was insufficient to induce MG proliferation in the uninjured retina, immune suppression significantly inhibited MGPC formation in all three paradigms. Enhancing inflammation promoted MGPC formation after stab-injury, while exhibiting a context-dependent role in the NMDA or insulin models. Furthermore, proper levels of inflammation promoted MG reprogramming and cell cycle re-entry after stab- or NMDA-injury, but excessive inflammation also suppressed MG proliferation in the latter model. Finally, while inflammation promoted retinal neuron regeneration after stab-injury, immune suppression surprisingly achieved the best regeneration in the NMDA model. Our study reveals the complex and context-dependent role of inflammation during retinal repair in fish, and suggests accurate inflammation management may be crucial for successful retina regeneration in mammals.

Introduction

Degenerative retinal diseases such as macular degeneration and retinitis pigmentosa often lead to blindness and currently there is no therapy available to restore the lost vision in these patients. Unlike mammals, lower vertebrates such as teleost fish are able to repair their damaged retinas and restore vision [1, 2]. Retina regeneration in zebrafish relies on Müller glia cells (MG) in the retina. After retinal injury, zebrafish MG undergo a reprogramming process and proliferate into multipotent progenitors (MG-derived progenitor cells, MGPCs)[3, 4]. These MGPCs further proliferate, migrate to different retinal layers and eventually differentiate into major types of retinal neurons[5]. Although mammals cannot spontaneously regenerate their damaged retina, their MG share many characteristics with those of zebrafish[6], and there are increasing evidences that they have neurogenic potential and could be used as an endogenous source for retinal repair[7–9]. A comprehensive understanding of the mechanisms governing retinal repair in fish could therefore provide important clues for retina regeneration in mammals in the future.

After tissue injury or infection in the central nervous system (CNS), inflammation is among the earliest responses detected in both mammals and fish[10–12]. Tissue inflammation is characterized by the expression of proinflammatory cytokines and chemokines, which recruit resident immune cells or those from the blood stream to the site of tissue injury. These activated immune cells will modulate the local environment by further secreting cytokines and chemokines, and removal of cell debris and metabolites[13]. It has been shown that acute inflammation in the CNS can activate tissue stem cells and promote their proliferation and neurogenesis [12, 14, 15]. On the contrary, chronic inflammation is

believed to be harmful because a prolonged inflammatory response could cause damage to the neurons and even lead to degenerative diseases[16, 17].

Inflammation and microglia are known to be required for MGPC formation and photoreceptor regeneration. In the postnatal chicken retina, microglia ablation suppressed the subsequent MGPC formation after NMDA-injury[18]. In the larval zebrafish, co-ablation of photoreceptor and microglia delayed MG proliferation and slowed rod cell replacement[19]. A recent study from our lab showed that microglia-mediated inflammation activated the mTOR signaling in the MG, and promoted MGPC formation after a stab-injury in adult zebrafish[20]. Similarly, immune suppression or microglia ablation impaired neuronal regeneration in other retinal injury models in adult zebrafish[21, 22]. However, most of these studies utilized a single injury model and lacked a comprehensive and comparative analysis of the roles of inflammation in different paradigms of retina regeneration. Furthermore, little is known about the effect of enhancing inflammation on MGPC formation and retina regeneration. In this study, we utilized three different paradigms (stab-injury, NMDA-injury, and insulin administration) and investigated the impact of immune manipulation on MG reprogramming and proliferation, MGPC formation and neuronal regeneration with or without injury in adult zebrafish. Our results show a surprisingly complex and context-dependent role of inflammation in the retinal repair in fish, and suggest that the optimization of inflammatory response according to injury paradigm, background inflammation level, and degree of tissue damage should be considered for a successful retina regeneration in mammals.

Methods

Animals

Wild type (AB) and *Tg(mpx:GFP)* transgenic zebrafish[23] were obtained from China Zebrafish Resource Center (CZRC). The *Tg(mpeg1:EGFP)* fish[24] was obtained from the Institute of Neuroscience, Chinese Academy of Sciences. All fish were treated in accordance with the Guidelines for Animal Use and Care at Nantong University. Fish was maintained at 28°C for a light/dark cycle of 14 hours/10 hours. Adult zebrafish of both sexes (4-6 months of age) were used in this study.

Retinal injury paradigms and insulin administration

The method of mechanical injury has been described previously[25]. Briefly, fish were anesthetized in 0.02% Tricaine methane sulfonate (Sigma-Aldrich, E10521) in system water. Under a dissecting microscope, the right eye was gently rotated in the socket and stabbed four times (once in each quadrant) through the sclera with a sterile 30 gauge needle to the length of the bevel. The uninjured left eye served as a negative control.

For chemical lesions, 1 µl of NMDA (N-methyl-D-aspartate, Sigma-Aldrich, M3262) was injected intravitreally through the front of the eye at the first day of experiment. The default concentration of NMDA is 50 mM unless otherwise indicated. Care was taken not to damage the retina or other tissues.

For insulin-induced retina regeneration[26], 2 μg of bovine insulin (Solarbio Life Science, China, I8040) in PBS was injected intravitreously through the front of the eye. Care was taken not to damage the retina or other tissues. Fish received daily insulin injection for 4 days before the sacrifice. PBS-injected eyes were used as a negative control.

Quantitative PCR (qPCR)

The methods for qPCR have been described previously[25]. Primers used in the study were listed in Table 1.

Table 1
Primers used for qPCR in the study.

Gene name	Forward primer (5'-3')	Reverse Primer (5'-3')
RT-ascl1a	GGCGTCCTGTCACCCACCAT	ACGCAGTGCTTTGTGTTCTTGGA
RT-hbegfa	ATGTCTGACCATCATTGGCCTCC	ACCATTCAGCTTGCTGTGCCC
RT-lin-28a	GGATGGGCTTCGGATTTCTGTC	TCCTCCACAGTTGAAGCATCGATC
RT-socs3a	CAGGGAAGACAAGAGCCGAGAC	GTCTTGAAGTGGTAAAACGGCAGC
RT-socs3b	CGTACTTCAACTCGATGGCACAAA	CACCGGAGAGCACCAACACAA
RT-p21^{cip1}	CCCGCATGAAGTGGAGAAAACC	CGGTGTCGTCTCTGGTTCCTGA
RT-p27^{kip1}	CGGGAATCACGACTGTAGGGTAAC	TGGGCGTTCGGGTCACTTC
RT-p57^{kip2}	cttcagtcctcagaaacagacggaag	catccgctctgcagataaacacaggtg
RT-ccna2	GCGTGCTCCAAGAAAGCACCTTTA	TTTCCCGCAAATGCGTGTG
RT-ccnb1	TGTGATGCAGCATATTGCCAAA	GGCAGTGAAGAAATCCGTAAAATAAA
RT-ccnd1	CTGGACAGGTTTTTATCTGTGGAGCC	GCTTGGAGCTCTGATGTATAGGCAGT
RT-ccne1	CACGTTAAGGCTCTCGACATTCAAG	GCATGGGCTTGTGTAACCTGTGT
RT-cdk1	CTGGCAGATTTCGGCTTAGCCCGTGC	CTTATAGTCTGGCAGAGACTCAACATCTGGC
RT-cdk2	CTTAAACCCCAGAATCTCCTCATCAA	CTTAAACCCCAGAATCTCCTCATCAA
RT-rp113	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
RT-lepa	TTTCCAGCTCTCCGCTCAACC	CGGCGTATCTGGTCAACATGC
RT-lepb	CATTGCTCGAACCACCATCAGC	TCTTTATGCACCGGGGTCTCG
RT-crlf1a	GGGATTCTGGGATCTAGGAAAGC	TCCTTGAAGAACCTGGTTGCG
RT-clcf1	GAAAGTTGGTCAGGTTGCTGTGC	CATAAGTCCACACGTGTTGCTGC
RT-il6	GCTATTCCTGTCTGCTACACTGG	TGAGGAGAGGAGTGCTGATCC
RT-il1	TGTGGGAGACAGACAGTGCTGTTT	TAAGACGGCACTGAATCCACCAC
RT-il11a	CTCCTCATCGCTGCTTCTCTCG	TTGCGAAGTCACTGGCTCTGC
RT-il11b	CTCCACCTTTCCCCTCATCA	CTGTAGTTCAGTGAGGGCAGGG
RT-tnfa	AAGACCCAGGGCAATCAACAAGA	GTGCAGCTGATGTGCAAAGACAC
RT-tnfb	GCATGTGATGAAGCCAAACG	ACCCATTTCAGCGATTGTCC
RT-igf1	TTAAGTGTACCATGCGCTGT	ATCCTGTCGGTTTGCTGAAA

Gene name	Forward primer (5’-3’)	Reverse Primer (5’-3’)
RT-ins	TCTTCTACAACCCCAAGAGAGAC	GTGGGCAAATCTCTTCAGTTACA

Immune suppression and activation

In order to suppress the retinal immune response, zebrafish were immersed in dexamethasone (Dex, Sigma-Aldrich, D4902) in system water or solvent control (0.1% DMSO) for a week prior to injury or insulin administration. The default concentration of Dex is 15 mg/L unless otherwise indicated. Dex treatment continued after retinal injury or insulin injection for 4 days and then fish were returned to the system water for recovery. Dex or DMSO was changed daily and zebrafish were fed two hours before the water change. To enhance the immune response in the retina, fish received an intravitreal injection of indicated amount of zymosan A (Zym, Sigma-Aldrich, Z4250) at the time of retinal injury or on the first day of the experiment.

Tissue preparation and immunofluorescence

Methods for tissue preparation have been described previously[25]. The following primary antibodies were used in the study: rat anti-BrdU (1:500, Abcam, AB6326); rabbit anti-PCNA (1:500, GeneTex, GTX124496); mouse anti-Zpr1 (1:500, Abcam, AB174435); mouse anti-HuC/D (1:500, Thermo Fisher Scientific, A-21271); The antigen retrieval method for BrdU and PCNA staining has been described previously[25]. The IB4 staining (DyLight® 594 Griffonia Simplicifolia Lectin-Isolectin B4, Vectorlabs, DL-1207) of the microglia/macrophage in the retina was performed as described previously[20].

TUNEL assay

The TUNEL experiment was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, 11684795910) according to manufacturer’s instruction. The DNAse I-treated sections were used as a positive control.

BrdU incorporation and lineage tracing

To label proliferating cells in the retina, 20 µl of 20 mM BrdU (Sigma-Aldrich, B5002) was injected intraperitoneally into the zebrafish 4 hours before sacrifice. In order to trace the lineage of proliferating MGPCs, a pulse of BrdU (20 µl of 20 mM) was intraperitoneally injected at 4 days post injury (dpi) before sacrificing at 30 dpi or at indicated time points to examine their distribution and differentiation.

Microscopy and cell counting

A Zeiss Imager M2 upright microscope (Carl Zeiss AG) equipped with an Axiocam 506 monochrome camera was used to capture the fluorescence images of retinal sections or flat mounts. For cell counting, fluorescence images were captured with a 10x or 20x objective and cells were counted using the ImageJ software (Cell Counter plugin) as previously described[27].

Quantification and Statistics

All of the experiments were performed at least in triplicate and repeated at least two times. The number of samples used in each experiment was indicated in the figures or figure legends. Student's *t-test* (2 tails, unpaired) was used for single comparison and a one-way analysis of variance (ANOVA) was used followed by a Tukey test for multiple comparisons. Error bars represent standard error (SEM). Statistical significance was defined as $p < 0.05$.

Results

Inflammatory responses in three different retinal regeneration paradigms

To assess the inflammatory responses within each paradigm, we first characterized the dynamics of inflammatory cells in the retina. For this purpose, retinal microglia/macrophage was labeled by an intravitreous injection of the isolectin IB4 [20, 28]. In the retina of *Tg(mpeg1:EGFP)* transgenic zebrafish in which GFP was specifically expressed by microglia/macrophage[24], the majority of IB4 signals co-localized with GFP (Figure 1A), further confirming the specificity of this staining. In the uninjured retina, a small number of IB4+ cells could be seen scattered in the retina and vitreous (Figure 1B, left panel, Figure 1E). Following stab injury, a large number of IB4+ cells accumulated at the injury site at 2 days post injury (dpi) (Figure 1B,C; $p < 0.001$), indicating a robust microglia/macrophage response in the retina as shown previously[20]. Intravitreal PBS injection into the uninjured eye did not significantly increase the total number of IB4+ cells in the retina and vitreous (Figure 1B,D,E; $p = 0.356$), though a small increase in the vitreous was observed at 2 d (Figure 1E, $p < 0.001$), indicating that some of the IB4+ cells in the retina migrated to the vitreous after PBS injection. Two days after the NMDA-injury or insulin-treatment, significantly increased numbers of IB4+ cells compared with the PBS control could be seen in the retina and vitreous (Figure 1D,E;[retina], NMDA, $p < 0.001$, insulin, $p = 0.009$; [vitreous], NMDA, $p < 0.001$, insulin, $p = 0.004$). The accumulation of IB4+ cells was most evident in the inner plexiform layer (IPL) in both paradigms (Figure 1D, lower panels). We have previously shown in the stab-injury model that the accumulated IB4+ cells could be seen as early as 6 hours post injury (hpi), and they peaked at 2 dpi before declining at 4 dpi[20]. Interestingly, similar time courses were observed following NMDA-injury or insulin-treatment, though the initial rise in the number of IB4+ cells occurred slightly later than following stab-injury (12 hpi vs 6 hpi, Figure 1F). These results indicate that an acute microglia/macrophage response with similar time courses occurred in the retina and vitreous in the three paradigms.

It has been previously shown that neutrophil infiltrates into the retina after stab injury, but not after sterile retinal cell loss in zebrafish larvae[19]. To characterize the potential neutrophil response in the three paradigms, we took advantage of the *Tg(mpx:GFP)* transgenic zebrafish in which GFP was selectively expressed in neutrophils but not microglia/macrophages[23]. 3 hours after stab injury, a large number of GFP+ cells were observed infiltrating the injured retina of the *Tg(mpx:GFP)* zebrafish (Figure 2A,B). The number of neutrophils at the injury site rapidly decreased at 6 hpi (Figure 2A-C), and by 12 and 24 hpi, only a few neutrophils could be seen in this region (Figure 2B,C and data not shown). In contrast to the early neutrophil response, an increased presence of microglia/macrophage within the injured retina was

not observed until 6 hpi (Figure 2B). Interestingly, numerous neutrophils were also observed accumulating in the space between the peripheral retina and iris (Figure 2A, white boxes) after stab injury at 3 hpi, and some appeared to migrate along the inner retinal surface toward the injury region (Figure 2A, upper left panel). After 6 hpi, neutrophil accumulation in the peripheral region decreased rapidly in a manner similar to that of the injury site (Figure 2A and data not shown). In contrast to the stab-injury paradigm, no obvious neutrophil infiltration into the retina or vitreous was found after NMDA injury or insulin injection at 3 and 6 hpi, though some neutrophils were seen in the iris (Figure 2A, arrowheads). These findings indicate that the early neutrophil response differs significantly between the stab-injury and other two models.

To investigate if the inflammation levels are comparable in the three models, RNA samples were extracted from the whole retina at different time points (0 h, 6 h, 12 h, 1 d, 2 d and 4 d), and qPCR was performed to determine the expression of typical inflammatory cytokines including *il1b*, *il6*, *il11a* and *il11b*. In the control retina, there was a relatively low induction of *il1b* and *il6* but not *il11a/11b* after PBS injection (Figure 2D-G), suggesting that the injection assay itself may cause a weak inflammatory response. qPCR showed that inflammatory cytokines were strongly induced in the retinas of all three models, but their highest levels were observed following NMDA-injury (Figure 2D-G). In insulin-treated retinas, the expression levels of *il1b* and *il11a* were higher than those of the stab injury, while their levels of *il6* and *il11b* were largely comparable (Figure 2D-G). Interestingly, qPCR also revealed that the expression of inflammatory cytokines peaked significantly earlier than the number of IB4+ cells (Figure 1F, Figure 2). Specifically, the expression of inflammatory cytokines peaked at 6 hours (NMDA and insulin groups) or 12 hours (stab-injury group). In contrast, the number of IB4+ cells peaked at 2 days in all three paradigms. This inconsistency indicates that cell subtypes or expression patterns of microglia/macrophage may vary between early and later time-points following injury or growth factor treatment.

Inflammation is essential for MGPC formation in all three paradigms

To investigate whether inflammation is required for the formation of MGPCs in each paradigm, glucocorticoid dexamethasone (Dex, 15 mg/L) was used to suppress retinal inflammation and previous studies have validated its efficacy in adult zebrafish retina[20, 22]. As it has been shown that 98% of the BrdU+ cells in the inner nuclear layer (INL) at 4 dpi are MGPCs[3], we quantified the number of INL BrdU+ cells in the retina with or without Dex-treatment at this time point. BrdU immunofluorescence showed that immune suppression significantly reduced the number of INL BrdU+ cells in all three paradigms (Figure 3A-F; $p < 0.001$). The suppression of MGPC formation after Dex treatment was not a secondary result of decreased cell death, as Dex treatment did not significantly change the number of TUNEL+ cells compared with the control (Figure S1)[20]. These results indicate that inflammation is essential for MGPC formation in all three models.

We next ask if inflammation is required for the cell cycle re-entry of MG. Since almost all of the INL BrdU+ cells are proliferating MG at 2 dpi in the stab-injury paradigm[3], we labeled these cells by BrdU and PCNA

immunostaining at this time point. Immunofluorescence microscopy showed that Dex-treatment significantly reduced the number of INL BrdU+ or PCNA+ cells at 2 dpi (Figure 4A-C; [BrdU], $p = 0.034$; [PCNA], $p = 0.032$), suggesting that inflammation is necessary for MG proliferation. Furthermore, qPCR was performed to examine the expression of regeneration-associated genes (RAGs) and cell cycle-related genes in control and Dex-treated retina. Our results showed that Dex-treatment significantly reduced the expression of RAGs such as *ascl1a*, *lin28a*, *hbegfa*, *igf1*, *socs3a* and *socs3b*, as well as several cell cycle-related genes including *ccna2*, *ccnb1*, *ccnd1*, *ccne1* and *cdk2* (Figure 4D). Together these results suggest that inflammation regulates the expression of RAGs and cell cycle-related genes and is necessary for MG proliferation and MGPC formation.

Inflammation alone is insufficient to elicit a regenerative response in the intact retina

The above data shows an essential role of inflammation in the regenerative response in zebrafish retina in different paradigms. To investigate whether inflammation alone is sufficient to induce the reprogramming and proliferation of MG, fish received intravitreal injection of zymosan A (Zym) to activate inflammation in the intact retina. The efficacy of Zym to induce inflammation in the zebrafish CNS has been reported[12] and our previous study also validated its capability to enhance inflammation in stab-injured zebrafish retinas[20]. To better understand the effect of Zym-treatment on retinal cell survival and inflammation, different doses of Zym were tested, and TUNEL experiment showed that 20 and 40 µg/eye of Zym caused significant cell death in the intact retina (Figure 5A; [ONL, 20 µg], $p = 0.001$; [INL, 20 and 40 µg], $p < 0.001$ and $p = 0.048$, respectively; [GCL, 20 and 40 µg], $p = 0.004$ and $= 0.022$, respectively). Therefore Zym doses at 10 µg/eye or lower (0.1 or 1 µg/eye) were used in the following experiments of this study. We first characterized the inflammatory time course after Zym injection. qPCR of typical inflammatory cytokines showed that intravitreal injection of 10 µg of Zym stimulated an acute inflammatory response which peaked at 12 hours in the uninjured retina (Figure 5B). Importantly, Zym injection significantly increased the number of IB4+ cells in the vitreous (Figure 5C; [0.1, 1, 10 µg], $p = 0.005$, < 0.001, and < 0.001, respectively), as well as the expression of inflammatory cytokines in the retina in a largely dose-dependent manner (Figure 5D). These findings suggest that Zym injection could dose-dependently induce the inflammatory response in the zebrafish retina.

We next investigated the effect of Zym injection on MG proliferation and MGPC formation in the intact retina. BrdU immunofluorescence showed that all three doses of Zym failed to induce MG proliferation or MGPC formation at 2 days and 4 days after injection (Figure 5E,F; [2 d; 0.1, 1 and 10 µg], $p = 0.944$, 0.959 and 0.933, respectively; [4 d; 0.1, 1 and 10 µg], $p = 0.961$, 0.908, and 0.716, respectively). Importantly, qPCR showed that Zym injection failed to induce the expression of key RAGs such as *ascl1a*, *lin28a* and *hbegfa* (Figure 5G), many of the cell cycle-related genes (Figure 5H), and critical cytokines required for MG reprogramming (Figure 5I). Zym injection did cause a significant induction of the Jak-Stat reporter *socs3a* (Figure 5G), suggesting that inflammation is sufficient to activate this signaling in the retina. Together these results indicate that inflammation alone is insufficient to drive MG into reprogramming and proliferation in the intact retina.

Enhancing inflammation influences MGPC formation in a context-dependent manner

As immune suppression inhibited MGPC formation, we predicted that enhancing inflammation would increase MGPC formation. Indeed, intravitreal injection of Zym (10 μg/eye) significantly increased the number of MGPCs in the stab-injury model at 4 dpi (Figure 3A,B, $p < 0.001$), consistent with the result from immune suppression (Figure 3A,B). Surprisingly, enhancing inflammation by intravitreous injection of the same dose of Zym following NMDA-injury or insulin-treatment significantly inhibited MGPC formation (Figure 3C-F, $p < 0.001$ for both groups). These results were unlikely caused by a significant change in retinal cell death, as Zym injection did not affect the number of TUNEL+ cells in the NMDA-treated retina (Figure S1), and very few TUNEL+ cells were seen in retinas of the insulin model (Figure S1). The unexpected results prompted us to further examine the influence of inflammation on MGPC formation. Since our previous data showed that the levels of typical inflammatory cytokines following NMDA-injury or insulin-treatment were higher than that of stab-injury in the retina (Figure 2), we hypothesize that Zym's inhibitory effect on MGPC formation in these two models was caused by an excessive level of inflammation. To answer this question, various doses of Zym were used and their effects on MGPC formation in the three paradigms were examined. In the stab-injury model, Zym injection significantly increased the number of MGPCs at 4 dpi in a dose-dependent manner (Figure 6A,D; [0.1, 1, 10 μg], $p = 0.012$, $p < 0.001$ and $p < 0.001$, respectively). In the NMDA-injury paradigm, low doses of Zym (0.01, 0.05, 0.1 and 1 μg/eye) dose-dependently increased MGPC formation (Figure 6B,E; [0.01, 0.05, 0.1 and 1 μg], $p = 0.007$, 0.003, 0.006, and 0.002, respectively). However, this promoting effect vanished at the dose of 2.5 μg (Figure 6E; $p = 0.415$), and was reversed at the dose of 10 μg (Figure 6B,E, $p < 0.001$). A similar dual effect of Zym on MGPC formation was also observed in the insulin model (Figure 6C,F). These results support our hypothesis and indicate a dual role of inflammation in MGPC formation in the NMDA and insulin models.

To investigate if the effect of enhancing inflammation also depends on the degree of retinal damage, retinas were treated with different concentrations of NMDA (0.5 mM, 5 mM, or 50 mM used in the above experiments) to manipulate the extent of tissue damage. TUNEL experiment showed a dose-dependent increase of cell death in NMDA-treated retinas (Figure 7A,B; [0.5 mM vs PBS], $p = 0.008$; [5 mM vs 0.5 mM], $p = 0.037$; [50 mM vs 5 mM], $p = 0.002$), suggesting the degree of retinal damage is correlated with the NMDA dose. BrdU immunofluorescence showed that enhancing inflammation with 0.1 μg of Zym increased the number of MGPCs in all three NMDA concentrations (Figure 7C,D; [50, 5 and 0.5 mM], $p = 0.003$, $p < 0.001$, and $p = 0.012$, respectively). 10 μg of Zym significantly inhibited MGPC formation in retinas treated with 50 mM of NMDA (Figure 7C,D, $p < 0.001$), consistent with the data above (Figure 6E). Surprisingly, this inhibitory effect of 10 μg of Zym was completely reversed in retinas treated with 5 mM or 0.5 mM of NMDA (Figure 7C,D; [5 mM and 0.5 mM], $p < 0.001$ and $p = 0.008$, respectively). The reversal of 10 μg Zym's effect was unlikely caused by a marked decrease in the overall background level of inflammation, as qPCR showed no significant difference in the levels of typical inflammatory cytokines between the 5 mM and 50 mM NMDA groups, and 4 out of 6 of these cytokines were also comparable

between the 0.5mM and 50 mM NMDA groups (Figure 7E). These results indicate that the influence of inflammation on MGPC formation also depends on the degree of retinal damage. Taken together, these data demonstrate a complex function of inflammation following retinal injury, and indicate the influence of inflammation on MGPC formation is dependent on the injury paradigm, the level of inflammation, as well as the degree of retinal damage.

Influence of enhancing inflammation on MG reprogramming and proliferation

To further understand how enhancing inflammation influences MGPC formation, the cell cycle re-entry of MG was examined by BrdU and PCNA immunofluorescence at 2 days post injury. In the stab-injury model, Zym injection had no effect on the number of INL BrdU+ cells at 2 dpi, but caused a significant increase of INL PCNA+ cells (Figure 8A,B; [BrdU], $p = 0.589$; [PCNA], $p < 0.001$). Since it has been shown that proliferating cells in the INL at 2 dpi are MG [3, 20], this suggests enhancing inflammation promoted the entry of more MG into the cell cycle as shown by the PCNA staining at 2 dpi, but they hadn't reached the S phase yet as indicated by the BrdU incorporation. Indeed, BrdU staining at 3 dpi showed a significant increase of INL BrdU+ cells in Zym-treated retinas (Figure 8C,D, $p < 0.001$), consistent with our notion. Importantly, qPCR analysis showed that Zym injection significantly increased the expression of key RAGs such as *ascl1a*, *lin28a*, *hbegfa, igf1 and socs3a*, as well as multiple cell cycle-related genes in the retina (Figure 4D). These results indicate that in the stab-injury paradigm, enhancing inflammation promotes MG reprograming and drives more MG from quiescence into the cell cycle, which in turn generates more MGPCs at 4 dpi.

We then investigated the effect of enhancing inflammation on MG proliferation by a low or high dose of Zym (0.1 µg or 10 µg) following NMDA injury (50 mM). In line with the aforementioned data, intravitreous injection of 10 µg Zym significantly reduced the number of INL BrdU+ or PCNA+ MG at 2 dpi (Figure 8E,F; [BrdU], $p = 0.009$; [PCNA], $p < 0.001$), suggesting a high level of inflammation prevents MG from entering the cell cycle in the NMDA model. Interestingly, no difference in MG proliferation measured by PCNA or BrdU immunofluorescence was observed between the 0.1 µg Zym group and the control at 2 dpi (Figure 8E-F, [BrdU], $p = 0.097$; [PCNA], $p = 0.167$; Figure 8G). We speculate there was a delay in MG response to Zym in the NMDA model compared with the stab injury, therefore the MG proliferation was further examined at 3 dpi. Indeed, 0.1 µg of Zym significantly increased the number of INL proliferating MG at 3 dpi (Figure 8H,I; [BrdU], $p = 0.027$; [PCNA], $p = 0.013$). To further explore if this phenomenon was caused by an increased proliferation of MG-derived progenitors, or a result of cell cycle entrance from a new population of MG, proliferating MG were labeled by a pulse of BrdU at 2 dpi, and their progeny cells were analyzed by BrdU/PCNA staining at 3 dpi (Figure 8J). In the control retina, most of the INL PCNA+ cells were also BrdU+ at 3 dpi (94.6±0.9%, Figure 8K). In contrast, a significantly lower proportion of INL PCNA+ cells at 3 dpi were BrdU+ in retinas received 0.1 µg of Zym (Figure 8K, 61.9±2.2%, $p < 0.001$), suggesting the increased proliferation was caused by the entry into the cell cycle of a new MG population between 2-3 dpi. Taken together, our findings indicate that enhancing inflammation following stab or

NMDA injury may drive more MG into the cell cycle, but if excessive inflammation is elicited, it may also exhibit an opposite effect in the NMDA model.

Inflammation promotes the regeneration of retinal neurons in the stab-injury model

To investigate the influence of inflammation on the regeneration of retinal neurons in the stab-injury paradigm, MGPCs were labeled with a pulse of BrdU at 4 dpi, and their distribution and differentiation were examined at 30 dpi (Figure 9A). BrdU immunofluorescence showed that Dex-treatment significantly reduced the total number of BrdU+ cells in the retina at this time point, while Zym injection had an opposite effect (Figure 9A,B; [Dex], $p = 0.002$; [Zym], $p = 0.002$). Further analysis revealed a similar effect of immune manipulation on the number of BrdU+ cells in different retinal layers, though the difference was only significant in the ONL and INL (Figure 9A,C; [Dex; ONL, INL and GCL], $p = 0.004$, 0.007 and 0.093, respectively; [Zym; ONL, INL and GCL], $p = 0.001$, 0.001 and 0.118, respectively). Interestingly, the percentage of BrdU+ cells in each layer in Dex- or Zym-treated retinas was comparable to that of control (Figure 9D; [Dex; ONL, INL and GCL], $p = 0.837$, 0.575, and 0.389, respectively; [Zym; ONL, INL and GCL], $p = 0.465$, 0.478 and 0.244, respectively), suggesting modulation of inflammation had no effect on MGPC fates. To examine the effect of inflammation on MGPC differentiation, immunofluorescence of retinal cell markers Zpr1 (photoreceptors) and HuC/D (amacrine cells in the INL and RGCs in the GCL) were performed and their co-localization with BrdU signal was examined. Our results showed that immune suppression decreased, while enhancing inflammation increased the number of BrdU+/Zpr1+ cells in the ONL (Figure 9E,F; [Dex], $p = 0.016$; [Zym], $p < 0.001$) and BrdU+/HuC/D+ cells in the INL (Figure 9E,F; [Dex], $p = 0.013$; [Zym], $p = 0.006$), respectively. Neither treatment had any effect on the number of BrdU+/HuC/D+ cells in the GCL (Figure 9E,F; [Dex], $p = 0.117$; [Zym], $p = 0.481$). These findings indicate inflammation promoted the regeneration of photoreceptors and amacrine cells, but not RGCs. Together these data demonstrated that inflammation increased the MGPC population and neuronal regeneration in the stab-injured retina, without affecting the cell fate decisions of MGPCs.

Immune suppression results in the best neuronal regeneration in the NMDA-injury model

Similar to the experiments above, we also labeled the proliferating MGPCs by a pulse of BrdU at 4 dpi in the NMDA-injury paradigm, and examined the influence of inflammation on their distribution and differentiation at 30 dpi. Immunofluorescence showed that the majority of BrdU+ cells in the NMDA control group were in the ONL at 30 dpi (Figure 10A,C). As expected, enhancing inflammation by 0.1 μg of Zym resulted in significantly more BrdU+ cells (Figure 10A,B, $p < 0.001$), with the most obvious increase in the INL (Figure 10A,C; [ONL], $p = 0.019$; [INL], $p < 0.001$; [GCL], $p = 0.655$). Surprisingly, early immune suppression by Dex generated the largest number of BrdU+ cells in the retina at 30 dpi (Figure 10A,B, $p < 0.001$). Analysis of their distribution showed that Dex treatment significantly increased the number of BrdU+ cells in the ONL and INL, with the most dramatic change observed in the latter layer (Figure 10A,C; [ONL, INL], $p < 0.001$; [GCL], $p = 0.085$). Zpr1 and HuC/D immunofluorescence showed that while

enhancing inflammation produced more retinal neurons compared with the NMDA control at 30 dpi (Figure 10D,E; [Zpr1+], $p = 0.005$; [INL HuC/D+], $p < 0.001$; [GCL HuC/D+], $p = 0.047$), immune suppression regenerated the highest number of photoreceptors and amacrine cells in the three groups (Figure 10D,E; [Zpr1+, INL HuC/D+], $p < 0.001$; [GCL HuC/D+], $p = 0.095$). As Dex treatment reduced the number of MGPCs in the NMDA model at 4 dpi (Figure 3), we asked how immune suppression could produce the best regeneration result. To address this question, MGPCs from the three groups were labeled with BrdU at 4 dpi, and their number and distribution were examined at 6, 14 and 30 dpi (Figure 10F, Figure S2). In the NMDA control and Zym-treated retinas, an initial increase of ONL BrdU+ cells was observed from 6-14 dpi (Figure 10F, left panel; [NMDA], $p < 0.001$; [NMDA+Zym], $p = 0.001$; Figure S2), but followed by a dramatic decrease from 14-30 dpi (Figure 10F, left panel; [NMDA], $p < 0.001$; [NMDA+Zym], $p < 0.001$; Figure S2). In contrast, there was no significant change in the number of MGPCs in the ONL in Dex-treated retina from 6-30 dpi (Figure 10F, left panel; [14 d], $p = 0.132$; [30 d], $p = 0.129$; Figure S2). In the INL, the number of BrdU+ cells in the NMDA control and Zym-treated retinas decreased dramatically from 6-14 dpi, with partial recovery in the latter group from 14-30 dpi (Figure 10F, middle panel, $p < 0.001$ for both groups; Figure S2). In contrast, there was an initial increase of INL BrdU+ cells from 6-14 dpi, followed by a slight decrease from 14-30 dpi in Dex-treated retinas (Figure 10F, middle panel; [14 d], $p = 0.044$; [30 d], $p = 0.128$; Figure S2). In the GCL, a gradual but significant decrease of BrdU+ cells was found in all three groups during this time period (Figure 10F, right panel; [NMDA; 14 d, 30 d], $p < 0.001$; [NMDA+Zym; 14 d, 30 d], $p = 0.004$ and $p < 0.001$, respectively; [NMDA+Dex; 14 d, 30 d], $p = 0.135$ and 0.013, respectively; Figure S2). Taken together, these results indicate a dramatic loss of MGPCs from 6-30 dpi in the NMDA control and Zym-treated retinas, presumably due to cell death. Importantly, immune suppression prevented MGPC depletion in the ONL and INL, resulting in the best neuronal regeneration in the NMDA paradigm.

Discussion

Inflammation plays important roles in MG-dependent retina regeneration[18–20, 22]. However, most of the previous studies employed a single injury paradigm, lacking comprehensive analysis and comparison of different models. In this study, we employed three different paradigms of retina regeneration in adult zebrafish, and investigated the influence of immune manipulation on MGPC formation and neuronal regeneration in these models. Our results show a context-dependent effect of inflammation on MG proliferation and retina regeneration, and may have important implications in designing future therapeutic strategies for mammalian retinal repair.

By IB4 staining, we found an acute microglia/macrophage response in all three models. The time course of microglia/macrophage accumulation was similar in these models, implying involvement of common mechanisms. Following NMDA injury, microglia/macrophages accumulated preferentially in the inner retina, correlating with a damage of inner retinal neurons. Similarly, ouabain treatment also resulted in microglia/macrophage accumulation in the damaged inner retina of adult zebrafish[29]. In our study, a marked difference in neutrophil response was found among the three models. Significant early neutrophil infiltration into the retina/vitreous was observed after stab injury, but not in the other two paradigms. This

finding is consistent with a previous study in which neutrophils entered the retina following puncture injury but not after sterile retinal cell loss in zebrafish larvae [19]. Excessive neutrophils recruitment to the site of injury has been traditionally thought to cause collateral tissue damage and chronic inflammation[30]. However, novel evidences are also emerging in recent years demonstrating their contribution to inflammation resolution and tissue repair[31, 32]. Indeed, relatively lower levels of inflammatory cytokines were expressed in the stab-injured retina compared with the other two, and the detrimental effect of high doses of Zym was not observed in this model. We speculate that the different neutrophil behaviors may contribute to these disparate results, and further functional investigation is required to address this question.

By immune suppression, we found inflammation was essential for MG proliferation and MGPC formation in all three paradigms in adult zebrafish. These results are consistent with previous studies carried out in chicken and fish[18–22], and highlight the beneficial effects of acute inflammation on retinal repair. Our further study showed that inflammation was required for the expression of key reprogramming factors *ascl1a* and *lin28a*[7, 33], as well as important growth factors *hbegfa* and *igf1* [26, 34]. In both the stab-injury and NMDA-injury paradigms, enhancing inflammation with a proper dose of Zym drove more MG into the cell cycle, which in turn generated more MGPCs. These results indicate an essential role of inflammation in MG reprogramming and proliferation.

Investigating the effect of Zym demonstrated that inflammation alone is insufficient to trigger a regenerative response in the intact retina without causing cell death. It is likely that inflammation-activated signaling pathways, such as mTOR[20], Jak-Stat, and MAPK[21] cooperate with other injury-induced signals to initiate the regeneration process. In adult zebrafish brain, however, Zym-induced sterile inflammation was sufficient to induce radial glia proliferation and subsequent neurogenesis[12]. The different response to Zym in the brain and retina could be attributed to the inherent differences between MG and radial glia, and/or distinct local environments. While Zym injection increased the number of L-plastin+ cells in the zebrafish brain, it was unclear what types of leukocytes they were, and whether their secretome was distinct to that of microglia/macrophage in the retina. Continuous intravitreal injections of recombinant human IL-6 were shown to be sufficient to promote MG proliferation in the uninjured zebrafish retina[35]. In our study, Zym injection resulted in a significant increase of *il6* expression in the uninjured zebrafish retina while no MG proliferation was observed. This discrepancy could be explained by a difference in the actual concentration and duration of IL-6 in the retina, and/or a different response to endogenous and recombinant IL-6 proteins. Additional experiments, such as daily Zym injections or treatment with endogenous zebrafish IL-6 proteins, are required to better understand the role of IL-6 in MG proliferation.

A key finding of this study is the complex and context-dependent effect of inflammation on retina regeneration. A previous study in zebrafish larvae also reported that the effect of immune suppression on rod replacement depends on the timing of treatment[19]. By manipulating inflammation in three different models, we show that the influence of inflammation on MGPC formation depends on many factors, including the injury paradigm, the level of inflammation, and the degree of retinal damage. The exact

molecules responsible for the adverse effect of inflammation in the NMDA and insulin paradigms remain unclear at this point, but studies in other systems have shown that some inflammatory cytokines such as interferons, TNFα and IL-1β could inhibit the proliferation of neural stem/progenitor cells [36–38]. In a recent study in mice, microglia ablation increased the neurogenic ability of MG in Ascl1-induced retina regeneration after NMDA-injury, and the authors suggest that the role of inflammation in retina regeneration is mechanistically different in fish and mice[39]. However, results from our study likely explain the difference. We show that by reducing the Zym dose or the degree of tissue damage in the NMDA-injured zebrafish retina, we could actually increase MGPC formation. It's possible that high levels of inflammation suppressed Ascl1-induced regeneration in NMDA-injured mice retinas, whereas microglia ablation reduced the inflammation to a more beneficial level and thus increased neurogenesis.

The most unexpected result of this study is the opposite outcome of immune manipulation on the regeneration of retinal neurons from the stab-injury and NMDA-injury paradigms. In the stab-injury model, inflammation promoted the regeneration of retinal neurons. In contrast, the best regeneration result was achieved after an early immune suppression in the NMDA model. Lineage tracing in the NMDA control and Zym-treated retinas revealed a dramatic loss of MGPCs from 6-30 dpi. The NMDA model possessed the highest level of inflammatory cytokine expression compared to the other two and, in particular, a sustained high level of IL-6 after injury. Since over-produced inflammatory cytokines are well known for their inhibitory effect on neurogenesis and neuronal survival[40, 41], this highly inflammatory environment likely caused the cell death of MPGCs and/or newborn neurons in the control and Zym-treated retinas. Consistent with this idea, an enhanced neuronal regeneration was found in Dex-treated retinas possibly due to improved survival of MGPCs and/or newborn neurons. Similarly, a recent study also found that late Dex treatment had regeneration-enhancing effects in the retina of *mmp9* mutant fish with elevated TNFα levels[22]. Given the fact that in our study fish were pre-treated with Dex for 7 days before NMDA injury, and Dex significantly reduced MGPC formation at 4 dpi, its final result in retina regeneration is indeed striking. It will be interesting to further investigate whether the combination of early Zym injection to expand the MGPC population and late Dex-treatment to improve cell survival will result in an even better regeneration in this model.

In summary, this study revealed a complex and context-dependent function of inflammation in retina regeneration. Inflammation clearly played an essential role in MG proliferation and MGPC formation, but in order to achieve the best regeneration, our results indicate that it needs to be optimized according to the context, including the injury paradigm, the inflammation level, and the degree of retinal damage. In some scenarios such as the NMDA-injury paradigm, immune suppression or early inflammation enhancement followed by late immune suppression may result in the best outcome. The findings of this study also suggest that in future retinal repairs in mammals including humans, accurate inflammation management may be key to successful regeneration.

Declarations

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Declaration of competing interests

The authors declare that they have no competing interests.

Data deposition and materials sharing

The data supporting the results reported in the article are available upon request to the corresponding authors.

Contributors

CZ, XZ and YC: performed the experiments; CZ, XZ, YC, ZL, SZ, ZZ, LC, HG, JL, and HX: analyzed the data; HX: designed the experiments and wrote the paper.

References

1. Goldman D (2014) Muller glial cell reprogramming and retina regeneration. Nat Rev Neurosci 15(7):431–442
2. Mensinger AF, Powers MK (1999) Visual function in regenerating teleost retina following cytotoxic lesioning. Vis Neurosci 16(2):241–251
3. Fausett BV, Goldman D (2006) A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci 26(23):6303–6313
4. Nagashima M, Barthel LK, Raymond PA (2013) A self-renewing division of zebrafish Muller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. Development 140(22):4510–4521
5. Lenkowski JR, Raymond PA (2014) Muller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. Prog Retin Eye Res 40:94–123
6. Hoang T et al., *Gene regulatory networks controlling vertebrate retinal regeneration*. Science, 2020
7. Roska B, Sahel JA (2018) Restoring vision. Nature 557(7705):359–367
8. Wilken MS, Reh TA (2016) Retinal regeneration in birds and mice. Curr Opin Genet Dev 40:57–64
9. Zhou H et al (2020) Glia-to-Neuron Conversion by CRISPR-CasRx Alleviates Symptoms of Neurological Disease in Mice. Cell 181(3):590–603 e16
10. Klein RS, Garber C, Howard N (2017) Infectious immunity in the central nervous system and brain function. Nat Immunol 18(2):132–141

11. Finsen B, Owens T (2011) Innate immune responses in central nervous system inflammation. FEBS Lett 585(23):3806–3812
12. Kyritsis N et al (2012) Acute inflammation initiates the regenerative response in the adult zebrafish brain. Science 338(6112):1353–1356
13. Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314–1318
14. Borsini A et al (2015) The role of inflammatory cytokines as key modulators of neurogenesis. Trends Neurosci 38(3):145–157
15. Kizil C, Kyritsis N, Brand M (2015) Effects of inflammation on stem cells: together they strive? EMBO Rep 16(4):416–426
16. Stephenson J et al (2018) Inflammation in CNS neurodegenerative diseases. Immunology 154(2):204–219
17. Furman D et al (2019) Chronic inflammation in the etiology of disease across the life span. Nat Med 25(12):1822–1832
18. Fischer AJ et al (2014) Reactive microglia and macrophage facilitate the formation of Muller glia-derived retinal progenitors. Glia 62(10):1608–1628
19. White DT et al (2017) Immunomodulation-accelerated neuronal regeneration following selective rod photoreceptor cell ablation in the zebrafish retina. Proc Natl Acad Sci U S A 114(18):E3719–E3728
20. Zhang Z et al (2020) Inflammation-induced mammalian target of rapamycin signaling is essential for retina regeneration. Glia 68(1):111–127
21. Conedera FM et al (2019) Retinal microglia signaling affects Müller cell behavior in the zebrafish following laser injury induction. Glia 67(6):1150–1166
22. Silva NJ et al (2020) Inflammation and matrix metalloproteinase 9 (Mmp-9) regulate photoreceptor regeneration in adult zebrafish. Glia 68(7):1445–1465
23. Renshaw SA et al (2006) A transgenic zebrafish model of neutrophilic inflammation. Blood 108(13):3976–3978
24. Ellett F et al (2011) mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117(4):e49–e56
25. Lahne M et al (2020) The Regenerating Adult Zebrafish Retina Recapitulates Developmental Fate Specification Programs. Front Cell Dev Biol 8:617923
26. Wan J et al (2014) Retinal injury, growth factors, and cytokines converge on beta-catenin and pStat3 signaling to stimulate retina regeneration. Cell Rep 9(1):285–297
27. Zhang S et al (2016) Antiviral Drug Ganciclovir Is a Potent Inhibitor of the Proliferation of Muller Glia-Derived Progenitors During Zebrafish Retinal Regeneration. Invest Ophthalmol Vis Sci 57(4):1991–2000
28. Stence N, Waite M, Dailey ME (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. Glia 33(3):256–266

29. Mitchell DM, Lovel AG, Stenkamp DL (2018) Dynamic changes in microglial and macrophage characteristics during degeneration and regeneration of the zebrafish retina. J Neuroinflammation 15(1):163
30. Castanheira FVS, Kubes P (2019) Neutrophils and NETs in modulating acute and chronic inflammation. Blood 133(20):2178–2185
31. Peiseler M, Kubes P (2019) More friend than foe: the emerging role of neutrophils in tissue repair. J Clin Invest 129(7):2629–2639
32. Sas AR et al (2020) A new neutrophil subset promotes CNS neuron survival and axon regeneration. Nat Immunol 21(12):1496–1505
33. Ramachandran R, Fausett BV, Goldman D (2010) Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12(11):1101–1107
34. Wan J, Ramachandran R, Goldman D (2012) HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell 22(2):334–347
35. Zhao XF et al (2014) Leptin and IL-6 family cytokines synergize to stimulate Muller glia reprogramming and retina regeneration. Cell Rep 9(1):272–284
36. Iosif RE et al (2006) Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. J Neurosci 26(38):9703–9712
37. Ben-Hur T et al (2003) Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells. Mol Cell Neurosci 24(3):623–631
38. Guadagno J et al (2015) Microglia-derived IL-1β triggers p53-mediated cell cycle arrest and apoptosis in neural precursor cells. Cell Death Dis 6(6):e1779
39. Todd L et al (2020) Microglia Suppress Ascl1-Induced Retinal Regeneration in Mice. Cell Rep 33(11):108507
40. Carpentier PA, Palmer TD (2009) *Immune influence on adult neural stem cell regulation function* Neuron 64(1):79–92
41. Glass CK et al (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140(6):918–934

Figures

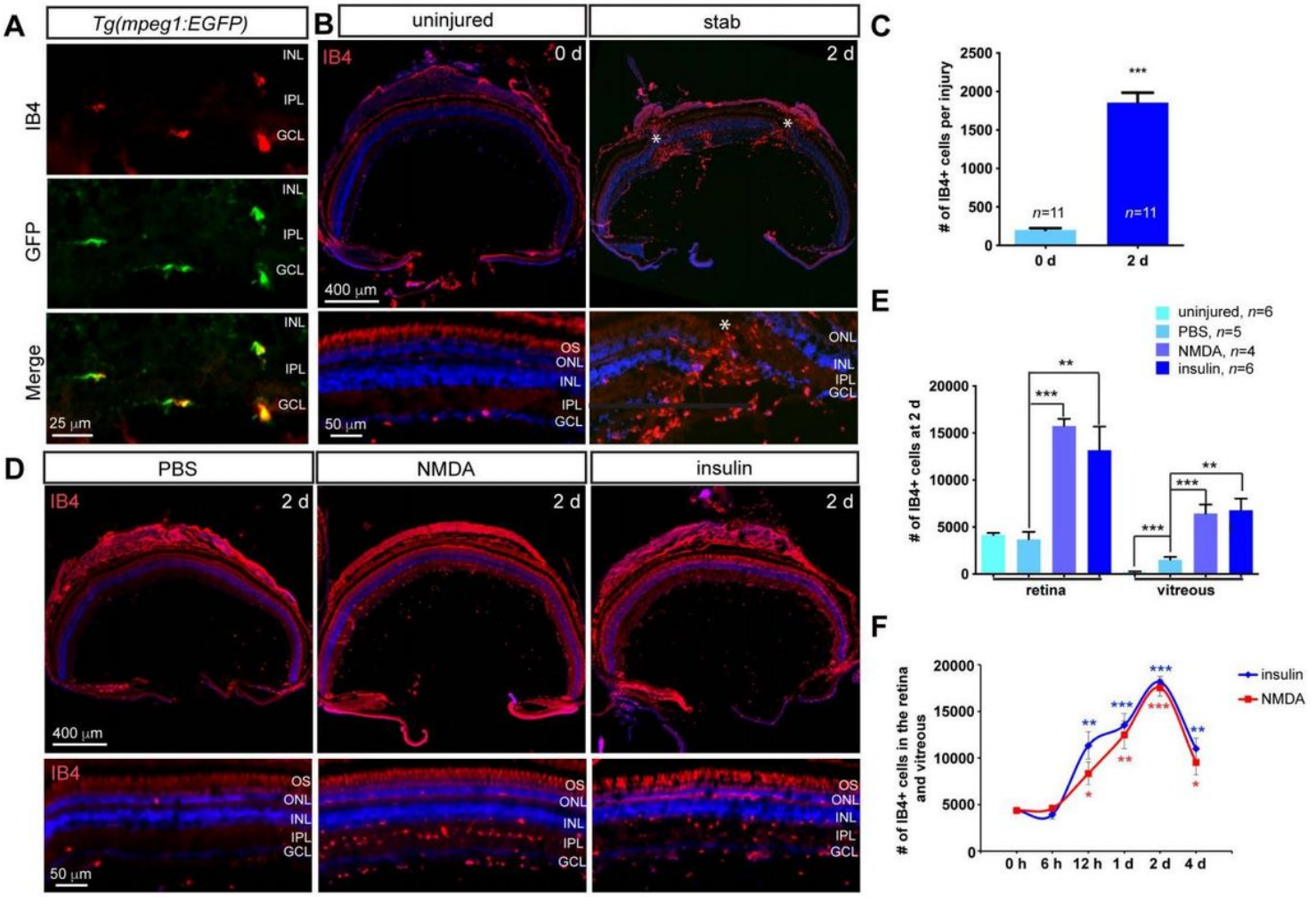

Figure 1

Microglia/macrophage response following stab-injury, NMDA-injury or insulin treatment in adult zebrafish retinas. (A) Fluorescence microscopy showing the co-localization of IB4 and GFP signals in the uninjured retina of the Tg(mpeg1:EGFP) zebrafish. (B,D) IB4 injection showing the presence of microglia/macrophage in the retina and vitreous of the three models. White asterisks, site of the stab injury. Note the autofluorescence in the outer segments. (C) Quantification of the number of IB4+ cells per injury in (B). (E) Quantification of the number of IB4+ cells in the indicated region of (D). **, $p < 0.01$, ***, $p < 0.001$ compared with PBS control. (F) Quantification of the number of IB4+ cells in the retina and vitreous at indicated time points in the NMDA-injury and insulin-treatment paradigms. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

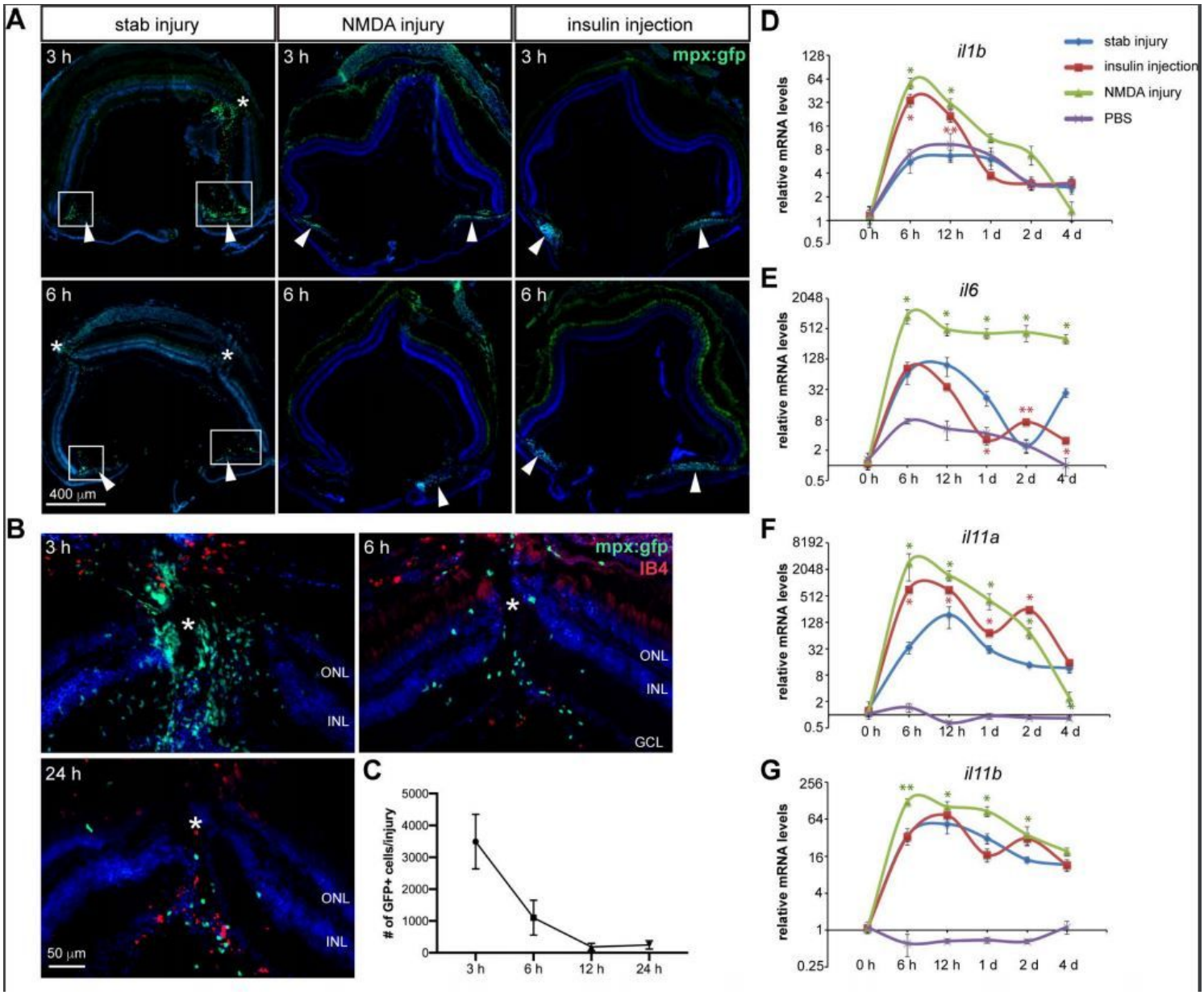

Figure 2

Neutrophil response and inflammatory cytokine expression in the retina of the three models. (A) Fluorescence microscopy showing the neutrophil response in the eyes of Tg(mpx:GFP) zebrafish. Arrowheads, the iris; White boxes, neutrophil infiltration in the space between the peripheral retina and iris. (B) Fluorescence microscopy showing the accumulation of neutrophil (green) and microglia/macrophage (red) in stab-injured retina at different time points in the Tg(mpx:GFP) fish. (C) Quantification of the neutrophil number at the injury site at indicated time points after stab-injury. (D-G) qPCR showing the relative mRNA expression of il1b, il6, il11a, and il11b in the retina at indicated time points in the three paradigms. n = 4 for each time point. White asterisks, site of the stab injury. *, $p < 0.05$, **, $p < 0.01$ compared with the stab-injury group at the same time point. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

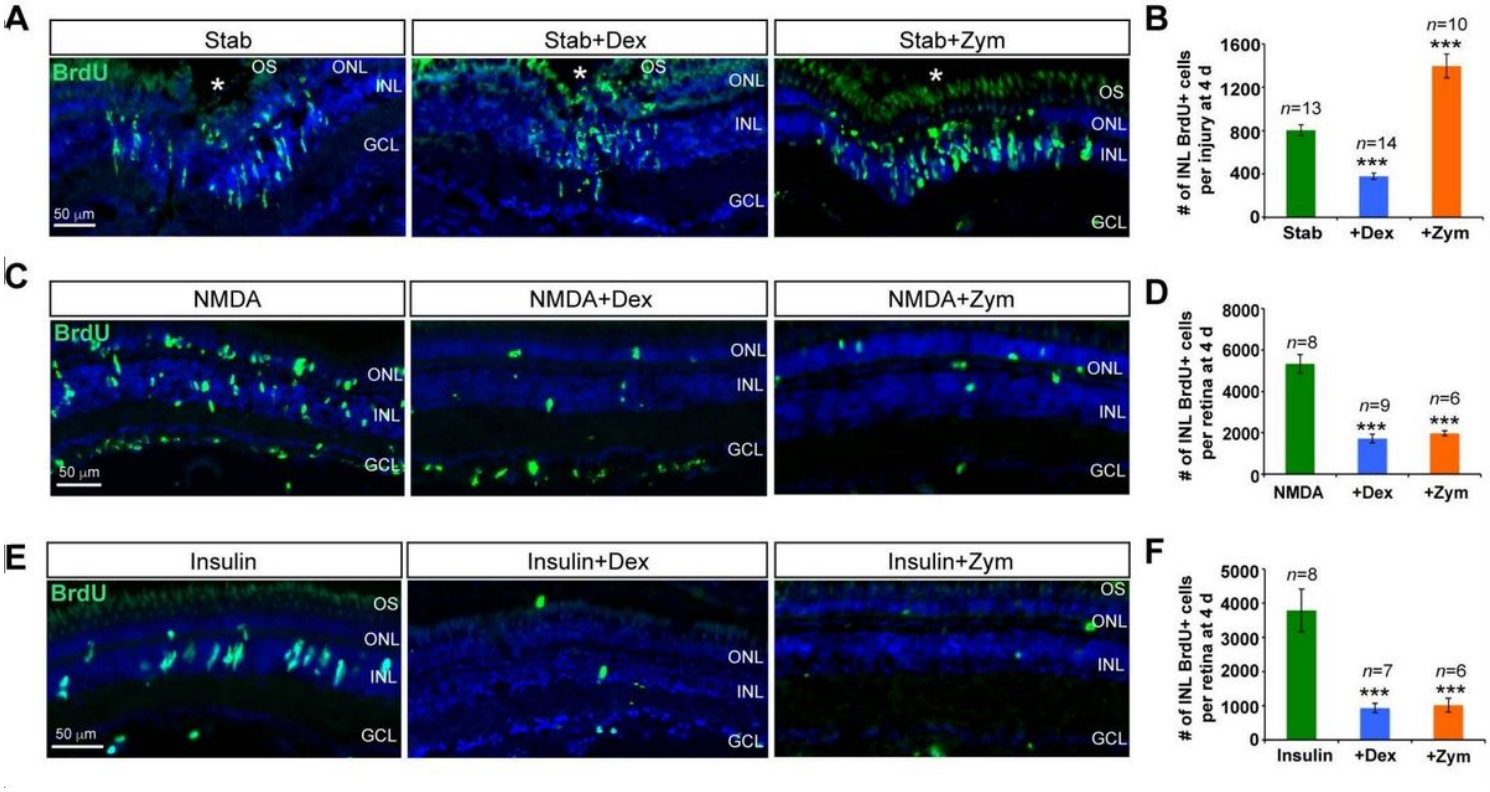

Figure 3

The effect of immune manipulation on MGPC formation in the three paradigms. (A,C,E) BrdU immunofluorescence showing the presence of proliferating cells in the retina at 4 days. For enhancing inflammation, 10 μg of Zym was injected into the vitreous at the time of retinal injury. MGPCs were BrdU+ cells in the INL at this time point. White asterisks in (A), site of the stab injury. Note the autofluorescence in the outer segments. (B,D,F) Quantification of the number of INL BrdU+ cells of (A,C,E). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Dex, dexamethasone; Zym, zymosan A.

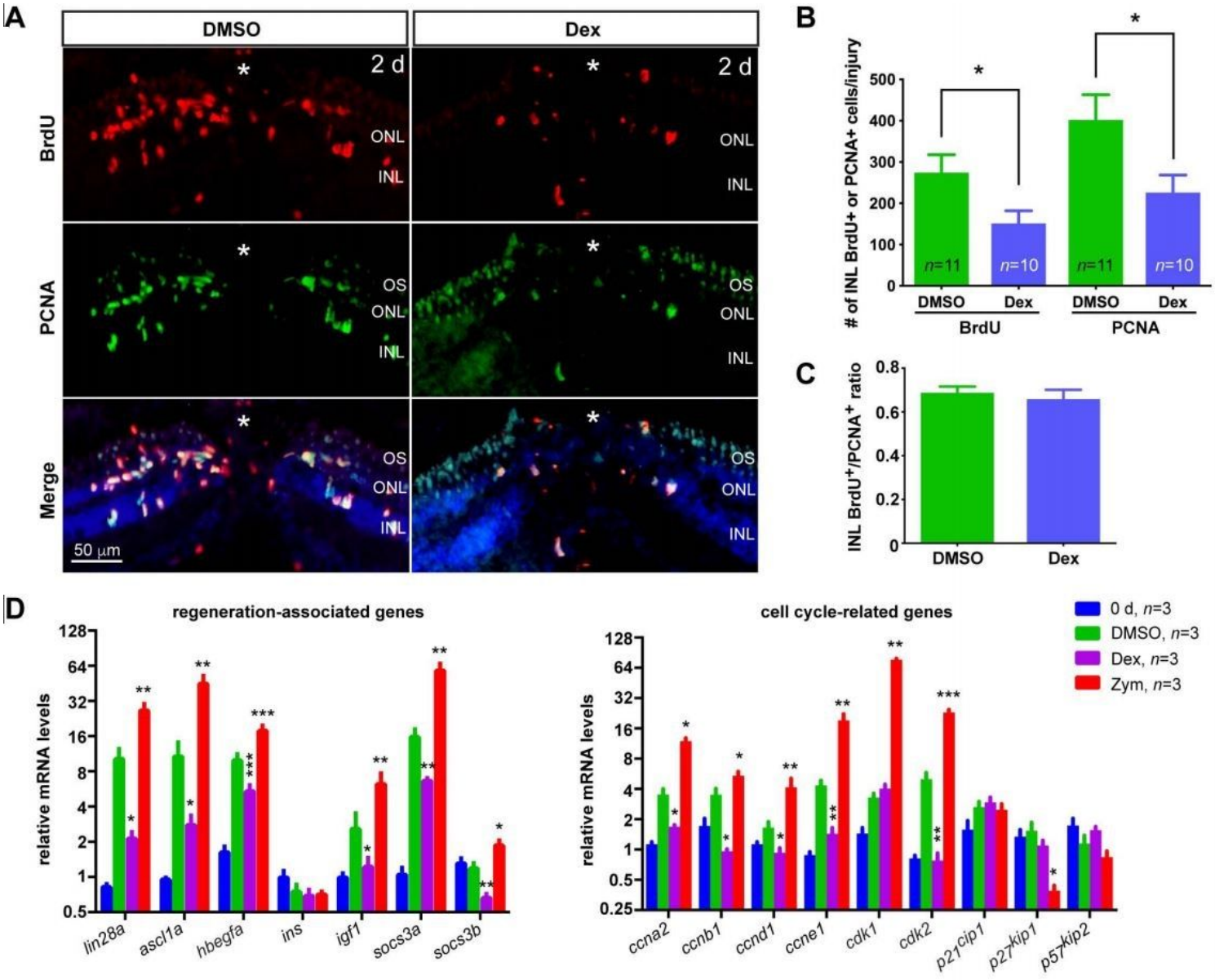

Figure 4

Effects of immune manipulation on MG proliferation and gene expression in retinas from the stab-injury model. (A) BrdU and PCNA immunofluorescence showing MG proliferation in control and Dex-treated retinas after stab injury at 2 days. White asterisks, site of the stab injury. Note the autofluorescence in the outer segments is not PCNA signal. (B,C) Quantification of cell proliferation of (A). (D) qPCR showing the expression of regeneration-associated genes (RAGs) and cell cycle-related genes in control, Dex- or Zym-treated retinas at 2 days. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the solvent control. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layers; GCL, ganglion cell layer.

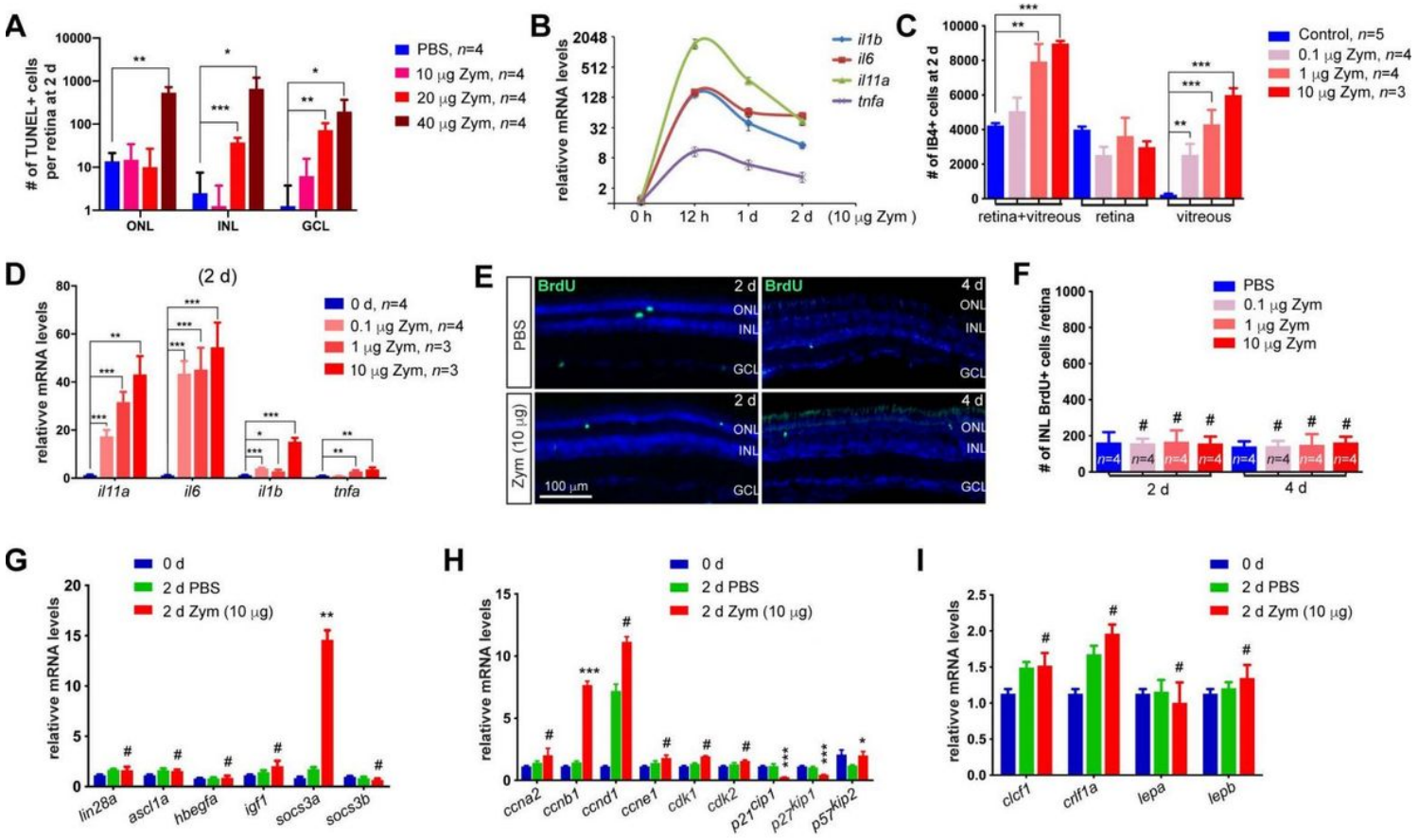

Figure 5

Zym treatment alone is insufficient to induce a regenerative response in the intact retina. At the beginning of the experiment, indicated doses of Zym were injected into the vitreous through the front of the eye to induce inflammation. (A) Quantification of the TUNEL+ cells in the uninjured retina treated with indicated doses of Zym or PBS control at 2 days. (B) qPCR showing the relative mRNA level of typical inflammatory cytokines at indicated time points after Zym injection in the uninjured retina. (C) Quantification of the IB4+ cell number in the retina and vitreous treated with indicated doses of Zym at 2 days. (D) qPCR showing the expression of typical inflammatory cytokines in the control and retinas received indicated doses of Zym at 2 days. (E) BrdU immunofluorescence showing the cell proliferation in the control and Zym-treated retinas at 2 and 4 days. (F) Quantification of the number of INL BrdU+ cells in the control retina and those treated with indicated doses of Zym at 2 and 4 days. (G-I) qPCR showing the expression of RAGs, cell cycle-related genes, and critical cytokines in control and Zym-treated retinas at 2 days. n = 3 for each group. #, $p > 0.05$ compared with PBS control. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

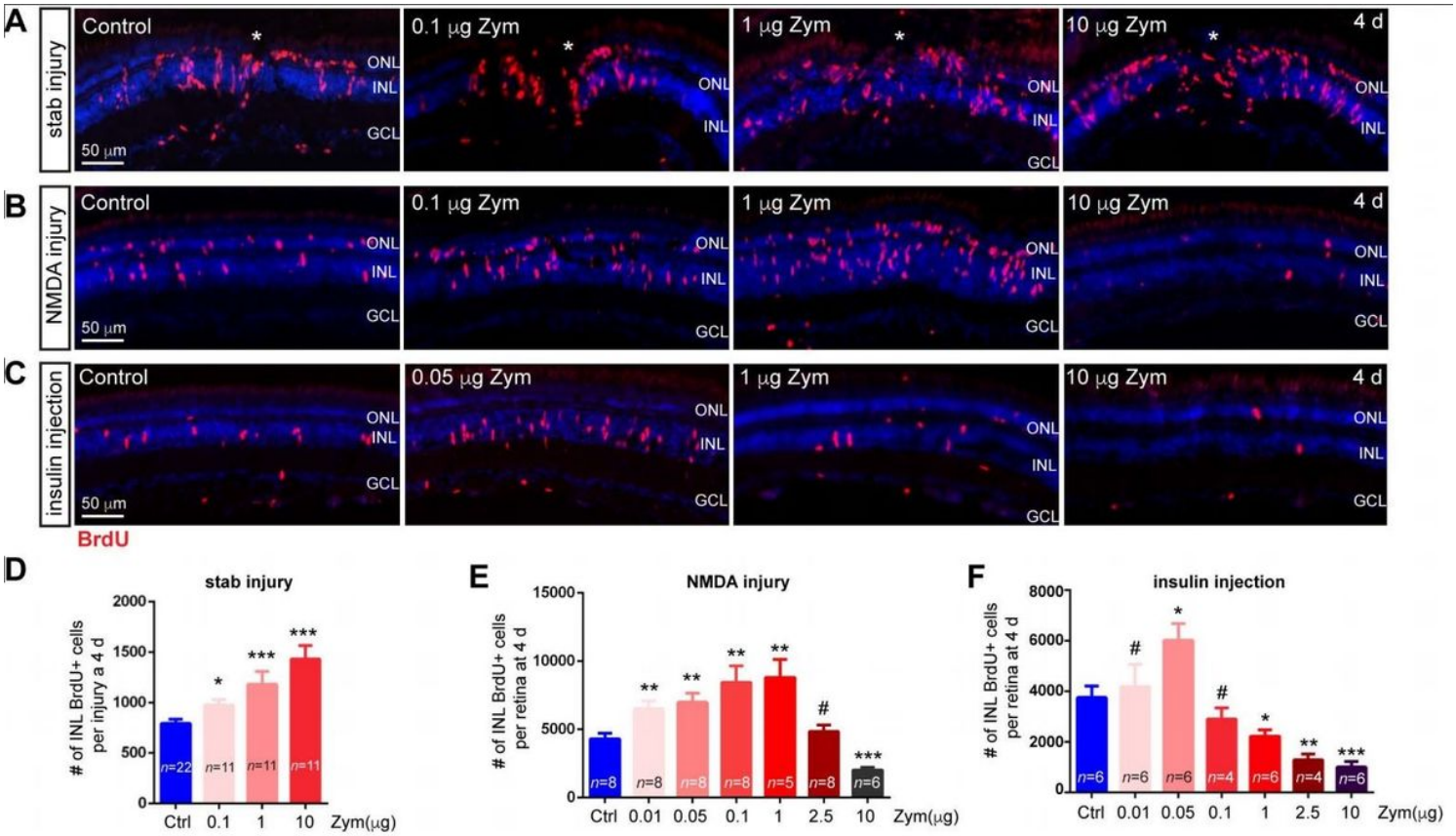

Figure 6

Context-dependent effects of enhancing inflammation on MGPC formation. (A-C) BrdU immunofluorescence showing MGPC formation in control and retinas treated with indicated doses of Zym at 4 days in the three models. White asterisks in (A), site of the stab injury. (D-F) Quantification of the number of INL BrdU+ cells at 4 days after treatment with indicated doses of Zym in the three paradigms. #, $p > 0.05$ compared with control. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control. Ctrl, control; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

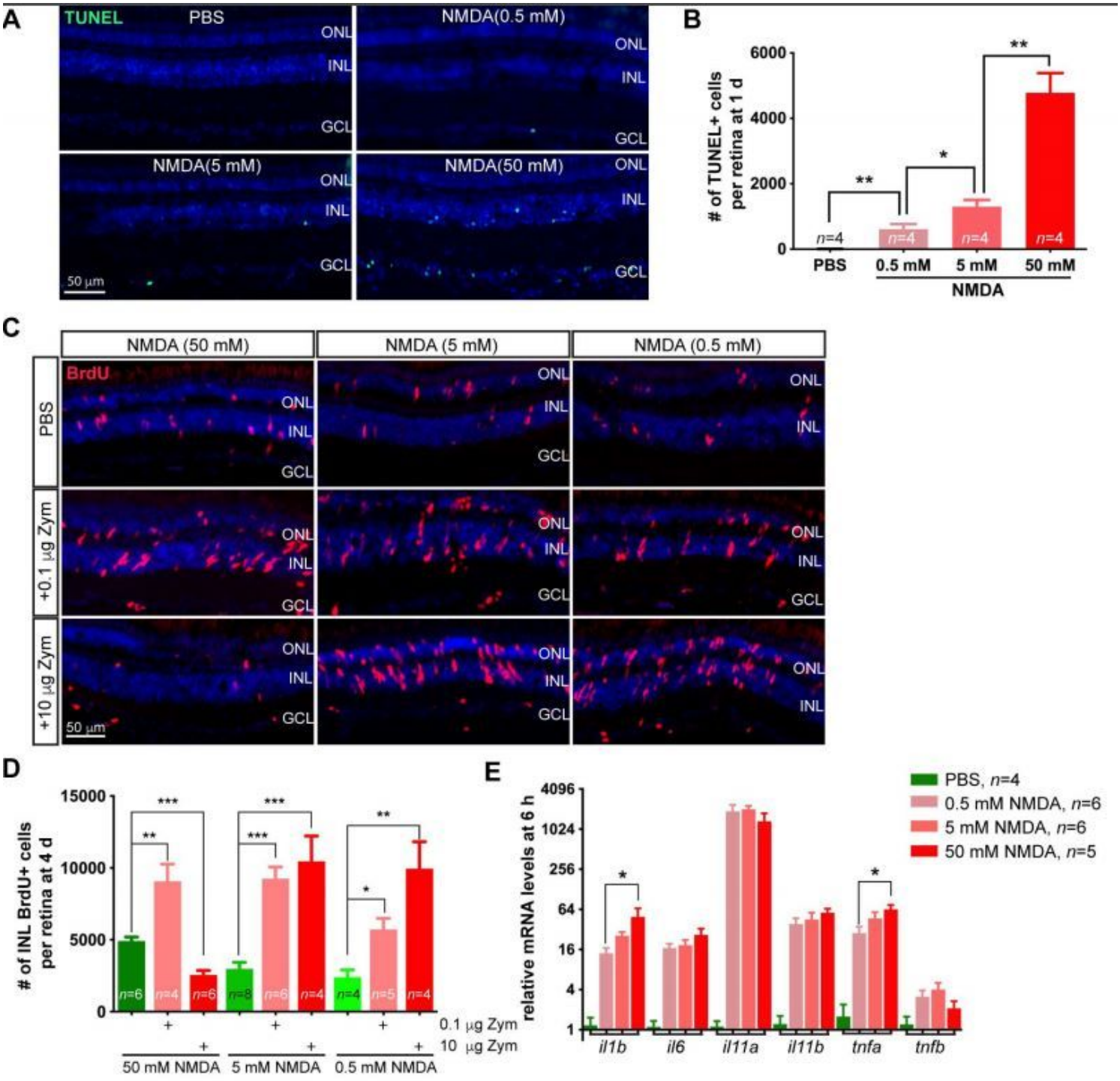

Figure 7

The influence of inflammation on MGPC formation also depends on the extent of retinal damage. (A) TUNEL assay showing the cell death in retinas treated with PBS or indicated concentrations of NMDA at 1 dpi. (B) Quantification of (A). (C) BrdU immunofluorescence showing the formation of MGPCs in retinas treated with indicated doses of Zym and NMDA at 4 dpi. (D) Quantification of (C). (E) Expression of typical inflammatory cytokines in retinas treated with PBS or indicated doses of NMDA at 6 hpi. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with PBS control. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

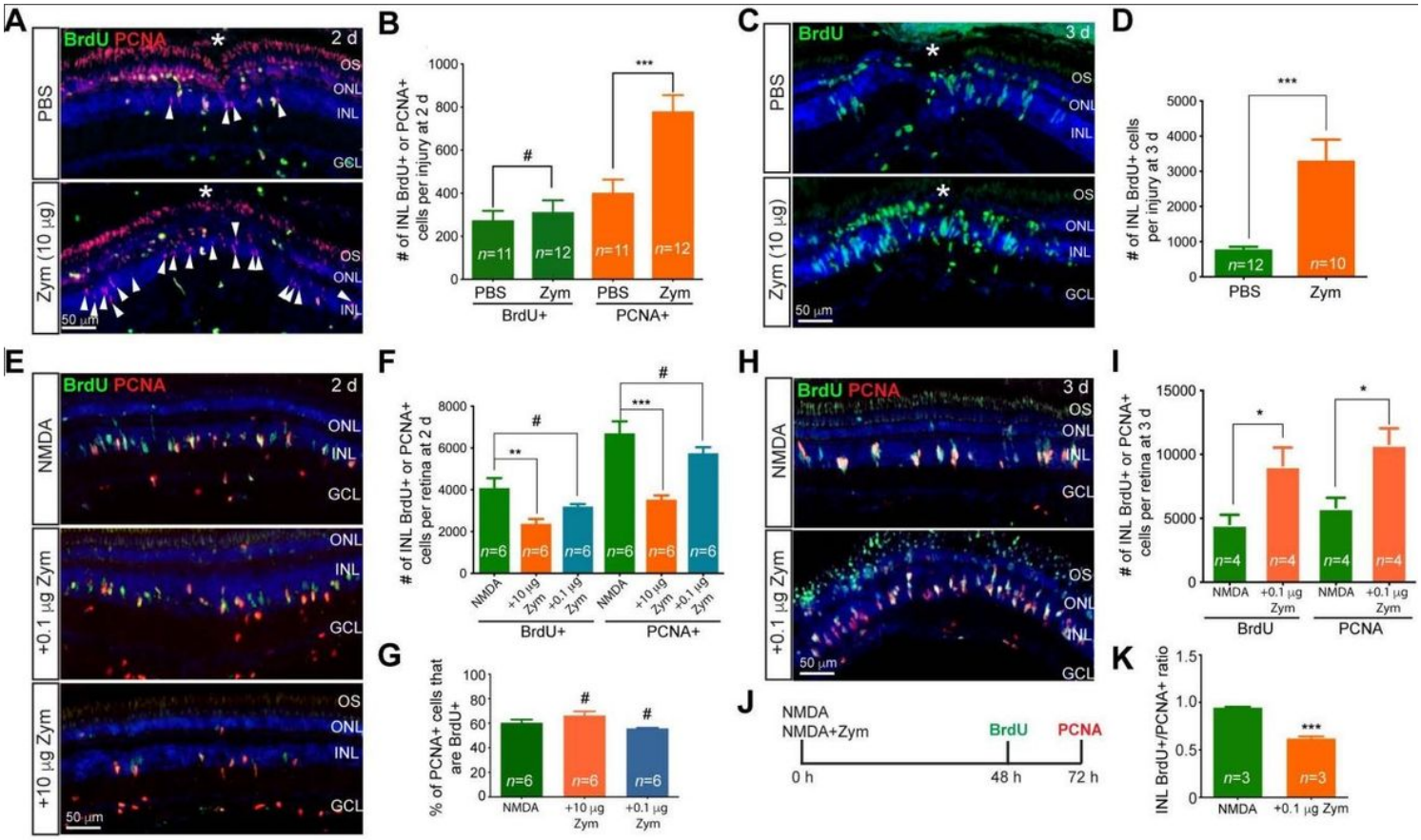

Figure 8

Influence of enhancing inflammation on MG proliferation in the stab-injury and NMDA-injury paradigms. (A) BrdU and PCNA immunofluorescence showing the cell proliferation in control and Zym-treated retinas after stab-injury at 2 dpi. Arrowheads, PCNA+/BrdU- cells in the INL. (B) Quantification of (A). (C) BrdU immunofluorescence showing the cell proliferation in control and Zym-treated retinas after stab-injury at 3 dpi. (D) Quantification of (C). (E) BrdU and PCNA staining showing the cell proliferation in control and Zym-treated retinas after NMDA-injury at 2 dpi. (F,G) Quantification of (E). (H) BrdU and PCNA staining showing the cell proliferation in control and Zym-treated retinas after NMDA-injury at 3 dpi. (I) Quantification of (H). (J) Timeline of the lineage tracing experiment in the NMDA-injury paradigm. (K) Quantification of the BrdU+/PCNA+ ratio in the INL at 3 dpi of (J). White asterisks in (A,C), site of the stab injury. #, $p > 0.05$ compared with control. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control. Note the autofluorescence in the outer segments is not BrdU or PCNA signal. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

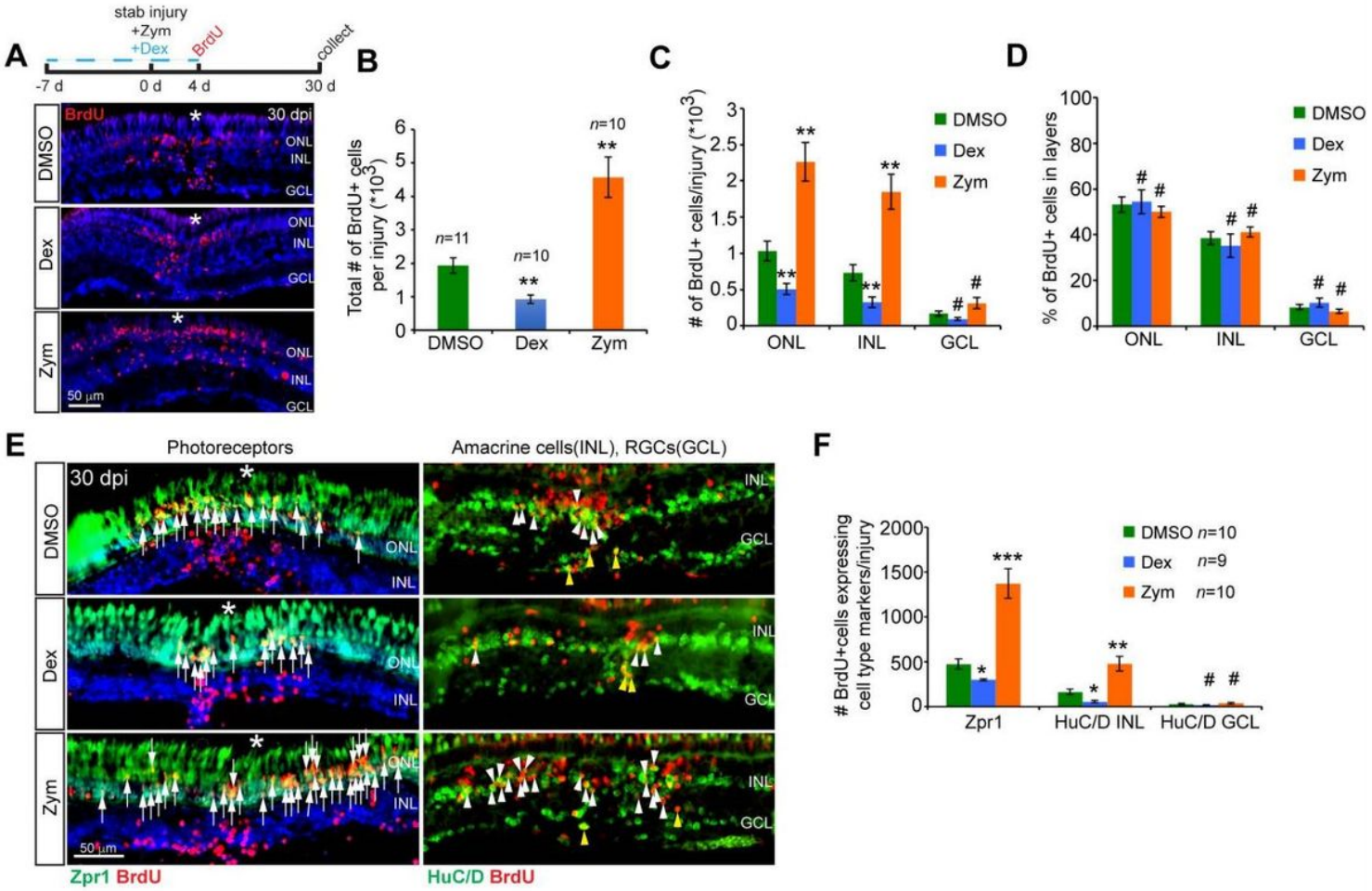

Figure 9

Inflammation promotes the regeneration of retinal neurons in the stab-injury model. (A) Timeline of the lineage tracing experiment and BrdU immunofluorescence at 30 dpi showing the distribution of MGPC descendants in retinas treated with solvent control, Dex or Zym (10 µg). (B) Quantification of the total number of BrdU+ cells per injury of (A). (C,D) Quantification of the number and percentage of BrdU+ cells in each retina layer of (A). (E) Zpr1, HuC/D and BrdU immunofluorescence showing the regenerated retinal neurons at 30 dpi in retinas treated with solvent control, Dex or Zym. Arrows, regenerated photoreceptors; White arrowheads, regenerated amacrine cells; Yellow arrowheads, regenerated RGCs. (F) Quantification of the number of regenerated photoreceptors (Zpr1+ BrdU+), amacrine cells (HuC/D+ BrdU+ in the INL) and RGCs (HuC/D+ BrdU+ in the GCL) in (E). White asterisks in (A,E), site of the stab injury. #, $p > 0.05$ compared with control. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

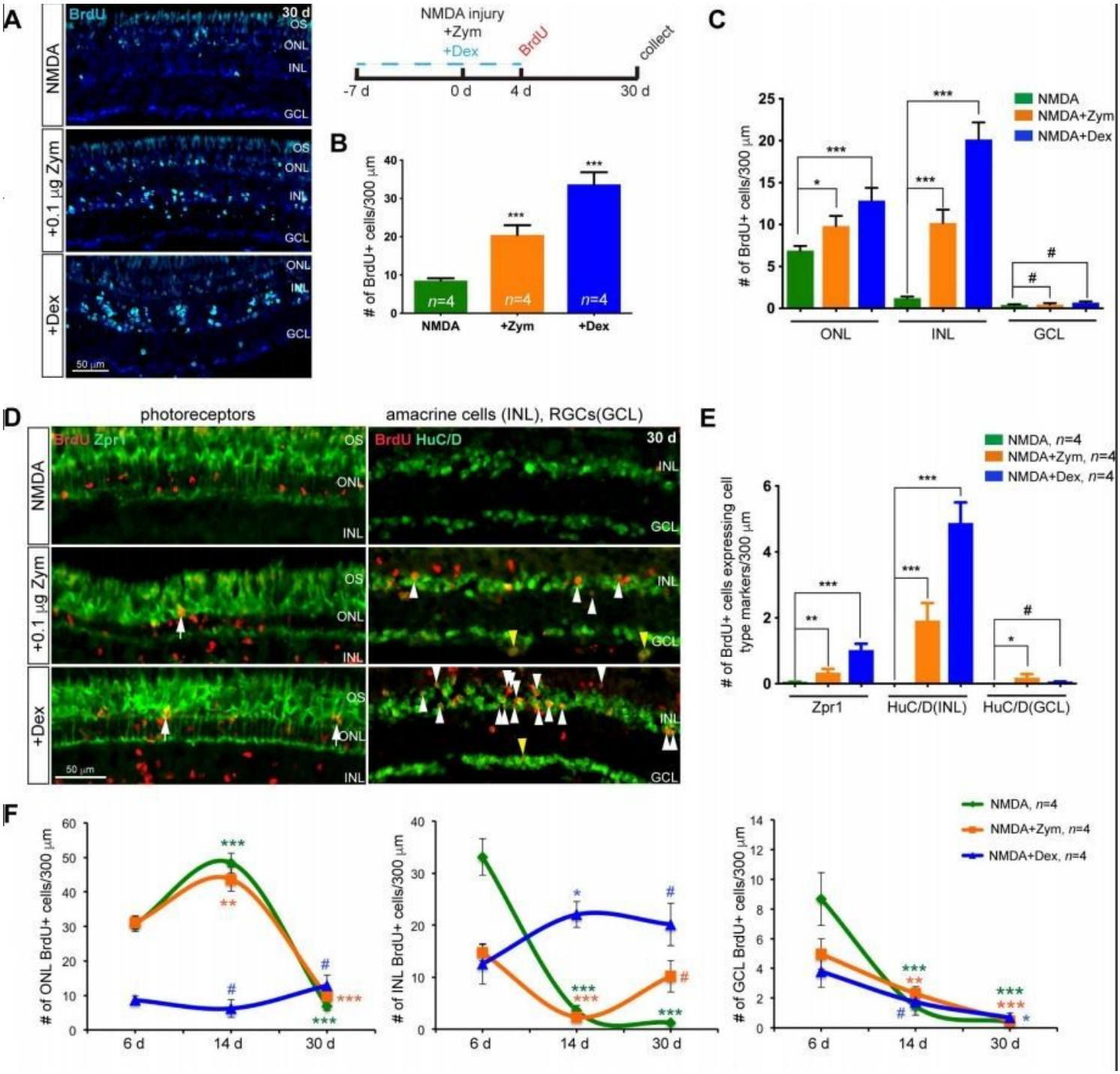

Figure 10

Influence of immune manipulation on retina regeneration in the NMDA-injury paradigm. (A) Timeline of the experiment and BrdU immunofluorescence at 30 dpi showing the distribution of MGPC descendants in control, Dex- or Zym-treated retinas after NMDA injury (50 mM). Note the autofluorescence in the outer segments. (B,C) Quantification of the number of BrdU+ cells in the retina. “Per 300 µm” is defined as the entire region of the neural retina with a span of 300 µm on 12 µm thickness cryosections. (D) Zpr1, HuC/D and BrdU immunofluorescence showing the regenerated retinal neurons at 30 dpi in control, Dex- or Zym-treated retinas. White arrows, Zpr1+/BrdU+ photoreceptors. White arrowheads, HuC/D+/BrdU+

amacrine cells. Yellow arrowheads, HuC/D+/BrdU+ RGCs. Note the autofluorescence in the outer segments is not BrdU signal. (E) Quantification of the number of regenerated retinal neurons of (D). (A-E) #, $p > 0.05$. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the NMDA control. (F) Quantification of the number of BrdU+ cells in each retinal layer at the indicated time points during the lineage-tracing experiment; #, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the result of 6 d in the same group. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Supplementary Files

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