

Reviewed Preprint

v1 • April 30, 2025

Not revised

Reviewed Preprint

v2 • December 9, 2025

Revised by authors

cxcl18b-defined transitional state-specific nitric oxide drives injury-induced Müller glia cell-cycle re-entry in the zebrafish retina

✉ For correspondence:

jjehe@ion.ac.cn

changchen@moon.ibp.ac.cn

* Lead contact

Competing interests: No competing interests declared

Funding: See page 35

Reviewing editor: Michael E Zuber, SUNY Upstate Medical University, United States

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Aojun Ye^{1,3}, Shuguang Yu², Meng Du⁴, Dongming Zhou⁴, Jie He²✉, Chang Chen^{1,3,*}✉

¹National Laboratory of Biomacromolecules, CAS Center for Excellence in Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China • ²Institute of Neuroscience, State Key Laboratory of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China • ³University of Chinese Academy of Sciences, Beijing, China • ⁴Tianjin Medical University, Tianjin, China

eLife Assessment

Following retinal injury, zebrafish Müller glia reenter the cell cycle and generate replacement cells; this potentially **valuable** study proposes that injury induces a *cxcl18b*+ transitional state in Müller cells, which then express nitric oxide, inhibiting Notch signaling and allowing Müller glial cells to reenter the cell cycle. However, the evidence supporting the claims is **incomplete**, and the authors have made interpretations and conclusions that are not supported by the data. Questions of the temporal expression and function of *cxcl18b*, as well as the source of potential inflammatory cues before *cxcl18b* expression, remain unanswered and technical limitations and data inconsistencies raise concerns. Using larval animals complicates the analysis since the retina is still forming, and distinguishing between injury-induced regeneration and ongoing development is complex. With more rigorous testing of the signaling pathways proposed and a clear demonstration of their interdependence, the link between nitric oxide signaling and Notch activity, particularly, would interest those investigating retinal regeneration.

<https://doi.org/10.7554/eLife.106274.2.sa3>

Abstract

In lower vertebrates, retinal Müller glia (MG) exhibit a life-long capacity of cell-cycle re-entry to regenerate neurons following the retinal injury. However, the mechanism driving such injury-induced MG cell-cycle re-entry remains incompletely understood. Combining single-cell transcriptomic analysis and *in-vivo* clonal analysis, we identified previously undescribed *cxcl18b*-defined MG transitional states as essential routes towards MG proliferation following green/red cone (G/R cone) ablation. Inflammation blockage abolished the triggering of these transitional states, which expressed the gene modules shared by cells of the ciliary marginal zone (CMZ), where life-long adult neurogenesis takes place. Functional studies of the redox properties of these transitional states further demonstrated the regulatory role of nitric oxide (NO) produced by *Nos2b* in injury-induced MG proliferation. Finally, we developed a viral-based strategy to specifically disrupt *nos2b* in *cxcl18b*-defined MG transitional states and revealed the effect of transitional state-specific NO signaling. Our findings elucidate the precision redox mechanism underlying injury-induced MG cell-cycle re-entry, providing insights into species-specific mechanisms for vertebrate retina regeneration.

Introduction

Unlike mammalian counterparts, retinal Müller glia (MG) in aquatic and amphibian species can enter injury-induced regeneration program (Goldman, 2014; Lahne et al., 2020b; Wan and Goldman, 2016), which unfolds a set of temporal events from glial reactivation, cell-cycle re-entry, new neuron generation, and circuit integration (Abraham et al., 2024; Powell et al., 2016). In zebrafish, MG respond to injury by glial reactivation, which refers to the initial response of quiescent MG, characterized by morphological changes and upregulation of reactive markers (*gfap*, *ascl1a*, *lin28a*) and activation of signaling pathways such as Notch, Jak/Stat, and Wnt (Lahne et al., 2020b; Pollak et al., 2013; Sifuentes et al., 2016; Yao et al., 2016). This is followed by the proliferation phase, in which MG initiate a transcriptional shift toward a progenitor-like state and cell cycle re-entry. Many genes induced by glial reactivation, such as *lin28a*, *sox2*, and *mycb/h*, are required for cell-cycle re-entry and subsequent neurogenesis (Gorsuch et al., 2017; Jorstad et al., 2017; Lee et al., 2024; Ramachandran et al., 2010). Diverse signaling pathways have been identified to participate in this initial regenerative response (Campbell et al., 2021; Wan and Goldman, 2017).

In contrast, although forced expression of factors like *Ascl1* or deletion of repressive chromatin regulators can partially reprogram MG into interneuron-like cells in the injured mouse retina (Hoang et al., 2020; Jorstad et al., 2017), the knowledge of the upstream signals that trigger injury-induced cell-cycle re-entry, a critical step for producing neurogenic progenitors, remains limited (Lenkowski and Raymond, 2014; Vihtelic and Hyde, 2000; Wu et al., 2001). The mechanistic understanding of MG cell-cycle re-entry in the injured zebrafish retina will provide critical knowledge for inspiring new strategies for *in-vivo* re-programming MG to repair the human retina.

The intrinsic and environmental factors contribute to injury-induced MG proliferation (Gao et al., 2021; Lahne et al., 2020b; Xiao et al., 2023). Recent efforts in single-cell RNA-sequencing (scRNA-Seq) have identified *ascl1a*, *clcf1/crlf1a* (Boyd et al., 2023), and *mycb/mych* (Lee et al., 2024) as key intrinsic drivers of injury-induced MG proliferation in the zebrafish retina. Also, the elegant study of MG injury responses across multiple species, including zebrafish, chicken, and mice, revealed the *hmg1/yap1* signaling network as a key regulator of MG reactivation and neurogenesis in the regenerating retina (Hoang et al., 2020). Interestingly, previous studies further reported that post-injury MG in the zebrafish is capable of re-acquiring the regeneration program that largely recapitulates the embryonic retinal developmental program (Celotto et al., 2023; Hoang et al., 2020; Lahne et al., 2020a; Lyu et al., 2023).

In terms of extrinsic cues, two sources, those from damaged neurons and injury-recruited microglia, are considered indispensable for injury-induced MG proliferation (Leach et al., 2021; White et al., 2017). Previous studies have shown that signals derived from various retinal neuron injury models trigger MG regeneration (Lyu et al., 2023). Meanwhile, accumulating evidence increasingly appreciates the role of inflammatory signals derived from injury-recruited microglia, such as *TNFα* (Conner et al., 2014; Nelson et al., 2013), cytokines IL-1 β , and IL-10 (Lu and Hyde, 2024). Notably, inflammatory responses are intrinsically associated with redox signaling, which is involved in the regeneration processes in various non-neuronal tissues (Breus and Dickmeis, 2021; Jaeschke, 2000; Zhang et al., 2024). However, the involvement of the redox signaling resulting from microglia-derived inflammatory signals in MG cell-cycle re-entry following retina damage remains to be clarified. Nitric oxide (NO), a redox signal, has participated in regenerating various tissues in zebrafish, including the heart (Rochon et al., 2020; Yu et al., 2024), spinal cord (Bradley et al., 2010), and fin (Matrone et al., 2021). Endogenous NO is derived from three forms of NO synthase (NOS) in mammals: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The zebrafish comprises *Nos1*, a form of neuronal *Nos*, and *Nos2a/2b*, two inducible *Nos*.

In this study, we performed single-cell RNA sequencing to characterize the post-injury MG states in the zebrafish retina following the specific ablation of green/red cone. This comprehensive analysis led us to define a set of previously unknown *cxcl18b*-positive transitional states as an essential

route of MG cell-cycle re-entry in response to injury, and the inflammatory was indispensable for the induction of these transitional states. Intriguingly, we found that these *cxcl18b*-defined transitional states exhibited gene patterns shared by cell states in the CMZ, favoring the idea that *cxcl18b*-defined MG transitional states might represent the developmental program conserved by neuronal regeneration beyond embryonic development. A remarkable phenotype of enriched redox features in these gene patterns suggested the importance of redox signaling. The subsequent screening of redox-related genes revealed the essential role of nitric oxide, produced by *Nos2b*, in triggering MG cell-cycle re-entry after the retinal injury. Notably, we developed a sophisticated viral-based approach to achieve the *cxcl18b*-defined MG transitional state-specific knockout of *nos2b* successfully and verified the requirement of transitional state-specific NO signaling in injury-induced MG proliferation. These findings provide novel cellular and molecular insights into this species-specific post-injury MG cell-cycle re-entry process, with potential implications for the development of regenerative medicine strategies.

Results

Single-cell transcriptome analysis reveals the landscape of injury-induced Müller glia states

Zebrafish Müller glia can respond to retina injury by cell cycle re-entry, a critical step evolutionarily absent from their mammalian counterparts but essential for neuron regeneration (Goldman, 2014; Powell et al., 2016). We created a zebrafish retina injury model by crossing *Tg(opn1lws2: nfsb-mCherry)^{uom3}* (referred to as *Tg(lws2: nfsb-mCherry)*) with *Tg(mpeg1: GFP)* fish, in which the bacterial nitroreductase (NTR) enzyme was specifically expressed in G/R cone. We selectively ablated the G/R cone starting at 5 days post-fertilization (dpf) by a subsequent 120 hours of metronidazole (MTZ) exposure (Curado et al., 2007; Curado et al., 2008) (Figure 1A and Figure 1-figure supplement 1A). G/R cone became significantly reduced in number since 48 hours post-injury (hpi) and was mostly depleted at 120 hpi (Figure 1B). Meanwhile, a number of microglia (marked by *Tg(mpeg1: GFP)*) migrated to the outer nuclear layer (ONL) as early as 48 hpi, peaked at 72 hpi, and began to reduce in number at 96 hpi and onward (Figure 1C). To confirm the identity of these proliferating cells, we performed BLBP immunostaining and observed that the PCNAC cells were also BLBPC (Figure 1-figure supplement 1B), indicating their MG origin. Notably, in response to G/R cone ablation, the proliferative MG population increased starting at 48 hpi, peaked at 72 hpi, and began to decline since 96 hpi and forward (Figure 1D). Considering the result that the number of proliferative MG peaked at 72 hpi, we focused on the 72-hpi time point for further exploration of MG proliferative behaviors following G/R cone ablation (Figure 1E).

By revisiting previously obtained scRNA-seq data of MG enriched from *Tg(lws2: nfsb-mCherry)* crossed with *Tg(gfap: EGFP)* and *Tg(her4.1: dRFP)* retina before and after G/R cone ablation at 72 hpi (Krylov et al., 2023), we selected 5,932 and 3,999 MG cells and their derived progenies from the uninjured and 72-hpi retina, respectively (Details in Methods; Figure 1-figure supplement 1C). By clustering these cells, we identified 13 clusters, 8 out of which (Clusters 2, 3, 5, 6, 9, 10, 11, and 12) with an increased proportion in response to the G/R cone ablation (Figure 1-figure supplement 1D). Subsequently, we re-clustered cells of these 8 clusters (695 cells of uninjured retinae and 3,477 cells of 72-hpi retinae), aggregating into 10 new clusters. After the quality control procedure, we did not consider Clusters 7/8/9 due to their small populations with ribosomal, dendritic, and doublet features, resulting in 7 clusters for further analysis. (Details in Methods; Figure 1F)

We performed the pseudo-time trajectory analysis to reveal the progression of these MG clusters following the cone ablation (Figure 1G). Cluster 4 cells were highly expressing genes related to mature MG (*glula*, *slc1a2b*, *apoeb*, and *rlbp1a*) (Bernardos and Raymond, 2006; Raymond et al., 2006; Thummel et al., 2008; Yurco and Cameron, 2005), the quiescent state (*cx43*) (Dermietzel et al., 2000; Janssen-Bienhold et al., 1998), and major MG population marker (*gf24*) (Krylov et al., 2023) (Figure 1-figure supplement 1E). Furthermore, Cluster 4 began to

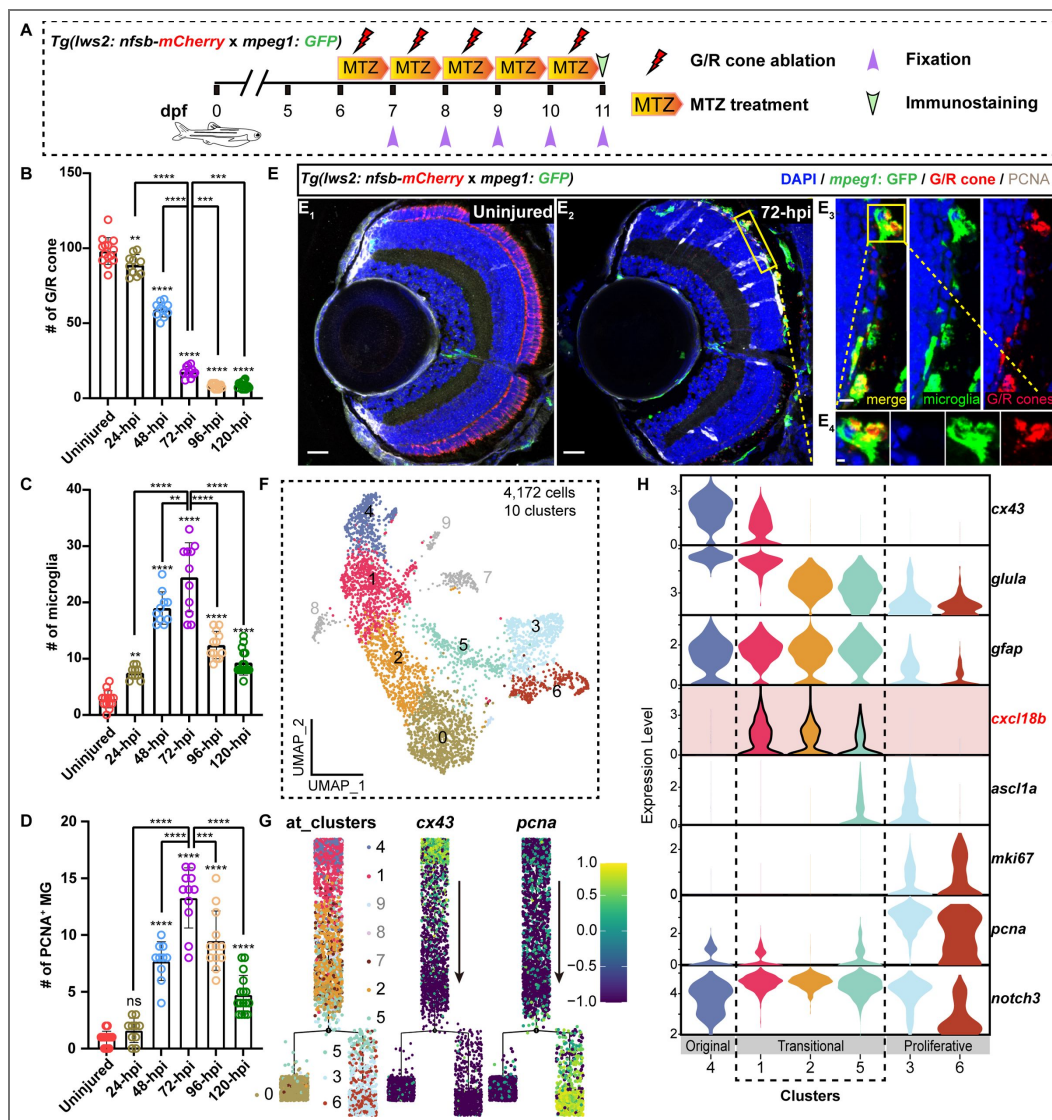


Figure 1. Single-cell RNA seq reveals injury-induced the *cxcl18b*-defined MG transitional states

(A) Schematic showing the experimental procedure: five consecutive days of MTZ treatment in *Tg(lws2: nfsb-mCherry x mpeg1: GFP)* fish to ablate green and red (G/R) cone, starting at 6 dpf and continuing until 11 dpf. The MTZ solution was refreshed every 24 hours, followed by fish fixation for further immunostaining. **(B-D)** Quantitative plots showing the dynamic changes in the number of G/R cone (**B**), recruited microglia (**C**), and proliferative MG (PCNA⁺) (**D**) at different time points after MTZ treatment (uninjured: collected retina number n=14; 24-hpi: n=10; 48-hpi: n=10; 72-hpi: n=11; 96-hpi: n=12; 120-hpi: n=16; mean ± SEM; ****p<0.0001, ***p<0.001, **p<0.01, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). **(E)** Representative images showing microglia recruitment (*mpeg1: GFP*, green), G/R cone ablation (*lws2: nfsb-mCherry*, red), and injury-induced MG proliferation (PCNA, white) in uninjured (**E₁**) and 72-hpi (**E₂**) retinas. The high-magnification images of the boxed area (**E₃**-**E₄**). Scale Bars: 20μm (**E₁**, **E₂**), 10μm (**E₃**), 2μm (**E₄**). **(F)** The UMAP plot of 4,172 MG cells was sorted with an increased proportion in response to the G/R cone ablation. Cells were further aggregated into 10 clusters based on previously published scRNA-seq data (Krylov et al., 2023). **(G)** Pseudo-time developmental trajectory of MG states identified by Monocle2 analysis shows a main developmental branch originating from Cluster 4 (*cx43*⁺), which diverges into two sub-branches: Cluster 0 and Clusters 5/3/6 (*pcna*⁺). **(H)** Violin plots showing the expression levels of key genes (*cx43*, *glula*, *gfap*, *cxcl18b*, *ascl1a*, *mki67*, *pcna*, and *notch3*) in the main developmental branch clusters, progressing from the most original MG states (Cluster 4) to transitional MG states (Clusters 1/2/5), and proliferative MG states (Clusters 3/6).

express *s100a10b* and *gfap*, reactive state makers (Celotto et al., 2023; Hoang et al., 2020), in the injured retinae, but not in the uninjured retina (Figure 1-figure supplement 1E). Thus, we set Cluster 4 as the early transitional MG state.

To examine the transition of these 7 MG clusters, the pseudo-time trajectory showed that the main developmental branch consisted of Clusters 4/1/2 and then became divergent into two sub-branches, including Cluster 0 and 5/3/6 (Figure 1G). In contrast to the sub-branch of Cluster 0, the sub-branch of Clusters 5/3/6 was highly expressing proliferative cell markers (*pcna*, *mki67*, and *mcm2*). Within the latter sub-branch, while Cluster 3 and 6 with the highest levels of proliferative cell markers, Cluster 6 began to express neuronal differentiation factors (*otx5*, *crx*, and *pde6gb*) (Abalo et al., 2020; Asaoka et al., 2014; Shen and Raymond, 2004) (Figure 1-figure supplement 1E). Thus, we identified 6 major post-injury MG states, from the early transitional state (Cluster 4) to three transitional states (Cluster 1/2/5), to finally two proliferative states (Cluster 3/6).

Remarkably, chemokine (C-X-C motif) ligand 18b (*cxcl18b*), an inflammatory chemokine, was uniquely expressed in three transitional states but largely absent from the early transitional Cluster 4 and two proliferative Cluster 3/6. Specifically, while the first *cxcl18b*⁺ transitional state (Cluster 1, was expressing *cx43*, a maker for MG quiescence (Dermietzel et al., 2000; Janssen-Bienhold et al., 1998), the last transitional state (Cluster 5) began to show a weak induction of *ascl1a* (Ramachandran et al., 2010). Immediately following this last transitional state, Cluster 3 started with high *ascl1a* expression and entered the proliferative state with the expression of *pcna* and *mik67* (Figure 1H). Our analysis highlighted a new set of *cxcl18b*-defined MG transitional states preceding *ascl1a* induction, bridging MG from the most original quiescence state to injury-induced proliferation.

Clonal analysis reveals injury-induced MG proliferation via *cxcl18b*-defined transitional states associated with inflammation

To directly verify the presence of *cxcl18b*-defined MG transitional states, we first examined the temporal relationship of *cxcl18b* expression and MG proliferation after the cone ablation using *in situ* hybridization combined with immunostaining of either BLBP (an MG maker) or PCNA (a proliferative cell maker) (Figure 2A and Figure 2-figure supplement 1A). The result showed that as early as 24 hpi, the number of *cxcl18b*⁺ MG was rapidly peaked with no emergence of proliferative MG (11 ± 4 , $n=10$ in *cxcl18b*⁺ MG; mean $\pm$ SEM), and then *cxcl18b*⁺ MG continued declining in number over time and reached the lowest level since 96 hpi (1 ± 1 , $n=7$; mean $\pm$ SEM; Figure 2B). In contrast, the number of proliferative MG (PCNA⁺) peaked at 72 hpi and decreased to the lowest level at 120 hpi (9 ± 2 , $n=11$ in 72-hpi retina; 1 ± 1 , $n=5$ in 120-hpi retina; mean $\pm$ SEM; Figure 2B). Note that *cxcl18b*⁺ MG was mostly proliferative at 72 hpi (Figure 2-figure supplement 1B).

To further verify the temporal expression of *cxcl18b* in MG following the cone ablation, we created a new transgenic reporter *Tg(cxcl18b: GFP)* by cloning a 3k-bp-long cis-element of 5'UTR with GFP, allowing real-time monitoring of injury-induced *cxcl18b* expression *in vivo* (Figure 2C and Figure 2-figure supplement 1C-D). Combining this line with PCNA immunostaining, we confirmed the remarkable increase of *cxcl18b* expression (GFP⁺) at 48 hpi (0 ± 1 , $n=4$ in uninjured retinae vs 11 ± 4 , $n=7$ in 48-hpi; mean $\pm$ SEM; Figure 2D and E). Due to the prolonged stay of GFP protein, we could also observe that some GFP⁺ MG were also PCNA⁺ (Figure 2D). The result of proliferative MG as a subpopulation of *cxcl18b*⁺ MG led to an outstanding question as to whether *cxcl18b*-defined MG transitional states represented an essential route to injury-induced proliferation.

To address it, we created a new transgene *Tg(cxcl18b: Cre-vmhc: mCherry:: ef1a: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)* to perform the clonal analysis of historical *cxcl18b*-expressing in MG after the G/R cone ablation (Details in Method; Figure 2-figure supplement 1E). After ablating the G/R cone by MTZ treatment at 6 dpf for 3 consecutive days, we found that the number of *cxcl18b* lineage-traced MG (marked by GFP⁺) and PCNA⁺ MG was significantly increased at 72 hpi (Figure

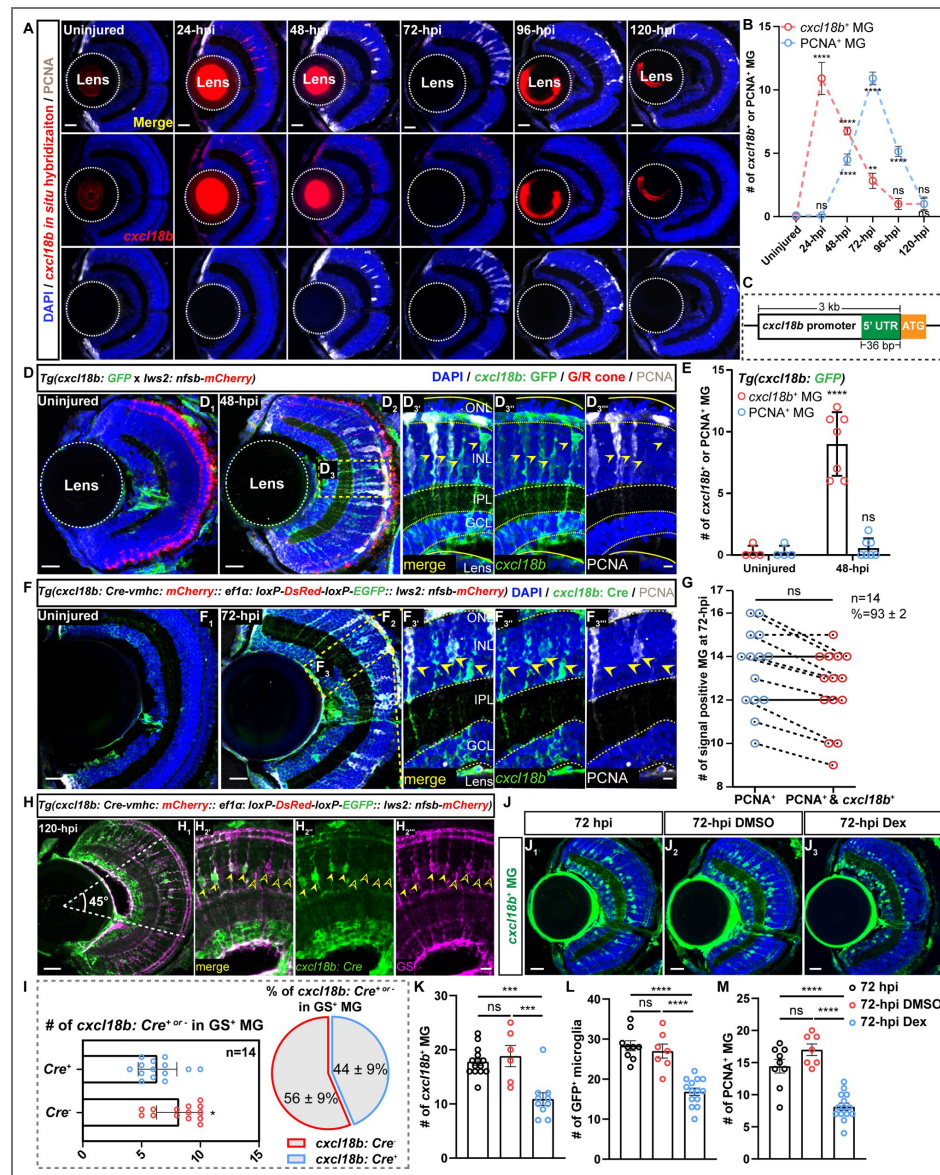


Figure 2. Clonal analysis reveals the proliferative MG mostly originated from *cxcl18b*⁺ MG transitional states

(A) Representative images show dynamic expression of *cxcl18b* (red, *in situ* hybridization) and PCNA (white, immunostaining) in *Tg(lws2: nfsb-mCherry)* retina at different time points following the G/R cone ablation. Scale bars: 20 μ m. **(B)** Quantitative plots showing the number of *cxcl18b*⁺ (red curve, significance shown above the curve) and PCNA⁺ MG (blue curve, significance shown below the curve) in uninjured (n=11) and injured retina at 24-hpi (n=10), 48-hpi (n=12), 72-hpi (n=11), 96-hpi (n=7), and 120-hpi (n=5). Each injured time point was compared to the uninjured retina (Mean $\pm$ SEM; ****p<0.0001, **p<0.01, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). **(C)** Schematic diagram of the *cxcl18b* promoter was used to construct the reporter fish line *Tg(cxcl18b: GFP)* and clonal analysis fish line *Tg(cxcl18b: Cre-vmhc-mCherry:: efla: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)*. **(D)** Immunostaining of injury-induced *cxcl18b*⁺ (green, indicated by *Tg(cxcl18b: GFP)*) and proliferative (PCNA⁺, white) MG showing overlapping in the central retina area (yellow arrows, GFP⁺/PCNA⁺ MG) at 48 hpi. The high-magnification images of the boxed area (**D**₃-**D**₃''). The area of the retina is labeled with a dashed line, and each layer structure is labeled with dashed lines and marked with the outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and lens. Scale Bars: 20 μ m (**D**₁-**D**₂) and 5 μ m (**D**₃-**D**₃''). **(E)** Quantitative plots showing the number of *cxcl18b*⁺ MG (red) and proliferative MG (PCNA⁺, blue) in (**D**₁) uninjured (n=4) and (**D**₂) 48-hpi retina (n=7) (Mean $\pm$ SEM; ****p<0.0001, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test). **(F)** Clonal analysis of injury-induced *cxcl18b*⁺ MG in transgenic fish line *Tg(cxcl18b: Cre-vmhc: mCherry:: efla: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)* at 72 hpi retina showing overlapping between proliferative (PCNA⁺, white) MG with *cxcl18b*⁺ (green, yellow arrows). The high-magnification images of the boxed area (**F**₃-**F**₃''). Scale Bars: 20 μ m (**F**₁-**F**₂) and 5 μ m (**F**₃-**F**₃'').

2F). Further analysis showed that all PCNA⁺ MG were *cxcl18b* lineage-traced MG, indicating the *cxcl18b* lineage-traced MG were the ones who could eventually enter into the cell cycle (13 ± 2 cells for PCNA⁺ vs 13 ± 2 cells PCNA⁺ and GFP⁺ MG; percentage of PCNA⁺ and *cxcl18b*⁺ vs PCNA⁺ = $93 \pm 2\%$; $n=14$; $p=0.19$; Mean $\pm$ SEM; Figure 2G). Further analysis showed that at 120 hpi, the time point that the MG proliferation has largely ceased, GFP⁺ MG with the lineage history of injury-induced *cxcl18b* expression constituted about 44% of GS⁺ MG at the central retina, indicating that only about half of MG could enter *cxcl18b*⁺ transitional states following the cone ablation (14 ± 3 in GS⁺ and GFP⁺ MG; 28 ± 4 in GS⁺ and GFP⁻ MG, $n=14$; $p<0.05$; mean $\pm$ SEM; Figure 2I). Together, our clonal analysis demonstrated that proliferative MG mostly originated from *cxcl18b*-defined MG transitional states, and 44% central MG could become *cxcl18b* positive. To investigate whether *cxcl18b* was required for MG proliferation following G/R cone ablation, we employed CRISPR-Cas9-mediated gene disruption, using two sgRNAs targeting *cxcl18b* (Figure 2-figure supplement 1F and G). We found that *cxcl18b* knockout did not reduce MG proliferation after G/R cone ablation at 72 hpi (13 ± 3 , $n=11$ in WT; 11 ± 3 , $n=7$ in *scramble* sgRNA-injected; 13 ± 3 , $n=7$ in *cxcl18b* sgRNA-injected; mean $\pm$ SEM), suggesting that *cxcl18b* per se does not regulate MG proliferation directly (Figure 2-figure supplement 1H and I). This led us to wonder about the induction of *cxcl18b*-defined MG transitional states.

As an inflammatory chemokine, *cxcl18b* serves as a reliable marker of inflammation and regulates neutrophil recruitment to injury sites (Goumenaki et al., 2024; Torraca et al., 2017). Inflammation has been previously shown to be critical for inducing regenerative responses in adult zebrafish, where it promotes reactive microglia/macrophages and Müller glial proliferation in the retina (Iribarne and Hyde, 2022; Kyritsis et al., 2012). Notably, suppressing the immune response using dexamethasone (Dex) in zebrafish retina reduced microglial reactivation and significantly decreased the number of proliferative MG (Silva et al., 2020; Zhang et al., 2020). In our study, we identified the *cxcl18b*-defined transitional states as the essential routing for MG proliferation after G/R cone ablation. These results prompted us to investigate whether the inflammatory responses mediated by recruited microglia are indispensable for the formation of these *cxcl18b*-defined transitional states.

To address this, we examined *cxcl18b* expression using *Tg(cxcl18b: GFP)* after inhibiting inflammation using Dex (Iribarne and Hyde, 2022), and observed a significant reduction in the number of *cxcl18b*⁺ MG (GFP⁺ cells) at 72 hpi (11 ± 4 , $n=10$ in Dex treated retina vs 19 ± 5 , $n=6$ in DMSO treatment; and 18 ± 3 , $n=14$ in the 72-hpi retina; mean $\pm$ SEM) (Figure 2K). Consistent with earlier reports, we observed that Dex treatment inhibited the migration of microglia (indicated by *Tg(mpeg1: GFP)*; 16 ± 4 , $n=14$ in Dex treated retina vs 27 ± 5 , $n=7$ in DMSO treatment; and 28 ± 4 , $n=9$ in the 72-hpi retina; mean $\pm$ SEM) to the ONL and significantly reduced the number of proliferative MG (PCNA⁺; 8 ± 2 , $n=14$ in Dex treated retina vs 17 ± 2 , $n=7$ in DMSO treatment; and 14 ± 3 , $n=9$ in the 72-hpi retina; mean $\pm$ SEM) at 72 hpi after G/R cone ablation (Figure 2L and M and Figure 2-figure supplement 1J). These findings suggest that microglia-mediated inflammation may contribute to the activation of *cxcl18b*-defined transitional states that precede MG proliferation, although a causal relationship remains to be established. While dexamethasone suppressed both microglial recruitment and *cxcl18b*⁺ MG generation, its broad anti-inflammatory action precludes definitive conclusions about microglial causality. Dissecting this relationship would require concurrent ablation of microglia and cone photoreceptors using a triple-transgenic strategy, which is beyond the scope of the current study. Targeted approaches will be necessary to resolve the specific role of microglia in initiating *cxcl18b* expression.

***cxcl18b*-defined MG transitional states recapitulate molecular features of retinal stem cells in the ciliary marginal zone**

Interestingly, we observed the *cxcl18b* expression in the CMZ after the cone ablation besides its high expression in the MG (Figure 2A). We were then curious about the *cxcl18b* expression in the developmental retina as well as in the CMZ without the injury. Notably, *in situ* results showed that *cxcl18b* was largely absent from the central region but presented in the peripheral region of 30-hpf retinae, whereas it was highly expressed in the most peripheral region of the CMZ, where

fabp11a and *col15a1b*, two putative markers for post-embryonic retinal stem cells (RSCs) are located (Gonzalez-Nunez et al., 2010; Raymond et al., 2006) (Figure 3A-B and E-F, and Figure 3-figure supplement 1A). The transgenic line of *Tg(cxcl18b: GFP)* also showed a robust *cxcl18b* expression in the CMZ (Figure 3-figure supplement 1D). Consistently, our scRNAseq data of CMZ cells also confirmed the co-expression of *cxcl18b*, *fabp11a*, and *col15a1b* (Figure 3C-D and G-H, and Figure 3-figure supplement 1B). Furthermore, cluster 1 MG, at the earlier stage of transitional states, has the highest *cxcl18b* expression with *col15a1b* expression (Figure 1F and Figure 3-figure supplement 1C). All these results suggested that *cxcl18b*-defined transitional states, at least to some extent, represent the developmental state of retinal stem cells in the CMZ, but not that of embryonic retinal progenitors.

Nos2b is required for MG entry into the proliferation via NO signaling

Previous studies have demonstrated the involvement of redox signaling in cell regeneration processes in various tissues across species (Han et al., 2014; Hunter et al., 2018; Matrone et al., 2021; Yoo et al., 2012). All these evidences led us to directly test the roles of redox genes in serving as the molecular mechanism underlying injury-induced MG proliferation. Thus, we first examined the expression levels of a comprehensive list of redox genes in *cxcl18b*-defined MG transitional states (Clusters 1/2/5) in our scRNA-seq data (Figure 1H) and screened the influence of 18 genes from each major category of redox signaling on injury-induced MG proliferation using CRISPR-Cas9-mediated gene disruption (Figure 4-figure supplement 1A). We focused on the nitric oxide (NO) signaling pathway, targeting three genes encoding nitric oxide synthases (*Nos*): neuronal *Nos* (*nos1*) and two inducible forms (*nos2a* and *nos2b*), as well as the gene encoding S-nitrosoglutathione reductase (*gsnor* or *adh5*), which modulates reactive NO signaling (Figure 4A and Figure 4-figure supplement 1B). The consequence and efficiency of gene disruption were verified by DNA sequencing (Figure 4-figure supplement 1C). Notably, the disruption of *nos2b* resulted in a significant reduction of PCNA⁺ MG at 72 hpi (6±2, n=22 in *nos2b*-disrupted vs 11±3, n=7 in *scramble* sgRNA-injected; Mean ± SEM) (Figure 4B). Noted that *nos* gene disruption did not significantly alter microglia recruitment or G/R cone ablation at 72 hpi, suggesting that the influence of NO on injury-induced MG proliferation was not via inflammatory reactions of recruited microglia or injury degree (Figure 4-figure supplement 1D-F).

To further examine the function of *Nos2b* via NO, we employed various *Nos* inhibitors (L-NG-nitro arginine methyl ester, L_NAME; L-NG-Monomethyl Arginine, L_NMMA; 1400W) and NO scavengers (carboxy-PTIO, C-PTIO) (Goldstein et al., 2003; Hong et al., 2012; Moore et al., 1990; Rees et al., 1990). We performed the intraocular injection of the drugs (PBS as control) into the zebrafish eye from 2 days before cell ablation until 72 hpi (Figure 4C). Notably, the NO scavenger C-PTIO mostly suppressed MG proliferation, indicating the involvement of NO (cell number of PCNA⁺ MG: 3±2, n=27 after blocking NO by C-PTIO vs 14±4, n=16 in PBS-injected retina; Mean ± SEM) (Figure 4D and E). Moreover, 1400W (an inhibitor specific to inducible *Nos*, 7±2, n=10; Mean ± SEM) and L_NAME (a broad inhibitor to all three *Nos* forms, 8±2, n=12; Mean ± SEM) could also significantly reduce the number of proliferative MG after the ablation, whereas L_NMMA (the inhibitor specific to neuronal *Nos*) did not influence MG proliferation (11±3, n=12; Mean ± SEM) (Figure 4E). Taken together, these results highlight the critical role of NO signaling in regulating injury-induced MG proliferation.

We further generated nitric oxide pathway mutant zebrafish (*nos1*, *nos2a*, *nos2b*, and *gsnor*) to investigate the role of NO in MG proliferation following G/R cone ablation. Utilizing CRISPR/Cas9-mediated gene disruption, we successfully screened out *nos1*, *nos2a*, *nos2b*, and *gsnor* mutants, characterized by deletions of 133-bp, 13-bp, 220-bp, and 11-bp coding sequences, respectively (see Methods for details, Figure 4-figure supplement 2A and B). Consistent with the gene disruption experiments described above, we observed a significant reduction in the number of proliferative (PCNAC) MG at 72 hpi following G/R cone ablation in both heterozygous and

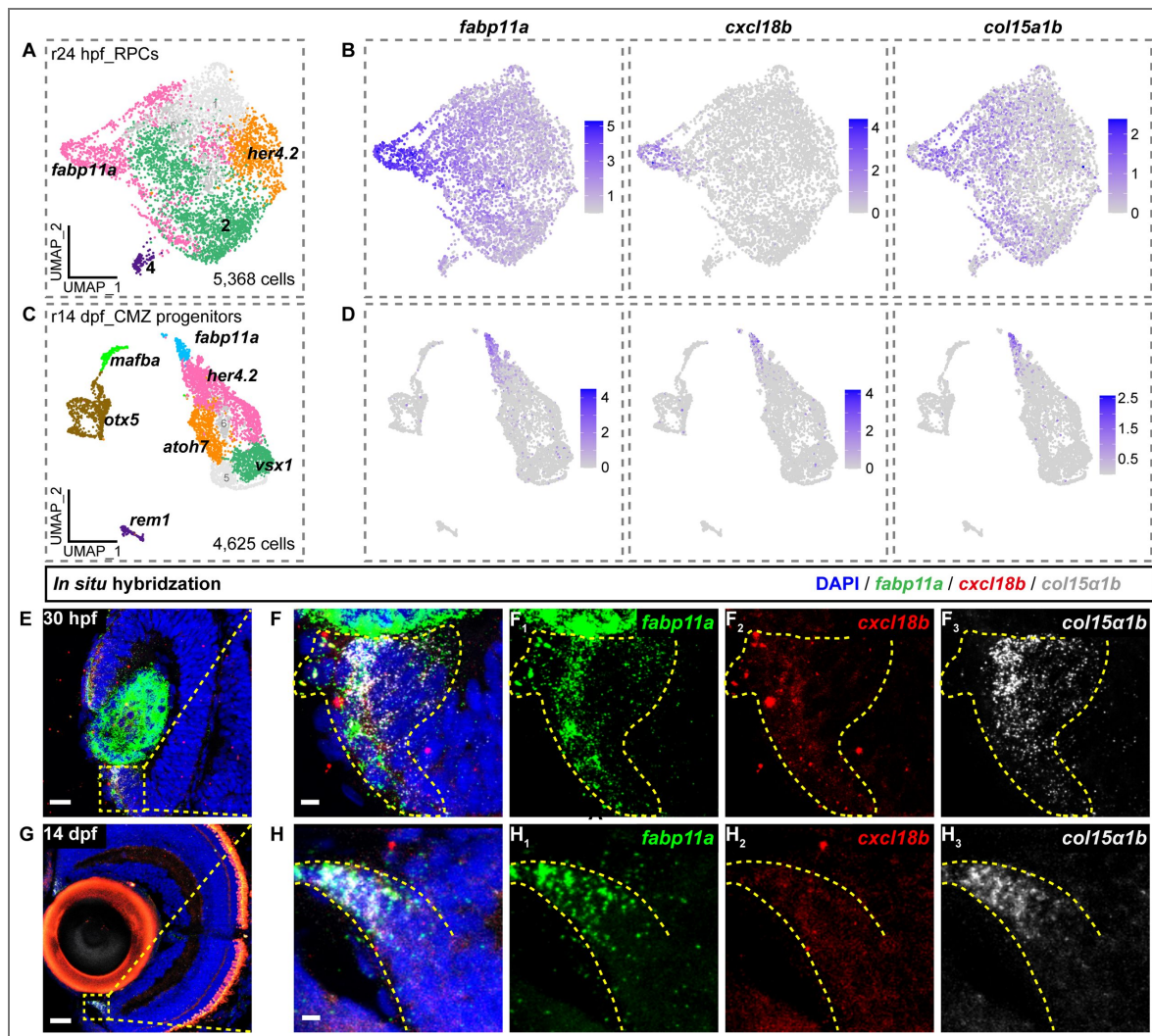


Figure 3. The *cxcl18b*-defined MG transitional states recapitulate the developmental states of RSCs in the CMZ

(A, C) UMAP plots display 5,368 retinal progenitor cells (RPCs) at 24 hpf and 4,625 CMZ progenitor cells at 14 dpf. Clusters are indicated by their cluster-specific marker genes based on previously published scRNA-seq data (Xu et al., 2020). (B, D) UMAP plots showing expression of *fabp11a*, *cxcl18b*, and *col15a1b* at r24-hpf RPCs (embryonic states) (B) and r14-dpf CMZ-progenitors (post-embryonic states) (D). (E-H₃) *In situ* hybridization images showing the expression of *fabp11a* (green), *col15a1b* (white), two putative markers for post-embryonic retinal stem cells (RSCs), and *cxcl18b* at 30-hpf (E-F₃) and 14-dpf (G-H₃) retina. The high-magnification images of the boxed area (F-F₃, H-H₃). The area of these three *in situ* signal trouble positive is labeled with a dashed yellow line. Scale Bars: 20Cμm (E, G) and 3 μm (F-F₃, H-H₃).

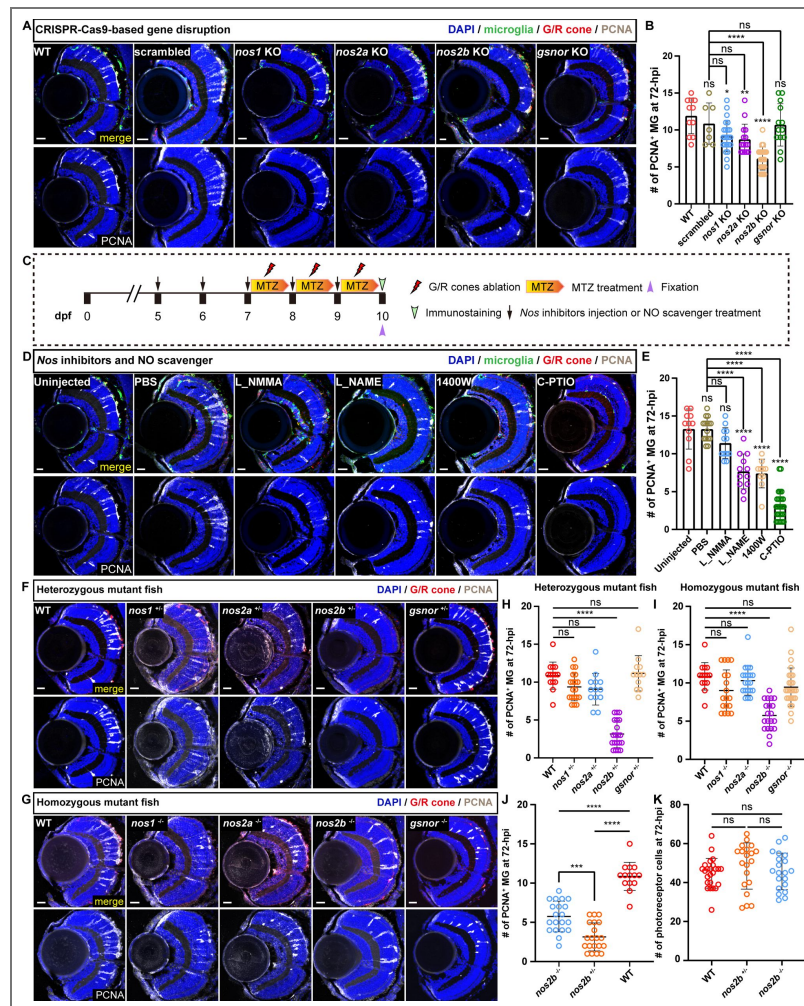


Figure 4. The nitric oxide metabolic pathway regulates injury-induced MG proliferation

(A) Representative images of microglia recruitment (green, marked by *Tg(mpeg1: GFP)*), G/R cone ablation (red, marked by *Tg(lws2: nfsb-mCherry)*) and proliferative MG (white, marked by PCNA⁺) at 72 hpi with nitric oxide metabolism pathway genes disruption (*nos1/nos2a/nos2b/gsnor*). Scale Bars: 20Cμm. **(B)** Quantitative analysis of the number of proliferative MG (PCNA⁺) at 72 hpi in **(A)**. In total, we collected 11 retinas for WT (n=11), *scramble* sgRNA-injection (n=7), *nos1* sgRNA-injection (n=23), *nos2a* sgRNA-injection (n=15), *nos2b* sgRNA-injection (n=22), and *gsnor* sgRNA-injection (n=13) (MeanC±CSEM; ****p<0.0001, **p<0.01, *p<0.05, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). **(C)** Schematic showing the experimental procedure of nitric oxide synthase (*Nos*) inhibitors injection or NO scavengers treatment in *Tg(lws2: nfsb-mCherry x mpeg1: GFP)* retina starting from 5 dpf for five consecutive days to 10 dpf with three consecutive days of MTZ treatment for G/R cone ablation from 7 dpf to 10 dpf. *Nos* inhibitors, NO scavengers, and MTZ solution were refreshed every 24 hours, and fish fixation was at 10 dpf for further immunostaining. **(D)** Representative images of microglial recruitment (green, marked by *Tg(mpeg1: GFP)*), G/R cone ablation (red, marked by *Tg(lws2: nfsb-mCherry)*) and proliferative MG (white, marked by PCNA⁺) at 72 hpi following L-NMMA (10 mM), L-NAME (10 mM), 1400W (200 nM) intraocular injection, and PBS as control, or C-PTIO (10 mM) treatment. Scale Bars: 20Cμm. **(E)** Quantitative plots showing the number of proliferative (PCNA⁺) MG at 72 hpi in **(D)**. Retinas analyzed WT (n=11), PBS-injected (n=16), L-NMMA-injected (n=12), L-NAME-injected (n=12), 1400W-injected (n=10), and C-PTIO treatment (n=27) (MeanC±CSEM; ****p<0.0001, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). **(F-G)** Representative images of proliferative MG (PCNA⁺, white) and G/R cone ablation (marked by *Tg(lws2: nfsb-mCherry)*, red) at 72 hpi in heterozygous (*nos1*^{+/-}, *nos2a*^{+/-}, *nos2b*^{+/-}, *gsnor*^{+/-}) **(F)** and homozygous mutants **(G)** of nitric oxide metabolism pathway genes (*nos1*^{-/-}, *nos2a*^{-/-}, *nos2b*^{-/-}, *gsnor*^{-/-}). Scale Bars: 20Cμm. **(H-I)** Quantitative plots showing the number of proliferative MG (white, PCNA⁺) at 72 hpi in *nos* and *gsnor* mutant fish. In heterozygous **(H)**, analyzed retinas include WT (n=14), *nos1*^{+/-} (n=19), *nos2a*^{+/-} (n=13), *nos2b*^{+/-} (n=20), *gsnor*^{+/-} (n=12). For homozygous **(I)**, analyzed retinas include *nos1*^{-/-} (n=18), *nos2a*^{-/-} (n=20), *nos2b*^{-/-} (n=20), *gsnor*^{-/-} (n=27) (MeanC±CSEM; ****p<0.0001, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). **(J-K)** Quantitative plots showing the number of proliferative MG (white, PCNA⁺) **(J)** and photoreceptor cells remain **(K)** at 72 hpi in *nos2b* hetero- or homozygous mutants. (MeanC±CSEM; ****p<0.0001, ***p<0.001, ns, p>0.05; one-way ANOVA followed by Tukey's HSD test).

homozygous *nos2b* mutants (*nos2b*L/L: $3C \pm C1$, $nC = C20$; *nos2b*L/L: $6C \pm C2$, $nC = C20$) compared to WT controls ($11C \pm C2$, $nC = C14$; mean $C \pm CSEM$). In contrast, no significant changes in MG proliferation were observed in *nos1*, *nos2a*, or *gsnor* mutants compared to wild type (Figures 4F–4I).

Interestingly, the reduction in proliferative MG was more pronounced in *nos2b* heterozygous mutants (*nos2b*^{+/−}) than in homozygous mutants (*nos2b*^{−/−}) (*nos2b*^{+/−} vs *nos2b*^{−/−}; $p < 0.001$; Mean $\pm$ SEM) (Figure 4J). We observed no significant difference in the loss of cone photoreceptor at 72 hpi between *nos2b* mutants and WT, indicating that the reduced MG proliferation observed in *nos2b* mutants is independent of the injury (WT: $45C \pm C8$ remaining cones, $nC = C24$; *nos2b*L/L: $49C \pm C12$, $nC = C20$; *nos2b*L/L: $46C \pm C9$, $nC = C20$; mean $C \pm CSEM$) (Figure 4K). This unexpected result suggests a concentration-dependent effect of NO on proliferative MG. Specifically, compared to homozygous mutants, heterozygous mutants with intermediate NO levels more effectively suppressed MG proliferation, whereas wild-type animals with higher NO levels promoted MG proliferation. This concentration-response pattern highlights the role of NO as a regulator, rather than a mediator, of injury-induced MG proliferation.

Specific *nos2b* expression in *cxcl18b*-defined transitional states MG after G/R cone ablation

To further examine whether the expression of *nos2b* in *cxcl18b*⁺ MG, we used the newly generated transgene fish line in our study *Tg(cxcl18b: GFP)*, enabling us to monitor *cxcl18b*⁺ MG after the G/R cone ablation in a real-time manner. By crossing different fish lines, we fluorescently sorted out three post-injury MG populations (72-hpi MG, 72-hpi PCNA⁺ MG, and 72-hpi *cxcl18b*⁺ MG) and three control groups (uninjured MG, 72-hpi retinal cells other than MG and 72-hpi G/R cone) (Figure 5A–C). The real-time quantitative PCR (RT-qPCR) analysis showed that compared to the three control groups, the expression of *nos2b* was significantly higher in *cxcl18b*⁺ MG than in 72-hpi MG and 72-hpi PCNA⁺ MG (relative expression of *nos2b*: 89 ± 32 , repeats $n = 7$ in *cxcl18b*⁺ MG; 14 ± 17 , repeats $n = 5$ in 72-hpi MG; and 1 ± 1 , repeats $n = 6$ in PCNA⁺ MG; Mean $\pm$ SEM) (Figure 5D and Figure 5-figure supplement 1D–F). Together with the fact that *cxcl18b*⁺ MG contained PCNA⁺ and PCNA[−] populations in *Tg(cxcl18b: GFP)* (Figure 2D), our result indicated that *nos2b* was specifically expressed in *cxcl18b*⁺ non-proliferative MG, which agreed with the scRNA-seq result of the specific expression of *cxcl18b* in three transitional states (Clusters 1, 2, and 5; Figure 1H). *In situ* hybridization using an HCR molecular probe in the *Tg(lws2: nfsb-mCherry:: cxcl18b: GFP)* fish line also suggests that injury-induced *nos2b* expression was specific in the *cxcl18b*-defined transitional state MG (Figure 5-figure supplement 1A–C). Thus, our analysis reveals that *nos2b* was specifically expressed in *cxcl18b*⁺ transitional MG states, particularly in non-proliferative cells.

NO produced by *nos2b* determines *cxcl18b*⁺ MG entry for proliferation

To further explore the role of *cxcl18b*⁺ MG-specific *nos2b* in regulating the entry of MG proliferation after the G/R cone ablation, we developed a sophisticated clonal analysis of *cxcl18b*⁺ MG with genetic disruption of *nos2b* using CRISPR-Cas9 method. To achieve glial type-specific gene manipulation, we employed an adenovirus strain that specifically infects radial glia in zebrafish (Jia et al., 2019; Liu et al., 2021), and confirmed that it could faithfully mark MG in the zebrafish retina (Figure 6A and B). For the clonal analysis, we performed the intraocular injection of two viruses packaged with elements of *cxcl18b: gal4* and *UAS: Cas9-T2A-Cre-u6: sgRNA (nos2b)* into the eye of *Tg(lws2: nfsb-mCherry:: ef1a: loxP-DsRed-loxP-EGFP)* at 5 dpf, virus packaged with the element of *UAS: Cas9-T2A-Cre-u6: empty* as the control (Figure 6C–D and Figure 6-figure supplement 1A). One day after the virus infection, the fish was treated with MTZ for 3 consecutive days to ablate the G/R cone (Figure 6E).

At 72 hpi, we collected the clones (GFP⁺) derived from *cxcl18b* lineage-traced MG and analyzed their proliferative property (Figure 6F and G). The analysis of virus-infected clones (GFP⁺) cell cycle re-entry (PCNA⁺) after G/R cone ablation revealed that only ~23% of GFP⁺ MG clones

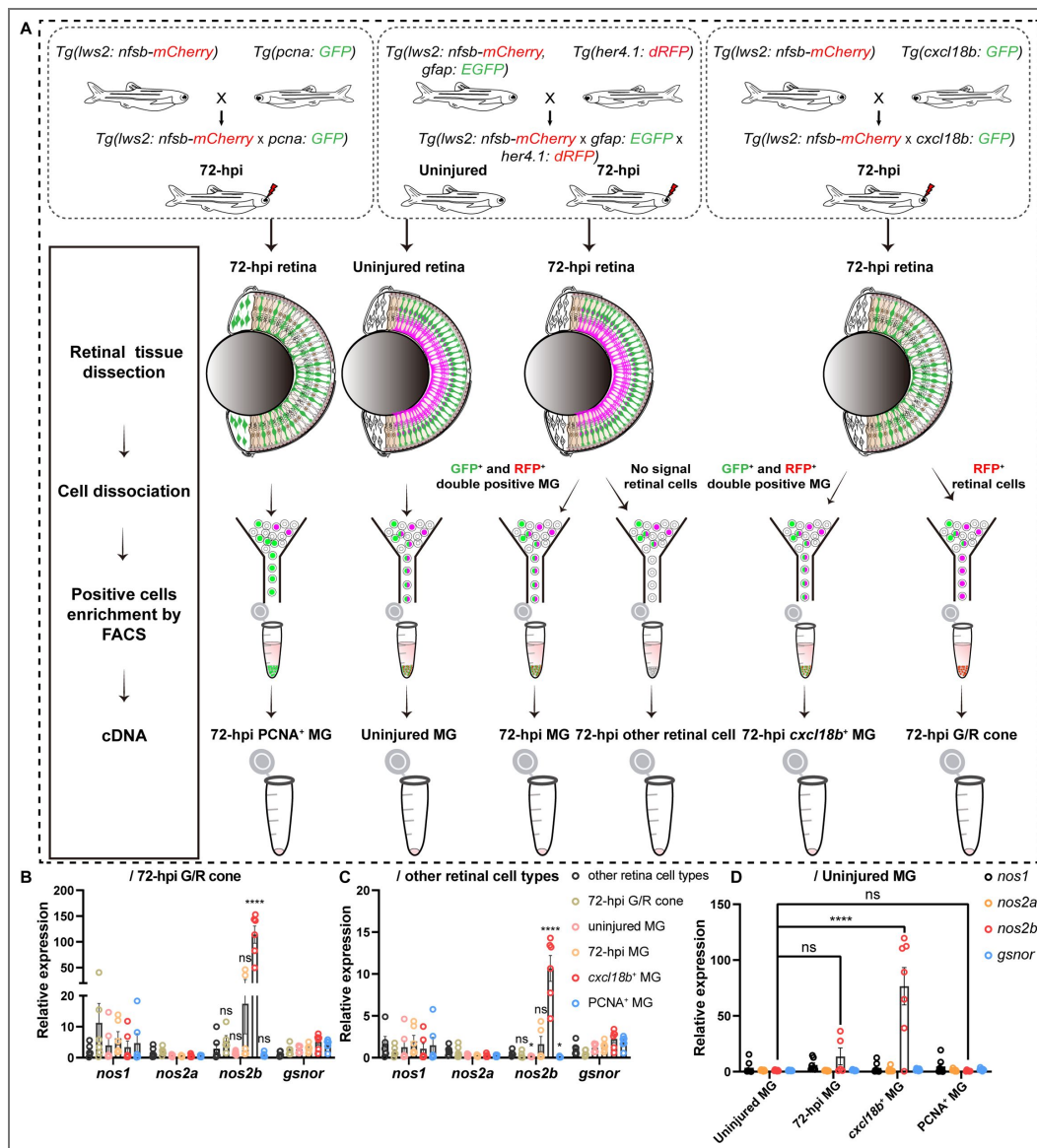


Figure 5. RT-qPCR analysis reveals *nos2b* cell-specific expression in the injury-induced *cxcl18b*⁺ MG

(A) Schematic showing the workflow for isolating and enriching three post-injury MG populations (72-hpi MG, 72-hpi PCNA⁺ MG, and 72-hpi *cxcl18b*⁺ MG) and three control groups (uninjured MG, 72-hpi retinal cells other than MG, and 72-hpi G/R cones) using fluorescence-activated cell sorting (FACS). (B–C) RT-qPCR analysis of *nos1*, *nos2a*, *nos2b*, and *gsnor* expression in different cell populations. Expression levels are shown relative to 72-hpi G/R cones (B) and other 72-hpi retinal cell types (C). A total of six independent replicates were performed for cell population enrichment and cDNA template preparation (n=6, Mean±CSEM; ****p<0.0001, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test). (D) RT-qPCR analysis comparing *nos1*, *nos2a*, *nos2b*, and *gsnor* expression in distinct MG states relative to uninjured MG (repeats n=7 in *cxcl18b*⁺ MG; n=5 in 72-hpi MG; and repeats n=6 in PCNA⁺ MG; n=6 in the uninjured retina; Mean±CSEM; ****p<0.0001, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test).

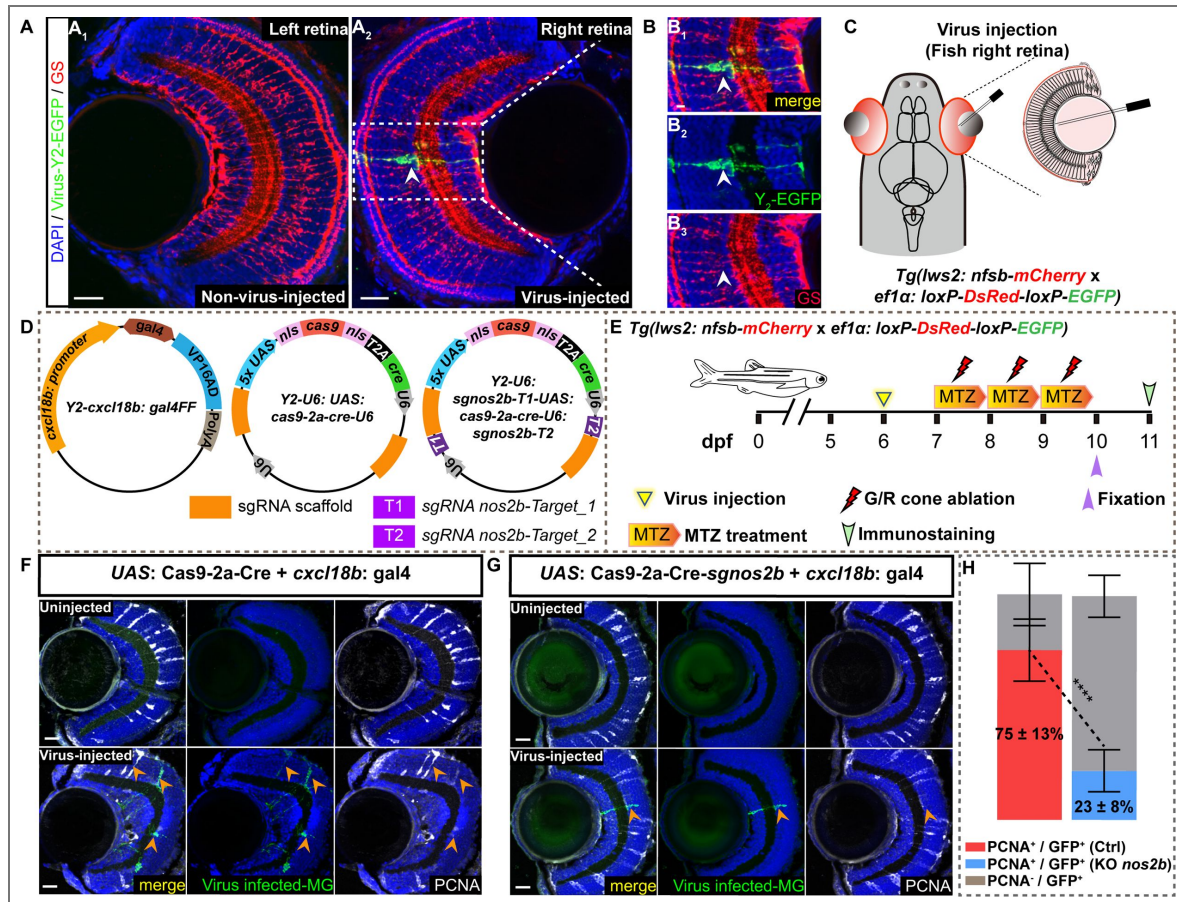


Figure 6. NO produced by *nos2b* in *cxcl18b*⁺ MG regulates injury-induced proliferation

(A-C) Representative images showing the adenovirus-mediated infection (green, indicated by Y₂-GFP) specifically target MG (red, GS staining) in the zebrafish retina. The virus was intraocularly injected into the right eye of *Tg(lws2: nfsb-mCherry x ef1a: loxP-DsRed-loxP-EGFP)* fish (A₂, C), with the left eye as a wild-type (WT) control (A₁). The high-magnification images of the boxed area (B-B₃). Scale Bars: 20Cμm (A₁, A₂) and 5 μm (B-B₃). (D) Schematic showing the design of the *cxcl18b*⁺ MG-specific *nos2b* knockout system. The viral construct consists of three plasmids: (1) *gal4* expression driven by the *cxcl18b* promoter; (2) UAS-derived *Cas9* and *Cre* elements, and (3) U6 promoters driving two sgRNAs targeting *nos2b*, with a non-targeting sgRNA as the control. (E) Schematic showing the procedure of injury process and intraocular viral injection in *Tg(lws2: nfsb-mCherry x ef1a: loxP-DsRed-loxP-EGFP)* fish. (F-G) Representative images showing proliferative MG (PCNA⁺, white) with *cxcl18b*⁺ MG specific knockout *nos2b* in (G) and control in (F), (GFP⁺, green, yellow arrows) are defined as virus-infected clones. Upper panels show WT retina (no virus injected), Bottom panels show retinas injected with the virus (two sgRNA targets as *nos2b* knockout and without sgRNA targets as control). Scale Bars: 20Cμm. (H) Quantification of proliferative (PCNA⁺/GFP⁺, red bars) and non-proliferative (PCNA⁻/GFP⁺, gray bars) MG clones in (F). For control, ~75% of virus-infected clones entered the cell cycle (PCNA⁺; red bars), with 90/120 clones analyzed across 8 independent experiments (n=8). For *nos2b* knockout clones, ~23% entered the cell cycle (PCNA⁺; blue bars), with 22/103 clones analyzed across 6 independent experiments (n=6) (MeanC±CSEM; ***p<0.0001; two-way ANOVA followed by Tukey's HSD test).

remained PCNA⁺ in the *nos2b* knockout group, compared to ~75% in the control group (Figure 6H [↗](#)). Note that the efficiency of *nos2b* sgRNAs was confirmed in terms of mutation types and knockout efficiency by sequencing (Figure 6-figure supplement 1B-D [↗](#)).

Taken together, these findings suggested that it is the NO produced by *Nos2b* within *cxcl18b*-defined transitional state MG that specifically drives MG from the transitional state into proliferation following injury, highlighting a pivotal mechanism underlying injury-induced regenerative processes.

NO decreased Notch activity that is responsible for injury-induced MG proliferation

Previous studies have shown that *ascl1a* and Notch signaling are essential for MG proliferation in the injured zebrafish retina (Conner et al., 2014 [↗](#); Wan et al., 2012 [↗](#)). Regarding Notch signaling, a high Notch3 expression is reported to maintain MG quiescence. In response to the injury, *notch3* expression is downregulated, but *notch1a* is necessary for the continued proliferation of the progenitors (Campbell et al., 2022 [↗](#)). In consistent, we observed that *notch3* and *hey* (the Notch downstream target) were highly expressed in uninjured MG clusters and became reduced from the early transitional state (Cluster4) to the proliferative states (Cluster 3 and 6), whereas *notch1a/1b* and *ascl1a* were prominently expressed in the late stage of *cxcl18b*⁺ transitional states (clusters 5) and the proliferative states (Cluster 3; Figure 1H [↗](#) and 7A [↗](#)). Interestingly, upstream regulators of Notch signaling activation, such as *fgf8a*, *fgf8b* (Wan and Goldman, 2017 [↗](#)), and *tgfb3* (Lee et al., 2020 [↗](#)), were predominantly expressed in clusters 4 and 1, preceding the expression of *cxcl18b* (Figure 7A [↗](#)). These results led us to wonder whether NO regulated *cxcl18b*-defined transitional state MG cell cycle re-entry via the Notch signaling pathway.

To examine the influence of NO signaling blockage on Notch activity dynamics following the cone ablation, we employed a reporter line *Tg(Tp1b:EGFP)* (referred to as *Tg(Tp1:EGFP)*), in which EGFP is driven by the TP1 element, the direct target of the intracellular domain of Notch receptors (NICD) that is generated upon Notch activation (Parsons et al., 2009 [↗](#); Quillien et al., 2014 [↗](#)). We treated fish with the NO scavenger C-PTIO and MTZ, starting at 5 dpf for 5 continuous days and at 7 dpf for 3 continuous days, respectively, followed by immunostaining for EGFP and PCNA (Figure 7B [↗](#)). Interestingly, *Tp1:EGFP*⁺ MG were significantly reduced at all three injury time points (cell number of EGFP⁺ clones: 34±3, n=9 in 24-hpi retina; 29±3, n=4 in 48-hpi retina; 33±4, n=6 in 72-hpi retina vs 42±1, n=4 in the uninjured retina; Mean ± SEM), demonstrating a decrease in Notch activity following G/R cone ablation (Figure 7C [↗](#)). Notably, this reduction in Notch activation was further rescued by NO blocking using C-PTIO (cell number of EGFP⁺ clones: 42±1, n=6 in C-PTIO-treated retina at 24 hpi; 42±2, n=5 at 48 hpi; 44±2, n=7 at 72hpi; Mean ± SEM), suggesting that NO modulates Notch signaling (Figure 7C [↗](#)). Meanwhile, C-PTIO treatment significantly reduced the number of proliferative MG (marked by PCNA) (Figure 7D [↗](#)). These findings indicated that injury-induced NO suppresses Notch signaling activation, which potentially drives MG to exit quiescence and enter proliferation. Together, our results highlight that NO signaling drove the injury-induced *cxcl18b*-defined transitional state MG to enter proliferation, which may be mediated by Notch pathway regulation.

Discussion

Our study provides the single-cell transcriptomic landscape of Müller glial state progression following the cone ablation at the larval stage. Combined with clonal analysis, we identified a previously unreported *cxcl18b*-defined transitional MG state as the essential routing for MG cell cycle re-entry. It led to our further genetic analysis, revealing the cell type-specific and concentration-dependent regulatory mechanism of nitric oxide derived from *nos2b* underlying injury-induced MG proliferation. Furthermore, NO signaling accounted for a decreased Notch activity, which has been previously reported to be essential for MG proliferation after the injury. Finally, cell state-specific gene disruption revealed that *cxcl18b*-defined MG transitional state-specific *nos2b* is required for injury-induced MG proliferation, which is a prime example of

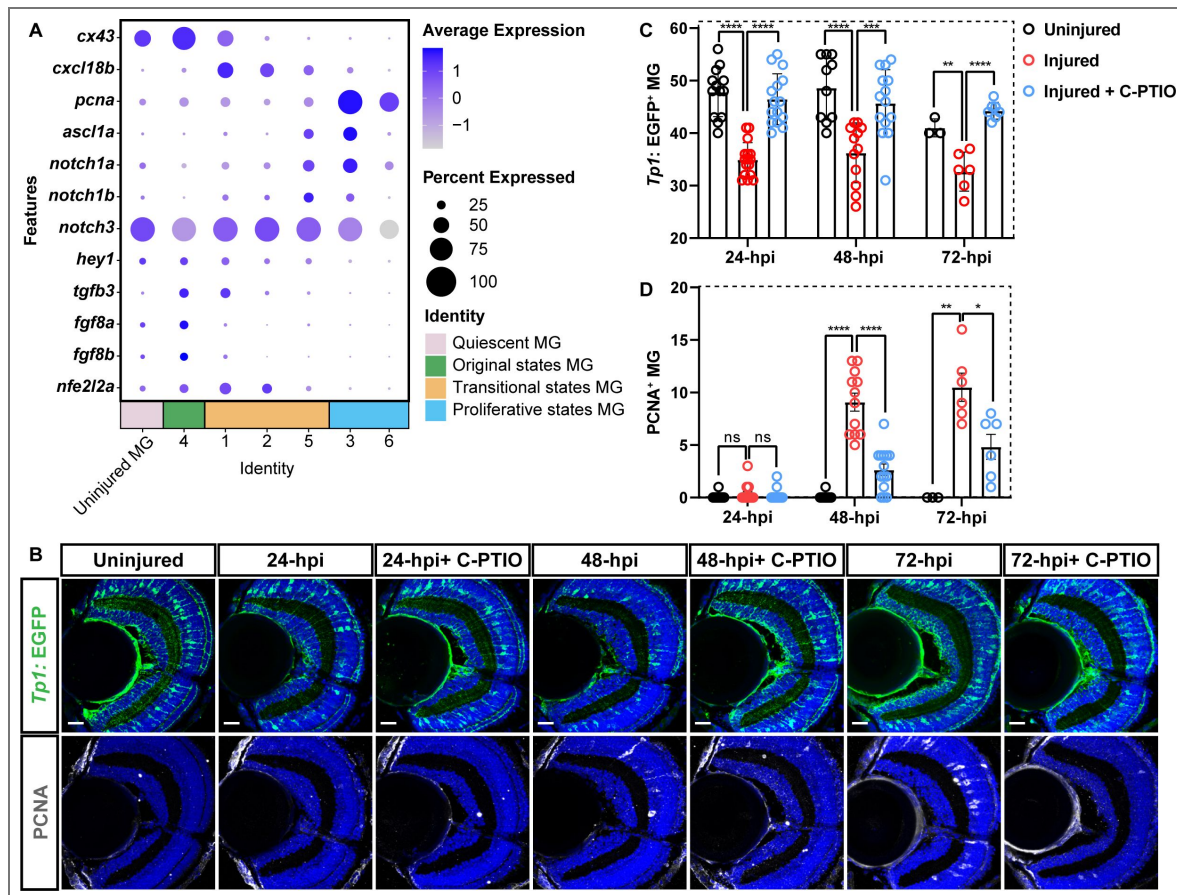


Figure 7. NO regulates MG proliferation by suppressing Notch signaling

(A) Dot plot showing the Notch signaling-related gene expression in different MG states. The average expression levels of these genes for all cells in each cluster are coded by the gray level. The percentage of cells expressing each gene within each cluster is coded by dot size. **(B)** Representative images showing the dynamic changes of Notch signaling activity (green, indicated by *Tg(Tp1: EGFP)*) and proliferative MG (white, PCNA⁺) following injury with nitric oxide (NO) blockade using C-PTIO. Scale Bars: 20Cμm. **(C-D)** Quantitative plots showing the number of Notch activation (*tp1: GFP*⁺ MG) in **(C)** and proliferative MG (PCNA⁺ MG) in **(D)** at different time points post-G/R cone ablation. We collected uninjured retina (24-hpi, n=4; 48-hpi, n=4; 72-hpi, n=3), injured retina (24-hpi, n=9; 48-hpi, n=4; 72-hpi, n=6), and retina treated with C-PTIO (24-hpi, n=6; 48-hpi, n=5; 72-hpi, n=7) (MeanC±CSEM; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test).

previously reported “5R” redox regulation (Meng et al., 2021 [↗](#)). Thus, our study provides the novel precision redox mechanism underlying MG proliferation in response to cone ablation in the zebrafish retina, opening exciting possibilities for future research and potential therapeutic interventions.

Identification of *cxcl18b*-defined MG transitional states following the cone ablation

Combined scRNA-seq analysis and clonal analysis, we identified a previously unreported transitional MG state, marked by the expression of *cxcl18b*, as the essential routing for MG to re-enter the cell cycle following the retina injury (Figure 1H [↗](#), 2F [↗](#), and 2G [↗](#)). To our knowledge, it is the first transitional state verified by *in vivo* clonal analysis to show a faithful prediction of injury-induced MG proliferation. Notably, this *cxcl18b* induction in MG depends on microglial recruitment in response to cone ablation, suggesting this transitional state is an MG response to the signals derived from the inflammatory reaction (Figure 2K [↗](#)). The underlying mechanism of this crosstalk is crucial to be addressed. Interestingly, the *cxcl18b*-containing gene module was also expressed in the CMZ, a region crucial for adult retina neurogenesis for a lifetime (Figure 3A-D [↗](#)). However, its expression is mainly absent from the central regions of the developing retina (Figure 3F [↗](#)). It suggests that the *cxcl18b*-defined transitional state might represent a developmental state used by constitutive neuron generation programs and injury-induced neuron regeneration programs beyond embryonic development. Furthermore, the *cxcl18b*-defined transitional state exhibits robust redox-related characteristics, such as the expression of *sod1*, *sod2*, and *catalase* (Figure 4-figure supplement 1A [↗](#)). It led to the critical discovery of this study, which showed the essential role of *nos2b* in regulating injury-induced MG proliferation. Unfortunately, our preliminary effects on gene disruption using CRISPR/Cas9 in F0 founders did not observe a significant reduction in the number of proliferative MG after *cxcl18b* disruption (Figure 2-figure supplement 1I [↗](#)). However, a recent study reported the essential role of *cxcl18b* in heart regeneration using mutant fish, providing a mechanism of *cxcl18b* as innate immune signaling in injury-induced tissue regeneration (Goumenaki et al., 2024 [↗](#)). Their results raised concern about the efficiency of *cxcl18b* disruption in our system. It is essential to use mutant fish to re-examine the role of *cxcl18b* in injury-induced MG proliferation in the future. Also, we cannot rule out the possibility that other co-factors are involved in the action of *cxcl18b* in MG regeneration, which is another critical issue that needs to be solved in the future.

The possible mechanism of NO signaling underlying injury-induced MG proliferation

Our study, for the first time, demonstrated an essential role of NO signaling in regulating MG proliferation after the cone ablation. However, we still need to understand more about the underlying mechanism. There are two well-characterized molecular events responsible for MG proliferation following the retina injury: a decreased Notch activity and an increased *ascl1a* expression (Fausett et al., 2008 [↗](#); Jorstad et al., 2017 [↗](#); Ramachandran et al., 2010 [↗](#)). Previous studies have shown that Notch3 is responsible for this decreased Notch activity, leading to increased *Ascl1a* through the de-depression mechanism (Campbell et al., 2021 [↗](#); Sahu et al., 2021 [↗](#)). Interestingly, unlike *notch3*, *notch1a* has been reported to stimulate MG proliferation (Campbell et al., 2021 [↗](#); Campbell et al., 2022 [↗](#)). Our scRNA-seq analysis also showed that as MG progressed into the proliferative states after the cone ablation, *notch3* expression gradually declined (Figure 7A [↗](#)). In contrast, *ascl1a*, *notch1a*, and *notch1b* expressions were up-regulated and peaked at the proliferative states (Figure 7A [↗](#)). Thus, both previous studies and our current analysis support the idea that the transcriptional regulation of Notch expression accounts for the decreased Notch activity after the injury. Intriguingly, NO has been reported to activate the Notch1 signaling cascade by promoting the release and accumulation of the Notch1 intracellular domain (NICD) through nitration reactions, subsequently enhancing tumorigenesis and stem-like features in various cellular systems (Charles et al., 2010 [↗](#); López-Juárez et al., 2017 [↗](#); Villegas et al., 2018 [↗](#)). It raises the possibility that NO signaling regulates injury-induced

MG proliferation through the post-translational modification of Notch3. Previous studies have demonstrated two significant forms of NO-mediated post-modification: S-nitrosylation on cysteines and nitration on tyrosine (Wu et al., 2014). Our preliminary analysis showed that all 11 cysteine residues within the putative γ -secretase-dependent cleavage sites are conserved between Notch1a and Notch3, while notable differences were observed at four tyrosine residues. It leads to an outstanding question of whether NO regulated injury-induced MG proliferation by decreasing Notch activity via tyrosine nitration of Notch3, which is worthwhile to elucidate.

The production of NO signaling in MG following retina damage

Previous studies reported that *cxcl18b* is a reliable inflammatory marker (Goumenaki et al., 2024; Torraca et al., 2017) and different inflammation responses modulate MG proliferation in the damaged zebrafish retina (Iribarne and Hyde, 2022). The induction of *cxcl18b* may represent the inflammatory responses of MG after the cone ablation, pointing out the potential link between the inflammatory response and the emergence of NO signaling in MG. Previous studies have demonstrated that iNOS is induced in various tissues by proinflammatory cytokines (Förstermann and Sessa, 2012; Pacher et al., 2007). One of the approaches to test the role of inflammatory responses is to manipulate the levels of inflammatory responses in MG to see the NO production and MG proliferative behaviors. Also, previous studies appreciate the essential role of electrical activity in tissue regeneration (Levin, 2009; Qin et al., 2023); in particular, calcium signaling has been shown to regulate various molecular pathways for liver regeneration, including the hepatocyte growth factor-Met-tyrosine kinase (HGF-Met) transduction pathway (Bedi et al., 2024) and the epidermal growth factor receptor (EGFR) signaling (Kimura et al., 2023). It is interesting to speculate that the abnormal electrical activity of MG in the injured retina may result in an elevated level of intracellular calcium, which activates calmodulin and induces the conformation change of NOS to NO production (Hanson et al., 2018; Jones et al., 2007). A similar mechanism has been proposed in the long-term potentiation of excitatory post-synaptic structure (Grover and Teyler, 1990; Kawamoto et al., 2012; Park, 2018) as well as in the glutamate neurotoxicity model (Ashpole et al., 2013; Ashpole et al., 2012). Thus, the production of NO derived from *Nos* may be the product of the interplay between the inflammatory responses and the electrical activity in MG after the retina damage.

Limitations of this study

A few limitations of this study should be acknowledged: 1. Our study focuses on larval zebrafish, in which the core features of MG and immune responses are conserved compared to the adult. However, we acknowledge that the adult retina—with its fully matured differentiated retina and immune response — provides irreplaceable biological insight. Nevertheless, larval models offer a powerful platform to uncover conserved regenerative mechanisms and serve as a valuable complement for identifying age-dependent differences in MG-mediated regeneration. 2. While our data suggest that injury-induced NO suppresses Notch signaling activation and promotes MG proliferation, the use of a general NO scavenger (C-PTIO) does not allow us to determine whether this regulation occurs in an autocrine or paracrine manner. The specific role of NO signaling within *cxcl18b* MG requires further validation using MG-specific NO depletion. 3. The current study's description of the landscape of MG transitional states is based on single-cell transcriptomic data obtained at a single time point (72 hpi) after the cone ablation, which may not provide a complete picture of the state transition of post-injury MG. Future scRNA-seq analysis of MG at multiple post-injury time points is necessary to clarify this issue. 4. While we demonstrated a critical role of NO in injury-induced MG proliferation, the potential contribution of microglia-derived NO was not directly examined. 5. We did not perform direct measurements of NO levels specifically within *cxcl18b*-defined MG cells, leaving open the question of localized NO signaling.

Methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (Danio rerio)	Wild type	Dr William		AB
strain, strain background (Danio rerio)	<i>lws2: nfsb-mCherry</i>	(Krylov et al., 2023)		<i>Tg(opn1lws2: nfsb-mCherry)^{uom3}</i>
strain, strain background (Danio rerio)	<i>her4.1: dRFP</i>	(Yeo et al., 2007)	ZDB-TGCONSTRC T-070612-2	<i>Tg(her4.1: dRFP)</i>
strain, strain background (Danio rerio)	<i>gfap: EGFP</i>	(Bernardos and Raymond, 2006)	ZDB-FISH-150901-29307	<i>Tg(gfap: EGFP)</i>
strain, strain background (Danio rerio)	<i>mpeg1: GFP</i>	(Ellett et al., 2011)	ZDB-TGCONSTRC T-170801-5	<i>Tg(mpeg1: GFP)</i>
strain, strain background (Danio rerio)	<i>Tp1bglob: EGFP</i>	(Yu and He, 2019)		<i>Tg(Tp1bglob: EGFP)</i>
strain, strain background (Danio rerio)	<i>pcna: GFP</i>	(Xu et al., 2020)	ZDB-LAB-070129-2	<i>Tg(pcna: GFP)</i>
strain, strain background (Danio rerio)	<i>ef1a: loxP-DsRed-loxP-EGFP</i>	(Hans et al., 2009)		<i>Tg(ef1a: loxP-DsRed-loxP-EGFP)^{z1284}</i>
strain, strain background (Danio rerio)	<i>cxcl18b: Cre-vmhc: ECFP</i>	This manuscript		<i>Tg(cxcl18b: Cre-vmhc: ECFP; ef1a: loxP-DsRed-loxP-EGFP; lws2: nfsb-mCherry)</i>
strain, strain background (Danio rerio)	<i>cxcl18b: GFP</i>	This manuscript		<i>Tg(cxcl18b: GFP)</i>

Key resources table.

antibody	Mouse monoclonal anti-PCNA	Abcam	Cat#Ab29; RRID: AB_303394	IF(1:500)
antibody	Rabbit polyclonal anti-BLBP	Abcam	Cat#ab32423; RRID: AB_880078	IF(1:1000)
antibody	Mouse monoclonal anti-Glutamine Synthetase	BD Biosciences	Cat# 610518; RRID: AB_397880	IF(1:1000)
antibody	Chicken monoclonal anti-GFP	Abcam	Cat# ab13970; RRID: AB_300798	IF(1:2000)
antibody	Rabbit polyclonal anti-GFPtag	Rabbit polyclonal anti-GFPtag	Cat#50430-2-AP; RRID: AB_11042881	IF(1:500)
antibody	Rabbit polyclonal anti-DsRed2	Takara Bio	Cat#632496; RRID: AB_10013483	IF(1:1000)
antibody	Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H+L)	Yeasten Biotech	Cat# 33206ES; RRID: AB_3662603	IF(1:1000)
antibody	Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Yeasten Biotech	Cat# 34206ES60; RRID: AB_2909605	IF(1:1000)
antibody	Alexa Fluor 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson ImmunoResearch Labs	Cat# 703-545-155; RRID: AB_2340375	IF(1:1000)
antibody	Alexa Fluor 594 AffiniPure Donkey Anti-Mouse IgG (H+L)	Yeasten Biotech	Cat# 34112ES; RRID: AB_3661960	IF(1:1000)

Key resources table. (continued)

antibody	Alexa Fluor 594 AffiniPure™ Goat Anti-Rabbit IgG (H+L)	Yeasen Biotech	Cat# 33112ES; RRID: AB_3661961	IF(1:1000)
antibody	Alexa Fluor 647-AffiniPure Goat Anti-Mouse IgG + IgM (H+L)	Jackson ImmunoResearch Labs	Cat# 115-605-044; RRID: AB_2338906	IF(1:1000)
recombinant DNA reagent	pTol2-cxcl18b: GFP	This manuscript		We made this plasmid by ligated the cxcl18b promoter and GFP element
recombinant DNA reagent	pTol2-cxcl18b: Cre-vmhc: mcherry	This manuscript		We made this plasmid by ligated the cxcl18b promoter and Cre element
recombinant DNA reagent	pTol2-cxcl18b: gal4FF	This manuscript		We made this plasmid by ligated the cxcl18b promoter and gal4FF element
recombinant DNA reagent	pUAS: Cas9T2ACre; U6: sgRNA1; U6: sgRNA2	(Di Donato et al., 2016)	Addgene plasmid #74010; RRID: Addgene_74010	
recombinant DNA reagent	pTol2-UAS: Cas9-T2A-Cre-U6: nos2b sgRNA1; U6: nos2b sgRNA2	This manuscript		We made this plasmid by insert two sgRNAs of <i>nos2b</i> in 10xUAS backbone
commercial assay or kit	MEGAscript™ T7 High Yield Transcription Kit	Invitrogen	Cat# AM1334	
commercial assay or kit	ClonExpress MultiS One Step Cloning Kit	Vazyme	Cat# C113-02	

Key resources table. (continued)

Key resources table. (continued)

commercial assay or kit	DIG RNA labeling kit	Roche	Cat# 11277073910	
chemical compound, drug	N(ω)-nitro-L-arginine methyl ester	Sigma-Aldrich	Cat# N5751-1G	10 mM
chemical compound, drug	N(ω)-Methyl-L-arginine acetate salt	Sigma-Aldrich	Cat# M7033-5MG	10 mM
chemical compound, drug	1400W dihydrochloride	MedChemExpress	Cat# HY-18730	200 nM
chemical compound, drug	Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide	Sigma-Aldrich	Cat# P5084-25MG	10 mM
chemical compound, drug	Dexamethasone	Sigma-Aldrich	Cat# D1756	10 mM
chemical compound, drug	Metronidazole	Sigma-Aldrich	Cat# M3761-100G	10 mM
software, algorithm	FV10-ASW 4.0 Viewer	Olympus	www.olympus-global.com	Analysis image
software, algorithm	GraphPad Prism V 9.0.0	GraphPad Software	www.graphpad.com	Data analysis
software, algorithm	Cell Ranger Single Cell Software Suite (v2.1.0)	10x genomics	https://support.10xgenomics.com	scRNA-seq data analysis
software, algorithm	RStudio	RStudio IDE	https://posit.co/	scRNA-seq data analysis
software, algorithm	R 4.4.1	R-project	https://www.r-project.org/	scRNA-seq data analysis
software, algorithm	Seurat	Satijalab	https://satijalab.org/seurat/	scRNA-seq data analysis

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chang Chen (changchen@moon.ibp.ac.cn [↗](#)).

Materials availability

All unique reagents generated in this study will be made available from the contact. We may require a completed MTA if there is potential for commercial application.

Experimental Model and Subject Details

Single-cell RNA sequencing data analysis

In this study, single-cell RNA sequencing (scRNA-seq) raw data of Müller glia enriched after G/R cone ablation at 72 hpi are from (Krylov et al., 2023 [↗](#)), embryonic RPCs at 24 hpf, and postembryonic retinal stem cells (RSCs) at 14 dpf are from (Xu et al., 2020 [↗](#)). We re-processed the single-cell FASTQ sequencing reads (Novogene) and converted to digital gene expression matrices using the Cell Ranger software (version 3.1.0) provided by 10X Genomics after mapping to the zebrafish GRCz11 (Ensembl release-96) genome assembly. An average of 47,545 mean reads per cell with 1,343 median genes per cell in no ablation control, 63,177 mean reads per cell with 1,556 median genes per cell in G/R cone ablation (lws2_72 hpi), 61,772 mean reads per cell with 2,474 median genes per cell in the embryonic RPCs at 24 hpf, and 49,243 mean reads per cell with 1,181 median genes per cell in the postembryonic retinal stem cells (RSCs) at 14 dpf were obtained.

We then used the *cellranger aggr* (version 6.0, <https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/aggregate> [↗](#)) to aggregate the *cellranger* counts from the two datasets and normalize them to the same sequencing depth. The feature barcode matrix was recalculated and analyzed on the combined data. This resulted in a dataset of 19,706 aggregated cells, with 100% of the no ablation control reads retained and 71.4% of the *lws2_3 dpi* reads retained. The aggregated matrix for the no ablation control and *lws2_72 hpi* samples was loaded into the Seurat R package (version 4.3.0, <https://satijalab.org/seurat/> [↗](#)). Cells with gene expression of more than 200 and less than 4000, lower than 5% mitochondrial content were filtered for further analysis. The filtered data were normalized, scaled, and clustered using principal component analysis (PCA) with a significance threshold of $p < 0.001$ (FindClusters, resolution = 0.5), and a UMAP was computed using scanpy.tl.umap, resulting in 20 distinct clusters. MG clusters were identified by high expression of marker genes, including *rlbp1a*, *fabp7a*, *slc1a2b*, *glula*, *glulb*, *gfap*, and *her4.1*. Proliferative MG clusters were identified from proliferative cell clusters by high expression of proliferative cell markers, including *pcna*, *mki67*, *gfap*, *her4.1*, and low expression of the CMZ markers, including *fabp11a*, *col15a1b*, *fabp7b*, *rx2*. We further analyzed the MG and proliferative MG clusters to obtain 13 clusters, including no ablation control 5,932 cells and G/R cone ablation 72 hpi 3,999 cells in the UMAP plot (Figure 1-figure supplement 1C [↗](#)). We compared the proportion of cell numbers in each cluster before and after injury and chose clusters with a high proportion of cell numbers in G/R cone ablation 72 hpi (clusters 2, 3, 5, 6, 9, 10, 11, 12, and 13) to further cluster into 10 clusters (Figure 1-figure supplement 1D [↗](#)).

Single-cell RNA sequencing data of embryonic RPCs at 24 hpf and postembryonic RSCs at 14 dpf were generated into 10 and 11 clusters, respectively. Each cluster is identified by the cluster-specific marker gene and different development stage markers, including *fabp11a*, *col15a1b*, *cxcl18b*, *her4.2*, *npm1a*, *her9*, *fabp7a*, *dla*, *atoh7*, *vsx1*, *mafba*, *otx5*, and *rem1* (Figure 3A [↗](#), 3C [↗](#) and Figure 3-figure supplement 1A [↗](#) and B [↗](#)).

Pseudo-time trajectory analysis

After the UMAP cluster analysis of the single-cell data, trajectory analysis was performed to investigate the pseudo-time transcriptomic change of these ten clusters using the ‘monocle 2’ R package, which revealed three distinct states (Figure 1G [↗](#)).

Zebrafish husbandry and transgenic fish lines

All experimental zebrafish embryos, larvae, and adults were produced, grown, and maintained at 28°C according to standard protocols. Embryos and larvae were kept in embryo medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.3×10^{-5} % w/v methylene blue in RO water) at 28.5 °C, under a 14:10 light: dark cycle. Animal procedures performed in this study were approved by the Animal Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences (NA-069-2023).

Published fish lines used in this study include the following: AB (Wild type, WT) and transgenic lines (Tg): *Tg(opn1lw2: NTR-mCherry)^{uom3}* (ZDB-ALT-201012-2)(Wang et al., 2020 [DOI](#)) in this study named *Tg(lws2: nfsb-mCherry)*, *Tg(gfap: EGFP)^{mi2001}* (ZDB-FISH-150901-29307)(Bernardos and Raymond, 2006 [DOI](#)), *Tg(her4.1: dRFP)* (ZDB-TGCONSTRCT-070612-2)(Yeo et al., 2007 [DOI](#)), *Tg(mpeg1: GFP)* (ZDB-TGCONSTRCT-170801-5)(Ellett et al., 2011 [DOI](#)), *Tg(pdna: GFP)* (ZDB-LAB-070129-2)(Xu et al., 2020 [DOI](#)), *Tg(ef1a: loxP-DsRed-loxP-EGFP)*(Hans et al., 2009 [DOI](#)), *Tg(Tp1bglab: EGFP)*(Yu and He, 2019 [DOI](#)).

Newly generated transgenic fishline contained: *Tg(cxcl18b: GFP)*, *Tg(cxcl18b: Cre-vmhc: mCherry:: ef1a: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)*, and mutants *nos1^{+/-}*, *nos2a^{+/-}*, *nos2b^{+/-}*, *gsnor^{+/-}*, *nos1^{-/-}*, *nos2a^{-/-}*, *nos2b^{-/-}*, *gsnor^{-/-}*.

Generation of Tg(cxcl18b: GFP)

The plasmid of *pTol2-cxcl18b: GFP* (10 ng/ μl) and *tol2* mRNA (50 ng/ μl) were co-injected into a wild-type embryo at the one-cell stage. Zebrafish embryos with green fluorescent were grown at the E3 medium according to standard protocol and selected as the founder (F0). The adult F0 fish crossed with the wild-type selected the fish with GFP^+ offspring as F1. In this stable transgenic line, *cxcl18b⁺* cells expressed the fluorescent protein GFP and the full name of this line is *Tg(cxcl18b: GFP)*.

Generation of Tg(cxcl18b: Cre-vmhc: mCherry:: ef1a: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)

The plasmid of *pTol2-cxcl18b: Cre-vmhc: mCherry* (10 ng/ μl) and *tol2* mRNA (50 ng/ μl) were co-injected into the embryo obtained from *Tg(lws2: nfsb-mCherry)* cross *Tg(ef1a: loxP-DsRed-loxP-EGFP)* at the one-cell stage. Zebrafish embryos were maintained at E3 medium according to standard protocol. Larvae with *mCherry* expression in the heart and eyes were selected and grown as the founder (F0) for further construction. The adult founder was crossed with wild-type (AB), considering the expression of *cxcl18b* during zebrafish development, larvae with *mCherry* and GFP double positive were selected and grown as the F1. In this zebrafish line, EGFP permanently marks all cells that have expressed *cxcl18b*, as well as their entire lineage of progeny. The full name of this line is *Tg(cxcl18b: Cre-vmhc: mCherry:: ef1a: loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry)*.

Generation of mutant fish lines

The CRISPR/Cas9 system was employed to efficiently and precisely generate mutant zebrafish lines(Wu et al., 2018 [DOI](#)). Two sgRNAs targeting gene coding sequences involved in nitric oxide metabolism signaling (nitric oxide synthase, *Nos*; and S-nitrosoglutathione reductase, *GSNOR*) were designed based on the zebrafish GRCz11 genome assembly.

Primers were designed approximately 200 bp from the sgRNA target sites for genotyping (sgRNAs and genotyping primers are listed in [Supplementary Table S1](#) [DOI](#)). We mixed the two sgRNAs (100 ng/ μl) for each target gene with Cas9 protein (400 ng/ μl) and injected them into transgenic fishline *Tg(lws2: nfsb-mCherry)* embryos at the one-cell stage. Adult fish were crossed with wild-type, and offspring displaying *mCherry* expression in the eyes at 5 dpf were selected for further genotyping. Mutants were confirmed by sequencing, and the fish with open reading frame (ORF) shift were collected as heterozygous fish for further studies.

Plasmid construction

We cloned the *cxcl18b* regulatory element (3048 bp upstream of the start codon including the 5'UTR) as the *cxcl18b* promoter from the zebrafish genomic DNA (Figure 2C). The primers used for amplification were 5'-GCATTTGTCTCCTCATGCATTGACTAC-3' (forward primer) and 5'-TTGCTGCAAACTATATGTAGGAAATGCTG-3' (reversed primer). For constructing the plasmids *pTol2-cxcl18b: GFP*, *pTol2-cxcl18b: Cre-vmhc: mCherry*, and *pTol2-cxcl18b: gal4FF*, each DNA elements cassette was inserted into the *pDestTol2pA2* vector (Kwan et al., 2007). The plasmid of *pUAS: Cas9T2AGFP*; *U6: sgRNA1*; *U6: sgRNA2* was kindly provided by Professor Filippo Del Bene (Di Donato et al., 2016). To prepare for plasmid *pTol2-UAS: Cas9-T2A-Cre-U6-empty* and *pTol2-UAS: Cas9-T2A-Cre-U6: nos2b sgRNA1*; *U6: nos2b sgRNA2*, the same two *nos2b* sgRNAs used to construct *nos2b* mutant fish were inserted into *pUAS: Cas9T2AGFP*; *U6: sgRNA1*; *U6: sgRNA2* plasmid, with the *GFP* element replaced by *Cre* using ClonExpressMultiS One Step Cloning Kit (Vazyme, C113C02). For the plasmid construction of the MG-specific knockout system based on recombinant adenoviral vectors, regulatory elements were inserted into adenoviral vectors *pAdC68-S* according to the standard protocol outlined by Jia et al. (Jia et al., 2019). The final plasmids generated included, *pAdC68XY2-E1-CMV-GFP* *pAdC68XY2-E1-10xUAS: Cas9-2a-Cre-U6: sgRNA1(nos2b)*; *U6: sgRNA2(nos2b)*, *pAdC68XY2-E1-cxcl18b: gal4FF* and *pAdC68XY2-E1-10xUAS: Cas9-2a-Cre-U6: empty* as the control. Plasmid construction primer sequences are listed in Supplementary Table S2.

Metronidazole treatment

Zebrafish larvae were exposed to a 10 mM metronidazole (MTZ) solution (Sigma-Aldrich, M3761-100G) in standard fish water. For *Tg(lws2: nfsb-mCherry)* larvae processed for immunohistochemical analysis, MTZ treatment was initiated at 6 dpf to ablate green and red cones. Larvae were maintained in the MTZ solution at densities of fewer than 50 larvae per 50 mL petri dish and kept at 28.5°C. The MTZ solution was refreshed every 24 hours to ensure continuous cone ablation until fixation in 4% PFA, while control larvae were kept in standard fish water.

Clone analysis of *cxcl18b* lineage-traced MG

Clone analysis of *cxcl18b*⁺ lineage-traced MG was restricted to cells located in the central and dorsal region of the zebrafish retina after G/R cone ablation in Figure 2, Figure 6, and their figure supplement. This spatial restriction strongly suggests that the proliferative MG originate from local mature MG, although we cannot completely rule out the possibility that CMZ-derived progenitors contribute to the generation of proliferative MG in the peripheral retina.

Gene disruption via CRISPR/Cas9 system

We used the CRISPR/Cas9 system for efficient gene disruption. Two sgRNAs were designed to target the coding sequences of genes involved in the nitric oxide signaling pathway (including *nos1*, *nos2a*, *nos2b*, *gsnor*) and *cxcl18b*. The sgRNAs were in vitro transcribed and purified using the LiCl precipitation approach (MEGAscript T7 Transcription Kit, Invitrogen, AM1334). A mixture of the two sgRNAs (in total 200Cng/μl) with Cas9 protein (400Cng/μl, Novoprotein, E365C01A) were co-injected into the embryo of *Tg(lws2: nfsb-mCherry)* at the one-cell stage. The efficiency of gene knockout for each sgRNA was validated, as presented in Figure 4-figure supplement 1C.

Pharmacological treatment with NO scavenger and dexamethasone or *Nos* Inhibitors intraocular injection

Zebrafish larvae at 6 dpf were anesthetized in 0.04% MS222 (Sigma, A5040) for 30 to 45 seconds. Inhibitor solutions of 2 μL were prepared as follows: 10 mM N(ω)-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, N5751-1G), a broad *Nos* inhibitor, diluted in sterile PBS from a 100 mM stock; 10 mM N(ω)-Methyl-L-arginine acetate salt (L-NMMA, Sigma-Aldrich, M7033-5MG), a specific inhibitor of *nNos*, diluted in sterile PBS from a 20 mM stock; and 200 nM 1400W dihydrochloride (SW1400, MedChemExpress, HY-18730), a specific inhibitor of *iNos*, diluted in sterile PBS from a 39.97 mM stock. PBS was used as the control. Each solution was loaded into glass capillaries prepared using a micropipette puller (Narishige, PC-10) and connected to a microinjector (Applied

Scientific Instrumentation, MPPI-3). Intraocular injection of the inhibitor solutions were administered to the zebrafish eyes starting 2 days before G/R cone ablation and continued until 72 hpi (Figure 4C [↗](#)).

Additionally, zebrafish were pre-treated with 10 mM of the NO scavenger, Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (C-PTIO, Sigma-Aldrich, P5084-25MG), diluted in standard water (from a 100 mM stock), or dexamethasone (Dex, Sigma-Aldrich, D1756) which diluted in DMSO, starting 2 days before G/R cone ablation and continuing until 72 hpi (Figure 2 [↗](#) and 4C [↗](#)).

The real-time quantitative PCR after FACS-sorted MG

We crossed *Tg(lws2: nfsb-mCherry)* with *Tg(pcna: GFP)*, *Tg(her4.1: dRFP)*, *Tg(gfap: EGFP)*, and *Tg(cxcl18b: GFP)* to collect retina cell. the retinas were dissected and the cells dissociated for sorting and enrichment of signal-positive cells using fluorescence-activated cell sorting (FACS, Beckman, MoFlo XDP). After 72 hours post G/R cone ablation, GFP⁺/RFP⁺ cells were collected from the retinas of *Tg(lws2: nfsb-mCherry x pcna: GFP)* as the proliferative MG group. For the uninjured MG group, GFP⁺/RFP⁺ cells were collected from *Tg(lws2: nfsb-mCherry x gfap: EGFP x her4.1: dRFP)* uninjured retinas, while GFP⁺/RFP⁺ cells from injured retinas represented the 72-hpi MG group, while GFP⁺/RFP⁻ cells were enriched as the other retinal cell types group in the 72 hpi retinas. Additionally, from *Tg(lws2: nfsb-mCherry x cxcl18b: GFP)* retinas, GFP⁺/RFP⁺ cells were collected as the *cxcl18b*⁺ MG group and single RFP⁺ cells as the G/R cone group at 72 hpi (Figure 5A [↗](#)).

Interesting cells were collected into a 1.5Cml tube containing lysis buffer (20Cmg/ml PK in TE buffer) and total RNA was isolated using this lysis buffer. cDNA synthesis was conducted by adding RT mix contains: 200CU Superscript II reverse transcriptase (Invitrogen, 18064C014), 1C×CFirstCstrand buffer (Invitrogen, 18064C014), 5CmM DTT (Invitrogen, 18064C014), 20CU Recombinant RNase inhibitor (Clontech, 2313A), 6CmM MgCl₂ (Sigma, M8266), 1CμM TSO(Picelli et al., 2013 [↗](#)); 8% PEG8000 (Sigma, P1458). PCR amplification was performed as previously described(Picelli et al., 2013 [↗](#)).

Real-time quantitative PCR (RT-qPCR) reactions were carried out using TB Green Premix Ex Taq (Takara, RR420A) on a LightCycler 480 II real-time PCR detection system (Roche). The qPCR primers are listed in Supplementary Table S3 [↗](#). The ΔΔCt method was used to determine the relative expression of mRNAs in different group retinae cells and normalized to *actin* mRNA levels. Each group comparison was performed using a two-way ANOVA followed by Tukey's HSD test. Error bars represented SEM. ****p<0.0001, ***p<0.001; **p<0.01; *p<0.05; ns, p>0.05.

Construction and injection of adenoviral-based MG-specific knockout system in the zebrafish retina

The type of AdC68 used in our study is a replication-deficient chimpanzee adenovirus. The plasmids used for viral packaging were described above, and the amplification and purification of recombinant chimpanzee adenovirus followed the standard protocol(Jia et al., 2019 [↗](#); Liu et al., 2021 [↗](#)). We generated four adenoviruses for this study, including *AdC68XY2-E1-CMV-GFP* *pAdC68XY2-E1-10xUAS: Cas9-2a-Cre-U6: sgRNA1(nos2b)*; *U6: sgRNA2(nos2b)* (referred to as *UAS: Cas9-2a-Cre-sgnos2b*, infectious titer: 2.00 x 10¹³ ifu/ml), *pAdC68XY2-E1-cxcl18b: gal4FF* (referred to as *cxcl18b: gal4*, infectious titer: 1.90 x 10¹³ ifu/ml) and *pAdC68XY2-E1-10xUAS: Cas9-2a-Cre-U6: empty* (referred to as *UAS: Cas9-2a-Cre*, infectious titer: 1.75 x 10¹³ ifu/ml) as a control.

All adenoviruses were diluted in sterile PBS to a final infectious titer: 1.00 x 10¹³ ifu/ml. The *UAS: Cas9-2a-Cre-sgnos2b* and *cxcl18b: gal4* adenoviruses were mixed at a 1:1 ratio for the MG-specific knockout system, while the *UAS: Cas9-2a-Cre* with *cxcl18b: gal4* adenoviruses were similarly mixed as a control. To achieve the *cxcl18b*⁺ MG cell-specific knockout *nos2b*, we performed the intraocular injection of the adenovirus mixtures into the eyes of *Tg(ef1α: loxP-DsRed-loxP-EGFP x lws2: nfsb-mCherry)* at 5 dpf. One day post-injection, the fish were treated with MTZ for 3 consecutive days to ablate the G/R cone (Figure 6E [↗](#)).

Tissue preparation and immunostaining

Zebrafish were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Services, 157–8) overnight, then cryoprotected in 30% sucrose for 6 hours, flash-frozen, and cryosectioned at a thickness of 14 μm . Immunostaining was performed following the protocol described by Tang et al. (Tang et al., 2017 [DOI](#)). The primary antibody including mouse anti-PCNA (Abcam, ab29) at a 1:500 dilution, as well as mouse anti-GS (glutamine synthase, BD Transduction Laboratories, 610518), rabbit anti-BLBP (Abcam, ab32423), rabbit anti-DsRed (Clontech, 632496), rabbit anti-TaqGFP (Proteintech, 50430-2-AP), and chicken anti-GFP (Abcam, ab13970) each at a 1:1000 dilution. Secondary antibodies conjugated to Alexa Fluor 488, 594, or 647 (Jackson ImmunoResearch Laboratories Inc.) were used at a 1:1000 dilution. Primary antibodies were incubated overnight at 4°C, while Alexa Fluor secondary antibodies were incubated at room temperature for 2 hours. DAPI staining was performed according to the standard protocol.

In situ hybridization

In this study, three digoxigenin (DIG)-labeled RNA probes targeting endogenous *cxcl18b*, *fabp11a*, and *col15a1b* were synthesized using the MEGAscript™ T7 High Yield Transcription Kit (Invitrogen, AM1334) and the DIG RNA Labeling Kit (Roche, 11277073910), following the manufacturer's instructions. The cDNA of each gene was amplified by PCR using the following primers: *cxcl18b* -F: 5'-ATGGCATTCACACCCAAAGCG-3'; *cxcl18b* -R: 5'-TAATACGACTCACTATAGGGATTGGCCCTGCTGTTTTGTG-3'; *fabp11a* -F: 5'-GTTGGAACCGGACCAACC-3'; *fabp11a* -R: 5'-TAATACGACTCACTATAGGGACGGCTCGTTGAGCTTGAAT-3'; *col15a1b* -F: 5'-CCTCAATGGAGGTCCTAAAGGT-3'; *col15a1b* -R: 5'-TAATACGACTCACTATAGGGACCAGCTTCTGAGACCAAGC-3'.

Following the *in situ* hybridization protocol described by Tang et al. (Tang et al., 2017 [DOI](#)), fresh zebrafish retinal sections were incubated overnight in the HybEZ system (Advanced Cell Diagnostics, 310013) at 65°C with 200 ng of probe for each slide. The next day, slides were sequentially washed in 5x SSC buffer and incubated overnight at 4°C with an anti-DIG-POD antibody (Roche, 11093274910) diluted 1:500 in TNB buffer (TN buffer with 0.5% blocking reagent; Roche). On the third day, the signal was detected using the TSA™ Plus Cyanine 3 (PerkinElmer, NEL744001KT) or Cyanine 5 (PerkinElmer, NEL745001KT)/Fluorescein System.

Imaging

Images were taken using an inverted confocal microscope system (FV1200, Olympus) confocal microscope using 40 × (silicon oil, 1.05 NA), or 60 × (silicon oil, 1.3 NA) objectives.

Quantifications and statistical analysis

All quantification and visualization were performed with FV31S-SW 2.3.1.163 Viewer (Olympus), and ImageJ. *Z intensity projection* was used to process the Z-stack images acquired from 14- μm -thick sections of zebrafish retina for statistical analysis. For cell counting, PCNA⁺ MG and their lineage in this study were defined as one proliferative MG, *cxcl18b*⁺ MG and their lineage were counted as one *cxcl18b*⁺ MG.

To perform the statistical analysis, p values were calculated with GraphPad Prism 8 (or Microsoft Excel). The unpaired, non-parametric Wilcoxon test was applied for comparison of two groups. The one-way ANOVA, followed by Tukey's HSD test was applied for comparison of different groups with one treatment. The two-way ANOVA followed by Tukey's HSD test was applied for comparison of four groups with two treatments. Error bars represent SEM. ****p<0.0001, ***p<0.001; **p<0.01; *p<0.05; ns, p>0.05.

Data availability

This paper does not report new raw scRNA-seq data and original code.

Supplementary figures and tables

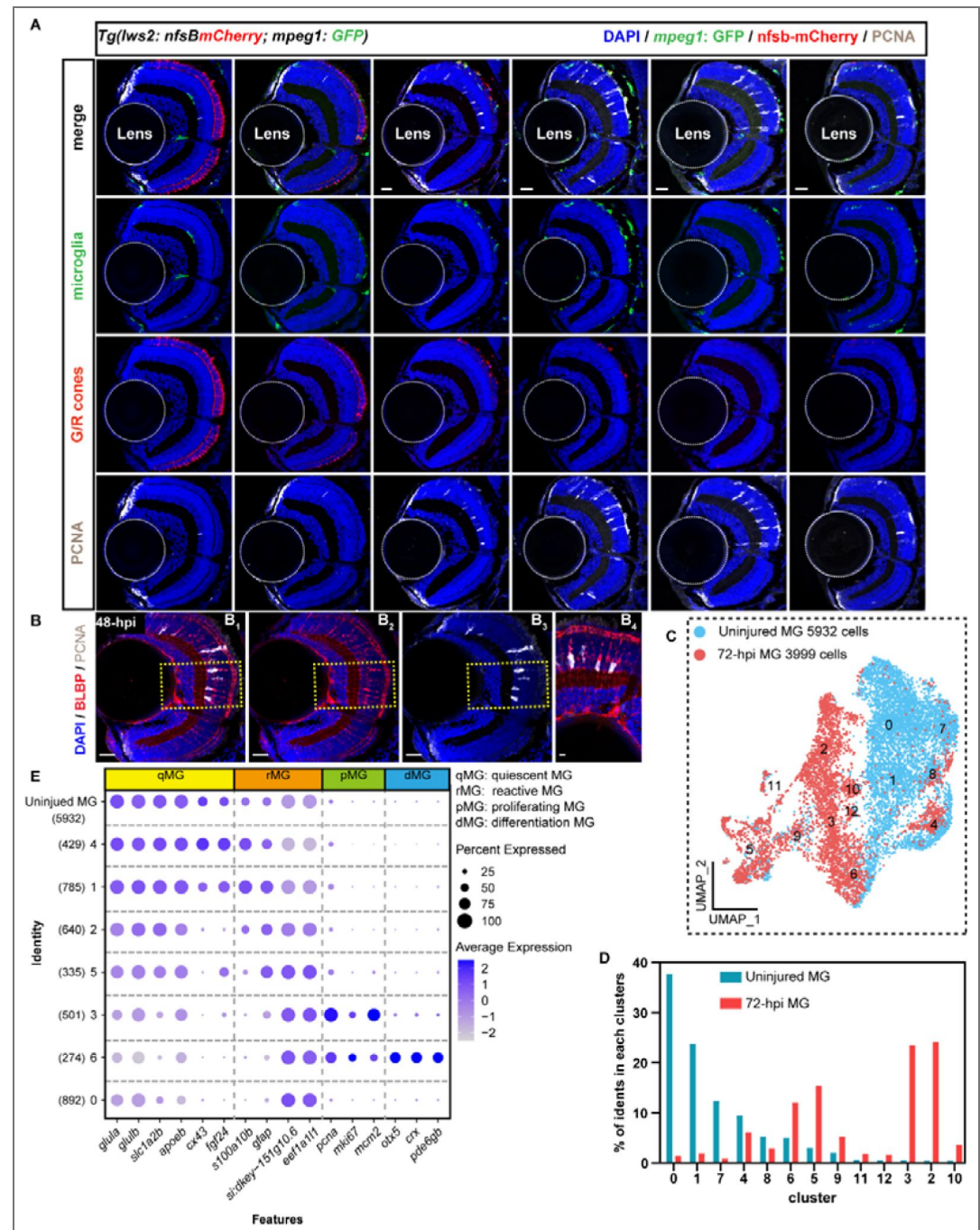


Figure 1-figure supplement 1. Clusters with increased proportion are identified from the scRNA-seq data
(A) Representative images showing microglia recruitment (green, indicated by *Tg(mpeg1: GFP)*), G/R cone ablation (red, *Tg(lws2: nfsb-mCherry)*), and injury-induced MG proliferation (white, marked by PCNA) following the injury process from uninjured retina to 120 hpi. Scale Bars: 20Cμm. **(B)** Representative images showing PCNA+ (white, immunostaining) cells co-expressed with BLBP (red, immunostaining) at 72 hpi. Scale Bars: 20Cμm (*B₁-B₃*) and 5 μm (*B₄*). **(C)** UMAP plot showing 5,932 cells from uninjured retinas (blue) and 3,999 cells from 72-hpi (red) retinas were obtained, which were further aggregated into 13 clusters. **(D)** Percentage of MGs from uninjured and 72-hpi retinas in each cluster. Eight clusters (6, 5, 9, 11, 12, 3, 2, 10) were identified and showed an increased proportion for further analysis. **(E)** Dot plot showing the expression levels of marker genes for quiescent MG (qMG), reactive

MG (rMG), proliferative MG (pMG), and differentiated MG (dMG). The average expression levels of these genes for all cells in each cluster are coded by the gray level. The percentage of cells expressing each gene within each cluster is coded by dot size.

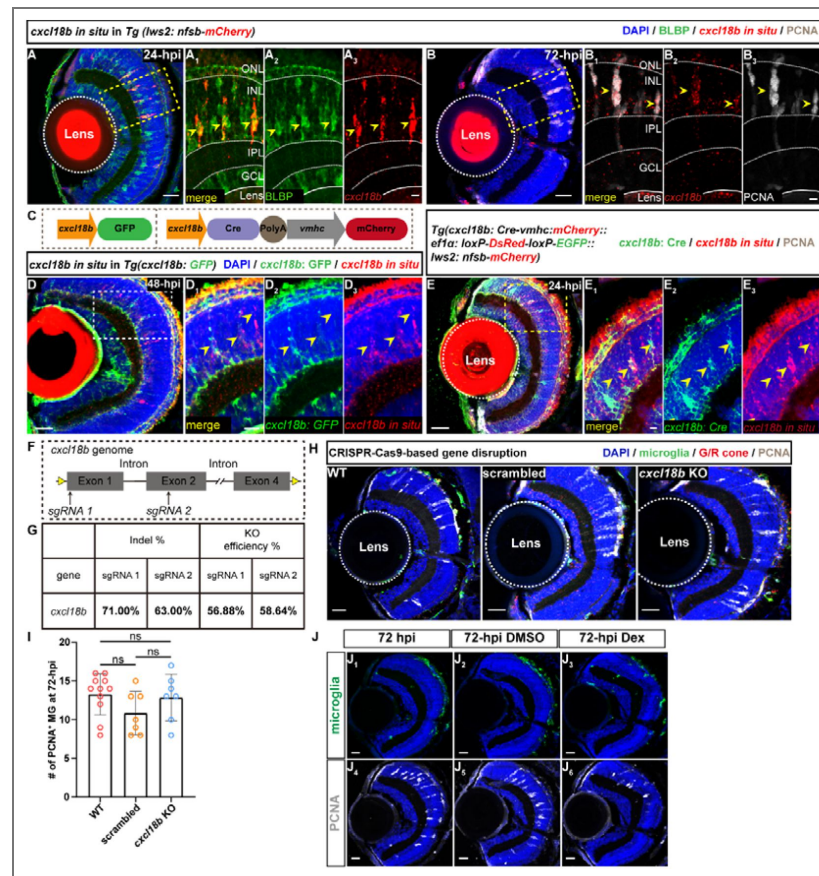


Figure 2-figure supplement 1. Gene description of *cxcl18b* does not reduce MG proliferation (A-A₃)

In situ hybridization images show injury-induced *cxcl18b* (red, marked by *in situ* hybridization) with MG-like morphology, cell-specific expression in the BLPB⁺ cells (green, labeled by immunostaining) at 24 hpi in the transgenic fish line Tg(*lws2: nfsb-mCherry*) retina. In these clones, *cxcl18b*⁺ MG are red⁺/BLPB⁺ cells (yellow arrowheads). The high-magnification images of the boxed area (A₁-A₃). The area of the retina structure is labeled with a dashed line, and each layer structure is labeled with dashed lines and marked with the outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and lens. Scale Bars: 20 μ m (A) and 5 μ m (A₁-A₃). (B-B₃) Representative images showing *cxcl18b* (red, *in situ* hybridization) co-expressed with PCNA (white, immunostaining) at 72 hpi. In these clones, *cxcl18b*⁺ MG was identified as red⁺/PCNA⁺ cells (yellow arrowheads). The high-magnification images of the boxed area (B₁-B₃). Scale Bars: 20 μ m (B) and 5 μ m (B₁-B₃). (C) Schematic showing the design of the *cxcl18b* reporter fish line and the Cre-loxP transgenic fish line used for clone analysis. The *cxcl18b* promoter drives GFP or Cre expression, with the heart labeled by mCherry under the *vmhc* promoter for fish screening. (D-E₃) Representative images showing *cxcl18b* expression labeled by Tg(*cxcl18b: GFP*) (green) in (D-D₃) at 48 hpi, or three transgenic fish Tg(*cxcl18b: Cre-vmhc: mCherry:: ef1 α : loxP-DsRed-loxP-EGFP:: lws2: nfsb-mCherry*) (green) in (E-E₃) at 24 hpi fish retina, merged with *cxcl18b* in situ hybridization signal (red) (D-E₃). The high-magnification images of the boxed area (D₁-D₃, E₁-E₃). Scale Bars: 20 μ m (D, E) and 5 μ m (D₁-D₃, E₁-E₃). (F) Schematic showing the two sgRNA target sites of the *cxcl18b* genome. (G) Summary table showing the indel and knockout efficiency of the two sgRNAs targeting *cxcl18b*. (H) Representative images of proliferative MG (PCNA⁺, white) at 72 hpi in Tg(*lws2: nfsb-mCherry*) fish retina with *cxcl18b* disruption. Scale Bars: 20 μ m. (I) Quantitative plots showing the number of proliferative MG (PCNA⁺) at 72 hpi in H. In total, retinas were collected from wild-type (WT, n=11), *scrambled* sgRNA-injected (*scrambled*, n=7), and *cxcl18b* sgRNA-injected (*cxcl18b* KO, n=7) (Mean $\pm$ SEM; ns, p>0.05; one-way ANOVA followed by Tukey's HSD test). (J₁-J₆) Representative images showing injury-induced microglia recruitment (green, marked by Tg(*mpeg1: GFP*)) (J₁-J₃), and proliferative MG (white, marked by PCNA) (J₄-J₆) after dexamethasone (Dex) or DMSO treatment in the in Tg(*mpeg1: GFP x lws2: nfsb-mCherry*) fish retina at 72 hpi. Scale bars: 20 μ m.

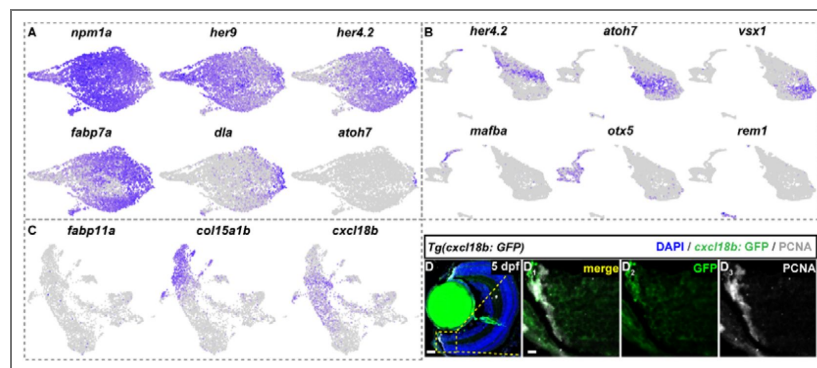


Figure 3-figure supplement 1. UMAP plots show the expression of cluster-specific marker genes utilized to identify developmental states

(A, B) UMAP plots showing the expression pattern of embryonic developmental stage cluster-specific marker genes (*npm1a*, *her9*, *her4.2*, *fabp7a*, *dla*, and *atoh7*) in (A) and post-embryonic developmental stage marker genes (*her4.2*, *atoh7*, *vsx1*, *mafba*, *otx5*, and *rem1*) in (B). (C) UMAP plots showing the expression pattern of *fabp11a*, *cxcl18b*, and *col15a1b* at MG clusters with increased population after G/R cone ablation at 72 hpi in Figure 1F. (D) Representative images showing the *cxcl18b* expression (green, marked by *Tg(cxcl18b: GFP)*) are located in the most peripheral region of the CMZ (white, marked by PCNA) at 5 dpf. The high-magnification images of the boxed area (D₁-D₃). Scale Bars: 20Cμm (D) and 5 μm (D₁-D₃).

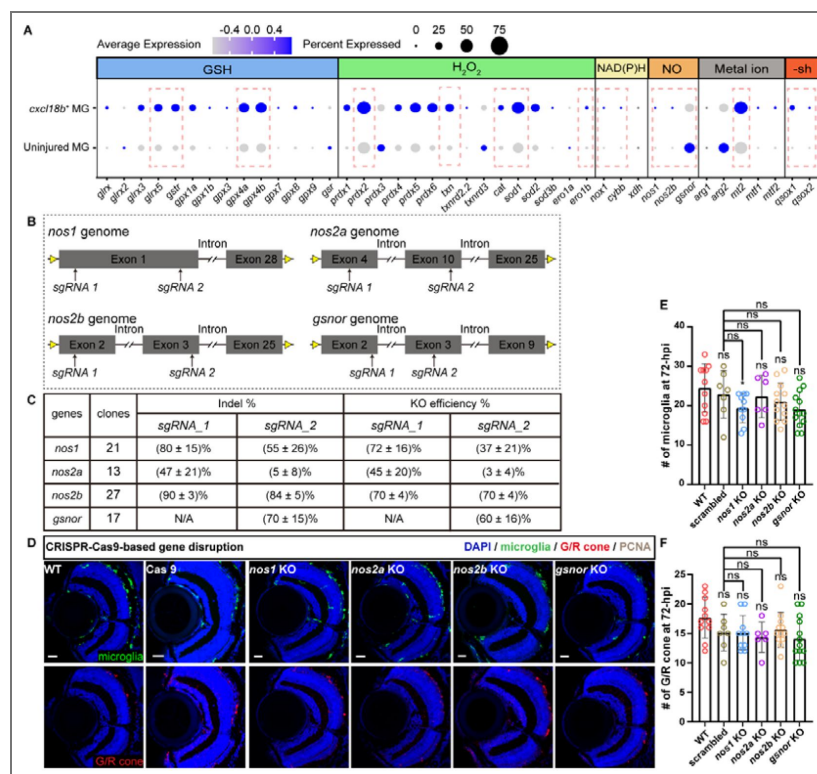


Figure 4-figure supplement 1. Genetic disruption of the nitric oxide pathway modulates G/R cone ablation and microglial recruitment

(A) Dot plot showing the redox gene expression profiles of regulating glutathione (GSH), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate (NADPH), nitric oxide (NO), metal ion, and sulfhydryl group (-SH) in the uninjured and *cxc118b*⁺ MG in 72-hpi scRNA-seq data (Krylov et al., 2023). The average expression levels of these genes for all cells in each cluster were coded by the gray level. The percentage of cells expressing each gene within each cluster was coded by dot size. **(B)** Schematic showing the two sgRNA target sites of the nitric oxide metabolism pathway genome (*nos1/nos2a/nos2b/gsnor*). **(C)** Summary table showing the indel and knockout efficiency of the two sgRNAs targeting *nos1/nos2a/nos2b/gsnor*. **(D)** Representative images of microglial recruitment (marked by *Tg(mpeg1: GFP)*, green) and G/R cone ablation (marked by *Tg(lws2: nfsb-mCherry)*, red) at 72 hpi with *nos1/nos2a/nos2b/gsnor* disruption, with scrambled sgRNA-injected as the control. Scale Bars: 20 μ m. **(E-F)**. Quantitative plots showing the number of recruited microglia (GFP⁺) **(D)** and G/R cone ablation (mCherry⁺) **(E)** at 72 hpi with *nos1/nos2a/nos2b/gsnor* disruption. In total, we collected 11 retinas for WT (n=11), scrambled sgRNA-injected (n=7), *nos1* sgRNA-injected (n=10), *nos2a* sgRNA-injected (n=6), *nos2b* sgRNA-injected (n=12), and *gsnor* sgRNA-injected (n=13) (Mean $\pm$ CSEM; *p<0.05; ns, p>0.05; one-way ANOVA followed by Tukey's HSD test).

Figure 4-figure supplement 2. The genotype of nitric oxide metabolism pathway mutants

(A) Sequence alignment of WT nitric oxide metabolism pathway genes (*nos1/nos2a/nos2b/gsnor*) and their CRISPR-cas9-induced mutant alleles. **(B)** Summary table showing the premature stop codon and unknown polypeptides sides of each mutant.

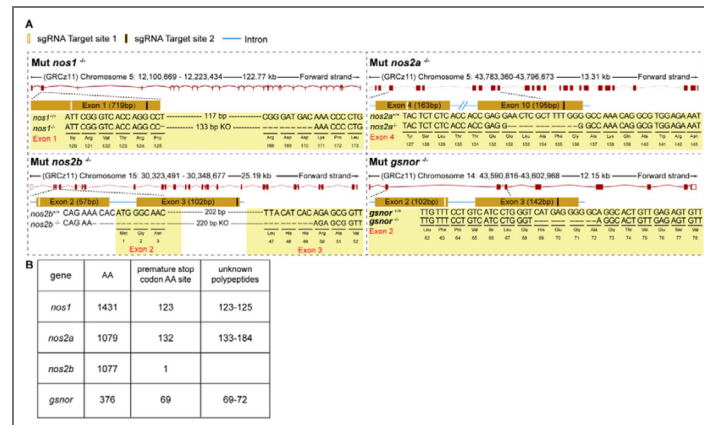


Figure 5-figure supplement 1. *In situ* hybridization reveals *nos2b* cell-specific expression in the *cxcl18b*-defined transitional MG states

(A-A₃) Representative *in situ* hybridization images showing *nos2b* expression (red, *in situ* hybridization) cell-specific in the injury-induced *cxcl18b*⁺ MG (GFP⁺, green) in *Tg(lws2: nfsb-mCherry x cxcl18b: GFP)* fish retina at 72 hpi. Clones with green/red/white trouble-positive are *cxcl18b*⁺/*nos2b*⁺/PCNA⁺ MG cells (yellow arrowheads). The high-magnification images of the boxed area (C-C₃). Scale Bars: 20Cμm (A, B) and 5 μm (C₁-C₃). (D-E) RT-qPCR analysis of *glula* (pink bar) and *glulb* (green bar) expression in different cell populations relative to 72-hpi G/R cone (D), or other retinal cell types (E). The significance of 72-hpi G/R cone as control is noted by * for comparisons with 72-hpi G/R cones and # for comparisons with uninjured MG. In total, we do 6 independent replicates to enrich the different cell populations and get the cDNA templates (MeanC±CSEM; **** and #####p<0.0001, **p<0.001, *p<0.01, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test). (F) RT-qPCR analysis of *cxcl18b* (blue bar) and *pcna* (orange bar) expression in different MG states. (MeanC±CSEM; ****p<0.0001, **p<0.01, *p<0.05, ns, p>0.05; two-way ANOVA followed by Tukey's HSD test).

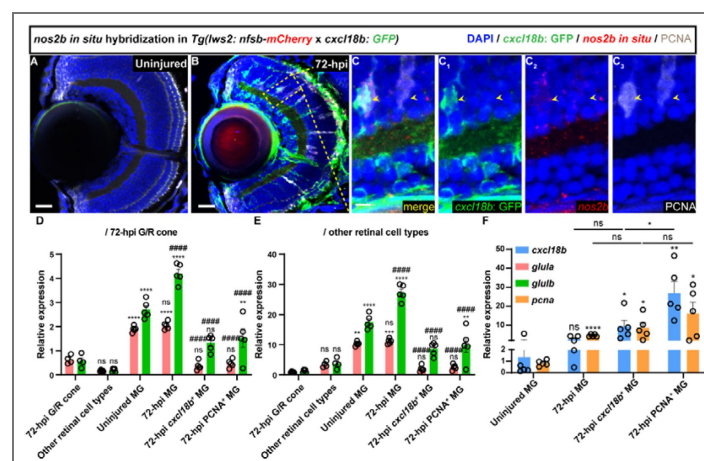
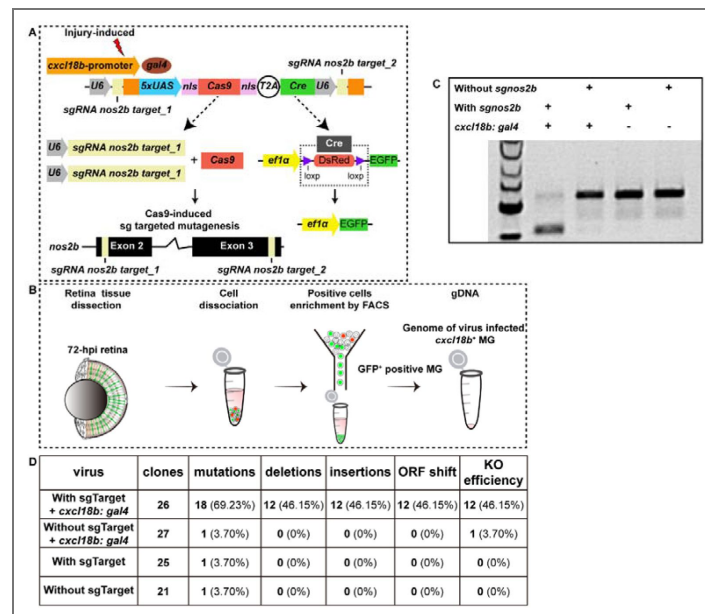


Figure 6-figure supplement 1. Analysis of cell-specific knockout efficiency for *nos2b* in virus-infected clones

(A) Schematic showing the cell-specific gene knockout system design. Injury-induced *cxcl18b* promoter directs the expression of *gal4*, which in combination with the *UAS* element, drives the cell-specific expression of Cas9 and Cre. The *U6* promoter enables the broad expression of sgRNAs targeting *nos2b*, and Cas9 mediates gene knockout within the targeted cells through these sgRNAs. The *ef1a* promoter controls the *loxP-DsRed-loxP-EGFP* reporter system, allowing for the switch from DsRed to EGFP upon Cas9 and Cre activity, and GFP⁺ MG clones are identified with successful *nos2b* knockout. This system operates with high specificity via double adenovirus infection. **(B)** Schematic showing the procedure of virus-infected clones are sorted and enriched by FACS for further knockout efficiency analysis. **(C)** Representative gel electrophoresis image showing *nos2b* deletions in knockout cells compared to controls. Single-virus injections were used to assess Cre system leakage. While minimal Cre system leakage was observed, it did not result in detectable *nos2b* knockout. **(D)** Summary table showing the knockout efficiency of each virus-injected clone.



Supplementary Table S1. sgRNA sequences and genotyping primers, related to STAR Methods.

sgRNA targets sequences	
Gene symbol	Primer forward
<i>nos1</i> sgRNA target-1	TAATACGACTCACTATAGGCTCCCAGGAAAGAGTCGCGTTTATAGCTAGAAATAGC
<i>nos1</i> sgRNA target-2	TAATACGACTCACTATAGGTCACCAGGCCTGTCAACCGTTTATAGCTAGAAATAGC
<i>nos2a</i> sgRNA target-1	TAATACGACTCACTATAGGAGGAACCTCGCTTTTGGGGGTTTATAGCTAGAAATAGC
<i>nos2a</i> sgRNA target-2	TAATACGACTCACTATAGGTGGCTGGTGCCACCGATGGTTTATAGCTAGAAATAGC
<i>nos2b</i> sgRNA target-1	TAATACGACTCACTATAGGAGTATTTTCAGAAACACATGTTTATAGCTAGAAATAGC
<i>nos2b</i> sgRNA target-2	TAATACGACTCACTATAGGATGTGTACATCACAGAGGTTTATAGCTAGAAATAGC
<i>gsnor</i> sgRNA target-1	TAATACGACTCACTATAGGCCCATGAAGTTCGAGTGAGTTTATAGCTAGAAATAGC
<i>gsnor</i> sgRNA target-2	TAATACGACTCACTATAGGTCATCCTGGGTCATGAGGGTTTATAGCTAGAAATAGC

Genotyping primers for gene disruption		
Gene symbol	Primer forward	Primer reverse
<i>nos1</i> -sgTarget-1	ATCGTCTCGGACCTCATCCG	ATAGGTTTTCTATTGCCCTGAGCTC
<i>nos1</i> -sgTarget-2	ATGCGAACGATGCTCGC	ATCCTTCTATCGATGACCCCTCTGG
<i>nos2a</i> -sgTarget-1	ATTACTGTTCTGTCGACTTGATAAACA CG	ATACCTTAAGATGAGTGACACAAGGCA TG
<i>nos2a</i> -sgTarget-2	ATTAGCCACTCTGAAATCAAGCC	ATCAGTCCTTTGAAGCTGACCTTGC
<i>nos2b</i> -sgTarget-1	ATTGACGACGTTATACCTCTTTAGC	AATGGGCACTGCTTGACTTTAT
<i>nos2b</i> -sgTarget-2	ACTCACAAGAGCGAACTACTGC	ATTGACCTTCACAAATCTTAGACG
<i>gsnor</i> -sgTarget-1	ATTAATTTTCAGGTGATCAAATGTAAGG C	ATGGGGTGAAGTTGAGTCAGC
<i>gsnor</i> -sgTarget-2	ATGTATTCAAAGTGTAATGTAGTCG G	TACCTGATTTCTGGCACAGG

Supplementary Table S2. Plasmid construction primer sequences, related to STAR Methods.

Plasmid construction primer sequences			
Plasmid	Elements	Primer forward	Primer reverse
<i>pTol2-cxcl18b: GFP</i>	<i>cxcl18b-promoter</i>	TAGGGCGAATTGGGCATTTGT CTCCTCATGCATTGACTAC	AATTGCTGCAAACATATATGT AGGAAATGCTG
	<i>GFP</i>	ATGGTGAGCAAGGGC	TTACTTGTACAGCTCGTCC ATGC
	<i>gal4FF</i>	ATGAAGCTACTGTCTTCTATC GAACAAGC	CTACCCACCGTACTCGTCA ATTCC
<i>pTol2-cxcl18b: Cre-vmhc: mCherry</i>	<i>Cre</i>	ATGGCCAATTTACTGACCGTA CACC	CTAATCGCCATCTTCCAGC AGGC
	<i>vmhc: mCherry</i>	CATGTGTCCTAAATTCTGAAA ACACAGTAAGCC	TTACTTGTACAGCTCGTCC ATG
<i>pTol2-UAS: Cas9-T2A-Cre-U6: nos2b sgRNA1; U6: nos2b sgRNA2</i>	<i>nos2b sgRNA1</i>	CTCGGGTCGCTCTTGTGAGT GACC	AAACGGTCACTCACAAGA GCGACC
	<i>nos2b sgRNA2</i>	AGCTCGGGCTGGTGCCGCCA ATGTCC	TAAACGACATTGGCGGCAC CAGCCCCG

Supplementary Table S3. qPCR primer sequences, related to STAR Methods.

Gene symbol	Primer forward	Primer reverse
<i>cxcl18b</i>	TGCTGCTCGCGGTAGTTTAC	TCTGCAGTAATTGGCCCTGC
<i>glul</i>	CGCATTACAGAGCCTGCCTA	ATTCCAGTTGCCTGGGATCG
<i>glulb</i>	ATGGTCCAGAACCCAGCATCC	TCACAAAGGCCCACTTGGAA
<i>pcna</i>	CAGAAACCTAGCCATGGGGG	GTCTTGGACAGAGGAGTGCC
<i>nos1</i>	TGAGCACGTTTGCATAGGGT	CACTGGATTCTGCCACACA
<i>nos2a</i>	CCAGATAACCACTGCTCTGCT	CTTGCTTCCCATCTTGCTAAAA
<i>nos2b</i>	GAGGCAAAATTCACCAACAG	TGCAAAGTGGTCATGGGACA
<i>gsnor</i>	ACAAGAAATCTCCACCCGGC	GACGGCTCGGATACTCTTCC
<i>β-actin</i>	ATTGCTGACAGGATGCAGAAAG	GATGGTCAGACTCATCGTACTC

Acknowledgements

This research was supported by grants from the National Key R&D Program of China (2022YFA1303000 to C.C.), the Chinese Academy of Sciences Strategic Priority Research Program B grants (XDB39000000 to C.C.), National Natural Science Foundation of China-Key program project (Grant No. 91849203 to C.C.), the Creative Research Groups of the National Natural Science Foundation of China (32321003 to J.H.), STI2030-Major Projects (2021ZD0204500 to J.H.), the National Key Research and Development Program of China (2020YFA0112700 to J.H.), National Natural Science Foundation of China (32471029 to J.H.).

Additional information

Author Contributions

A.Y., design and experimental investigation, visualization, data analysis, methodology, writing-original draft, writing-review and editing. S.Y., M.D., and D.Z., investigation, methodology. J.H. and C.C., conceptualization, experimental design, data analysis, supervision, funding acquisition, writing-original draft, project administration, writing-review and editing.

Funding

Funder	Grant reference number	Author
National Key Research and Development Program of China	2022YFA1303000	Chang Chen
National Natural Science Foundation of China	91849203	Chang Chen
Chinese Academy of Sciences	XDB39000000	Chang Chen
National Natural Science Foundation of China	32321003	Jie He
Ministry of Science and Technology of the People's Republic of China	2021ZD0204500	Jie He
National Key Research and Development Program of China	2020YFA0112700	Jie He
National Key Research and Development Program of China	32471029	Jie He

Author ORCID iDs

Aojun Ye: <http://orcid.org/0009-0008-4702-5914>

Jie He: <http://orcid.org/0000-0002-2539-2616>

Chang Chen: <http://orcid.org/0000-0003-1008-1062>

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Peer reviews

Reviewer #1 (Public review):

Summary:

This study presents a valuable contribution of NO signaling in zebrafish retinal regeneration in larval animals. The data on NO signaling are solid. There are multiple limitations to the study, but these are largely acknowledged by the authors in the revised text.

Strengths:

New data on NO signaling is valuable to the field but may be limited to larval "regeneration".

Weaknesses:

A weakness of the approach is testing cone ablation and regeneration in early larval animals. A near identical study was already done by Hoang et al 2020 in the adult zebrafish, a more relevant biological timepoint.

<https://doi.org/10.7554/eLife.106274.2.sa2>

Reviewer #2 (Public review):

Summary:

In this manuscript Ye et al. examine the sequence of events that occur in the damaged zebrafish Muller glia (MG) in states between quiescence and the onset of proliferation. Using an inducible metronidazole (MTZ) and nitroreductase system to ablate red/green cones in

larval zebrafish, they identify a novel transitional MG state that is characterized by the expression of *cxcl18b*. Using trajectory analysis from single-cell RNA-seq datasets, they find that *cxcl18b* is expressed before MG expression PCNA and become proliferative. They find that *cxcl18b* expression peaks in MG at approximately 24 hours post injury (hpi) and rapidly declines as MG proliferate following injury. In a most interesting finding, the authors find a link between *nos2b*-dependent nitric oxide signaling and *cxcl18b*-mediated proliferation. Mutagenesis of *nos2b* decreases MG proliferation. The mechanism linking NO signaling to proliferation was suggested to function via notch signaling as pharmacological inhibition of nitric oxide signaling resulted in elevated Notch activity, thus preventing MG proliferation. The authors suggest a model whereby *cxcl18b* induces autocrine NO signaling in MG to reduce activity of Notch3, thereby promoting MG proliferation.

Strengths:

The authors utilize a number of sophisticated transgenic approaches and generate novel lines that will have value to the field. The identification of a novel *cxcl18b* transition state is exciting and the putative link between NO signaling and Notch activity would provide new insight into the drivers of Muller glia proliferation.

Weaknesses:

While the overall model is appealing and may serve as a foundation for future studies, some information gaps remain and certain conclusions rely on correlational data. The cellular expression of *nos2b* remains unclear as the single-cell RNA-seq data cannot provide expression data that matches RT-PCR results. The temporal sequence of events are based on transgene expression in the Tg(*cxcl18b*:GFP) lines, where persistence of the GFP fluorescence may not reflect endogenous *cxcl18b*. The identity of putative *cxcl18b* receptors on MG to support an autocrine signaling pathway remains unclear. Nevertheless, this is an interesting study that should open new avenues of exploration.

<https://doi.org/10.7554/eLife.106274.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1:

(1) The authors state that more is known about glial reactivation than cell-cycle re-entry. They are confusing many points here. More gene networks that require cell-cycle re-entry are known. Some of the genes listed for "reactivation" are, in fact, required for cell cycle re-entry/proliferation. And the authors confuse gliosis vs glial reactivation.

We thank the reviewer for this important and constructive comment. We fully agree that clearly distinguishing between the concepts of glial reactivation, glial proliferation, gliosis, and neurogenesis is essential to avoid conceptual confusion in our study.

Injury-induced retinal regeneration in zebrafish:

Glial reactivation refers to the initial response of quiescent Müller glia (MG) to injury, characterized by morphological changes and upregulation of reactive markers (e.g., *gfap*, *ascl1a*, *lin28a*) and activation of signaling pathways such as Notch, Jak/Stat, and Wnt (Lahne et al., 2020; Pollak et al., 2013; Sifuentes et al., 2016; Yao et al., 2016).

Glial proliferation refers to the clonal expansion of these MG-derived progenitor cells, which undergo rapid cell-cycle re-entry and amplify to generate sufficient progenitors for regeneration (Iribarne and Hyde, 2022; Lee et al., 2024; Wan and Goldman, 2016)

Gliosis vs neurogenesis represents a divergent fate decision following proliferation. In zebrafish, MG-derived progenitor cells differentiate into retinal neurons that can replace those damaged or lost due to retinal injury. In contrast, mammalian MG tend to undergo an initial gliotic surge and rapidly revert to a quiescent state, exhibiting gliosis and glial scarring (Thomas et al., 2016; Yin et al., 2024). Thus, we totally agreed that gliosis cannot be confused with glial reactivation because glial reactivation is the very first step of glial injury responses, whereas gliogenesis is the very last glial response to the injury.

We agree with the reviewer that many genes typically described as “reactivation markers” (e.g., *ascl1a*, *lin28a*, *sox2*, *mycb*, *mych*) are also essential regulators of cell-cycle re-entry (Gorsuch et al., 2017; Hamon et al., 2019; Lee et al., 2024; Lourenço et al., 2021; Pollak et al., 2013; Thomas et al., 2016). Because the glial reactivation is a leading event for glial proliferation, the regulators of glial reactivation are expected to be responsible for glial proliferation as well.

In our study, we focused on the states preceding glial proliferation to understand the mechanism underlying injury-induced glial cell-cycle re-entry. We defined these transitional states and the subsequent proliferative MG states based on single-cell RNA-seq trajectory analysis. (revised lines: 41-58)

(2) A major weakness of the approach is testing cone ablation and regeneration in early larval animals. For example, cones are ablated starting the day that they are born. MG that are responding are also very young, less than 48 hrs old. It is also unclear whether the immune response of microglia is a mature response. All of these assays would be of higher significance if they were performed in the context of a mature, fully differentiated, adult retina. All analysis in the paper is negatively affected by this biological variable.

We thank the reviewer for raising this important point regarding the developmental stage of the retina in our model system. We have carefully considered this concern and now provide additional clarification and justification, as follows:

(1) The glial responses in larval and adult retina:

Previous studies have demonstrated that injury-induced glial responses are largely conserved in larval and adult zebrafish retina, including reactive gliosis marked by *gfap* upregulation and proliferation (Meyers et al., 2012; Sarich et al., 2025). In our study, G/R cones were ablated beginning at 5 dpf using metronidazole (MTZ), and we observed robust induction of PCNA⁺ MG in the inner nuclear layer, consistent with injury-induced proliferation (Figure 1E). These findings align with previous studies showing that key features of MG regenerative responses are conserved across larval and adult stages.

(2) The microglial responses in larval and adult retina:

Retinal microglia functionally mature at 5 dpf in the zebrafish retina (Mazzolini et al., 2020; Svahn et al., 2013), and prior studies have demonstrated that microglia in larval and adult zebrafish exhibit similar responses to injury, including migration, morphological activation, and phagocytosis (Nagashima and Hitchcock, 2021; White et al., 2017). In our experiments using Tg(*mpeg1*: GFP) larvae, we observed clear microglial recruitment to the outer nuclear layer (ONL) following cone ablation (Figure 1E and Figure 1-figure supplement 1A), supporting the functional competence of larval microglia in injury-induced immune responses

(3) The contribution using larval animals to study the regeneration program:

We agree that regeneration studies in the adult retina can provide important biological insights, particularly in a fully differentiated tissue environment. Accordingly, we have

acknowledged this limitation in our revised manuscript “limitations of this study” section (revised lines 534-540: “1. Our study focuses on larval zebrafish, in which the core features of MG and immune responses are conserved compared to the adult. However, we acknowledge that the adult retina—with its fully matured differentiated retina and immune response—provides irreplaceable biological insight. Nevertheless, larval models offer a powerful platform to uncover conserved regenerative mechanisms and serve as a valuable complement for identifying age-dependent differences in MG-mediated regeneration.”) and have stated our intention to extend future analyses to adult zebrafish, especially to explore age-dependent differences in redox signaling and MG proliferation. At the same time, we believe that the larval model offers unique advantages for uncovering fundamental, conserved mechanisms of regeneration and enables characterization of age-dependent regulatory differences. Thus, our study in larval animals serves as a complementary and informative platform for understanding both the conserved and developmental stage-specific features of injury-induced regeneration.

(4) Related to the above point, the clonal analysis of cxcl18b⁺ MG is complicated by the fact that new MG are still being born in the CMZ (as are new cones for that matter).

We thank the reviewer for raising this important point regarding potential contributions from CMZ-derived progenitors to the lineage-traced cxcl18b⁺ MG clones. To address this concern, we have implemented evidence to rule out a CMZ origin for the clones analyzed:

Spatial restriction of clones: All clones included in our analysis were located exclusively within the central and dorsal retina, as shown in Figure 2H. From the spatial distribution of reactive MG populations across the retina, we observed a patterned organization in which the vast majority of proliferating MG arose from local mature MG-derived progenitors, rather than from peripheral CMZ-derived progenitors. However, we acknowledge that we cannot entirely exclude the possibility that CMZ-derived progenitors contribute to injury-induced MG proliferation, particularly in the peripheral retina.

We have clarified this point in the revised Methods section (revised lines 756–762: “Clone analysis of cxcl18b⁺ lineage-traced MG was restricted to cells located in the central and dorsal region of the zebrafish retina after G/R cone ablation in Figure 2, Figure 6, and their figure supplement. This spatial restriction strongly suggests that the proliferative MG originate from local mature MG, although we cannot completely rule out the possibility that CMZ-derived progenitors contribute to the generation of proliferative MG in the peripheral retina.”) and updated the corresponding figure legends.

(4) A near identical study was already done by Hoang et al., 2020, in adult zebrafish, a more relevant biological timepoint. Did the authors check this published RNA-seq database for their gene(s) of interest?

We thank the reviewer for pointing out the relevance of the study by Hoang et al., 2020, which characterized the transcriptional dynamics of MG reactivation in the adult zebrafish retina. We agree that comparisons with their single-cell RNA-seq dataset are important to confirm the conservation of our findings in larval vs adult zebrafish.

To this end, we examined the adult zebrafish MG dataset reported by Hoang et al., and confirmed that cxcl18b is also present and enriched in their analysis, particularly in activated MG populations under various injury paradigms:

(1) cxcl18b is listed as a differentially expressed gene (DEG) in Supplementary Table ST2, enriched in GFP⁺ MG following injury. It is also significantly upregulated in both NMDA-induced and light damage conditions, as shown in Supplementary Table ST3.

(2) In Supplementary Table ST5, cxcl18b is identified as a classifier of activated MG, with classification power scores of 0.552 (NMDA), 0.632 (light damage), and 0.574 (TNFα + γ-

secretase inhibitor treatment), indicating its consistent expression across multiple injury models.

(3). In their pseudotime analysis (Figure 4C and Supplementary Table ST8), *cxcl18b* is specifically expressed in Module 5, which is expressed earlier along the trajectory than *ascl1a*. This temporal pattern of *cxcl18b* preceding *ascl1a* expression is consistent with our trajectory analysis in larval MG (Figure 1H), further supporting its role as an early marker of the transitional state before proliferation.

These findings underscore the robustness and biological relevance of *cxcl18b* as a conserved marker of injury-responsive MG in both larval and adult zebrafish. Our data expand upon the prior work by specifically characterizing a *cxcl18b*-defined transitional MG state preceding cell-cycle re-entry, thereby offering additional insights into the temporal staging of MG activation during regeneration.

(5) KD of cxcl18b did not affect MG proliferation or any other defined outcome. And yet the authors continually state such phrases as "microglia-mediated inflammation is critical for activating the cxcl18b-defined transitional states that drive MG proliferation." This is false. Cxcl18b does not drive MG proliferation at all.

We thank the reviewer for raising this concern. We agree with the reviewer and have revised this statement as "These findings suggest that microglia-mediated inflammation may contribute to the activation of *cxcl18b*-defined transitional states that precede MG proliferation, although a causal relationship remains to be established." (revised lines 251-253).

(6) A technical concern is that intravitreal injections are not routinely performed in larval fish.

We appreciate the reviewer's technical concern regarding the use of intravitreal injections in larval zebrafish. In our study, we performed intraocular injection according to previously established methods (Alvarez et al., 2009; Giannaccini et al., 2018; Rosa et al., 2023). This approach involves carefully delivering a small volume of viral suspension into the intraocular space by a glass micropipette. To address this concern, we will revise the Materials and Methods section to clearly describe the injection procedure and will cite the relevant references accordingly.

Reviewer #2:

(1) The authors note a peak of PCNA+ Muller glia at 72 hours post injury. This is somewhat surprising as the MG would be expected to generate progenitor cells that would continue proliferating and stain with PCNA. Indeed, only a handful of PCNA+ cells are seen in the INL/ONL layer in Figure 1E2 with few clusters of progenitors present. It would be helpful to stain with a Muller glia marker to confirm these PCNA+ cells are Muller glia. It's also curious that almost all the PCNA+ cells are in the dorsal retina, even though G/R cone loss extends across both dorsal and ventral retina. Is this typical for cone ablation models in larval zebrafish?

We thank the reviewer for their insightful comment regarding the spatial distribution and identity of PCNA⁺ cells following injury.

In our study, we observed that the injury-induced proliferating cells (PCNA⁺) were predominantly located in the central and dorsal regions of the retina at 72 hours post-injury (hpi) (Figure 1E). To verify the identity of these proliferating cells, we performed additional immunostaining using BLBP, and confirmed that the majority of PCNA⁺ cells also express BLBP (Figure 1-figure supplement 1B in our revised Data), these results supporting their MG origin.

The regional bias of MG proliferation towards the central and dorsal retina is consistent with previous findings. Notably, (Krylov et al., 2023) demonstrated that MG exhibit region-specific heterogeneity in their regenerative responses to photoreceptor ablation. Their study identified proliferative MG subpopulations predominantly in the central (fgf24-expressing) and dorsal (efnb2a-expressing) domains, whereas ventral MG showed limited proliferative capacity (Krylov et al., 2023). These observations provide a plausible explanation for the spatially restricted PCNA⁺ MG population observed in our model following cone ablation.

(2) In Line 148: What is meant by "most original MG states" in this context? Original meaning novel? Or original meaning the earliest state MG adopted following injury? The language here is confusing.

We thank the reviewer for pointing out the ambiguous phrasing in our original manuscript. The term “most original MG states” was imprecise and misleading, as it could be interpreted as referring to the quiescent state of MG. In our context, we intended to describe the earliest transitional states in MG respond to injury, as they begin to exit quiescence and enter reactive characteristics. These early transitional MG populations co-express quiescent markers such as cx43 and early reactive markers gfap, as shown in Figure 1H.

To avoid confusion and improve conceptual clarity, we have revised the manuscript by replacing “most original MG states” with “early transitional MG state” (revised line 154) and have added a clearer explanation in the corresponding Results section to define this population more accurately.

(3) Perhaps provide a better image in Figure 2A of the cxcl18b at 48 hpi and 72 hpi. The current images appear virtually identical, with very little cxcl18b expression observed, especially compared to the 24 hpi. This is in contrast to the Tg(cxcl18b:GFP) transgenic line shown in Figure 2D, which indicates either much higher expression in proliferating cells at 48 hpi or the stability of GFP protein. Can the authors provide guidance on the accurate temporal expression of cxcl18b? Does expression peak rapidly at 24 hpi and then rapidly decline or is there persistence of expression to 48-72 hpi?

We appreciate the reviewer’s careful observation regarding the apparent similarity of cxcl18b expression at 48 hpi and 72 hpi in the in situ hybridization (ISH) images (Figure 2A), and the differences compared to the Tg(cxcl18b: GFP) reporter line shown in Figure 2D.

(1) The similarity of ISH images at the 48 hpi and 72 hpi (Figure 2A):

The cxcl18b mRNA signal peaked at 24 hpi, suggesting a rapid transcriptional response after retina injury. By 48 hpi, cxcl18b expression had already declined substantially, and by 72 hpi, the signal was further reduced to near-background levels. This temporal expression pattern explains why the ISH images at 48 hpi and 72 hpi appear nearly identical and much weaker compared to 24 hpi.

(2) The discrepancy between ISH and GFP reporter signal (Figure 2D):

The Tg(cxcl18b: GFP) reporter line shows persistent GFP expression beyond the transcriptional window of cxcl18b mRNA. This may be due to the prolonged stay of GFP protein, which remains detectable even after the endogenous transcription of cxcl18b has diminished. This explanation is also noted in the manuscript (revised lines 198–200). As a result, GFP⁺ MG cells are still visible at 48–72 hpi, and some of them co-label with PCNA.

These findings are consistent with our Pseudotime analysis based on scRNA-seq data (Figure 1H), which shows that cxcl18b expression precedes the induction of proliferative markers such as pcna and ascl1a.

(4) Line 198: The establishment of the Tg(cxcl18b:Cre-vmhc:mcherry::ef1a:loxP-dsRed-loxP-EGFP::lws2:nfsb-mCherry) is considerable but the nomenclature doesn't properly fit. Is the mCherry fused with Cre and driven by the cxcl18b promoter? What is the vmhc component? Finally, while this may provide the ability to clonally track cxcl18b-expressing MG, it does not address the prior question of what is the actual temporal expression of cxcl18b? If anything, this only addresses whether proliferating MG expressed cxcl18b at some point in their history, but does not indicate that cxcl18b expression co-exists in proliferating cells. The most convincing evidence is in Supplemental Figure 2B.

The "vmhc" component refers to the ventricular myosin heavy chain promoter, commonly used to label atrial cardiomyocytes (Jin et al., 2009). We cloned the vmhc upstream region containing its promoter and fusing with mCherry for selection during transgenic fish line construction.

Clone analysis using the Tg(cxcl18b: Cre-vmhc: mCherry::ef1a: loxP-DsRed-loxP-EGFP::lws2: nfsb-mCherry) further indicates that cxcl18b-defined the transitional state is the essential routing for MG proliferation. We have clarified in the revised text that this lineage tracing indicates a "history of injury-induced cxcl18b expression" rather than its ongoing expression during proliferation (revised line 205).

(5) Line 203: The data shown in Figure 2F do not indicate that these MG are cxcl18b+. Rather, the data are consistent with the interpretation that these MG expressed Cre at some prior stage and now express GFP from the ef1a promoter rather than DsRed. Whether these MG continue to express cxcl18b at the time these fish were collected is not addressed by these data. It is not accurate to conclude that these cells are cxcl18b+.

We thank the reviewer for pointing out this important issue. We agreed that the GFP⁺ MG shown in Figure 2F represents cells that have previously expressed cxcl18b and thus belong to the cxcl18b-expressing cell lineage, but this does not indicate that they continue to express cxcl18b at the time of sample collection. Performing clonal analysis using the Cre-loxp system, the GFP signal reflects historical cxcl18b promoter activity rather than ongoing transcription. We have revised the relevant sentence in our manuscript to clarify this point and now refer to these GFP⁺ cells as "cxcl18b lineage-traced MG" rather than "cxcl18b⁺ MG" to avoid any misinterpretation (revised line 207).

(6) Line 213: The statement that proliferative MG mostly originated from cxcl18b+ MG transitional states is a conclusion that does appear fully supported by the data. Whether those MG continue to express cxcl18b remains unanswered by the data in Figure 2 and would likely be inconsistent with the single-cell data in Figure 1.

We thank the reviewer for this valuable comment. We agree that the original statement on Line 213 regarding the lineage relationship between cxcl18b⁺ transitional MG and proliferative MG required clarification.

(1) The cxcl18b expression dynamics:

Our single-cell RNA-seq and ISH analyses consistently show that cxcl18b expression peaks as early as 24 hpi and declines rapidly, with significantly reduced expression by 48 and 72 hpi. These findings suggest that cxcl18b marks an early transitional MG state, rather than being maintained in proliferative MG. Indeed, in our scRNA-seq pseudotime trajectory analysis (Figure 1H), cxcl18b expression is highest in early transitional MG clusters (Clusters 1) and downregulated as cells progress toward proliferative states (Clusters 3/6), supporting a model in which cxcl18b is downregulated before cell-cycle re-entry.

(2) Prolonged stability of GFP protein:

The GFP signal observed in Tg(cxcl18b: GFP) retinas at 72 hpi may be because of the prolonged stability of GFP protein, rather than sustained cxcl18b transcription. The actual expression dynamics of cxcl18b are more directly reflected by our in situ hybridization and single-cell RNA-seq data, both showing a rapid decline after its early peak at 24 hpi. This explanation is also noted in the manuscript (revised lines 196–197).

(7) Line 246: The use of Dexamethasone to block inflammation is a widely used approach. However, dexamethasone is a broad-spectrum anti-inflammatory molecule that works through glucocorticoid signaling that may involve more than microglia. The observation that microglia recruitment and cxcl18a expression are both reduced is correlative but does not prove causation. Thus, the data are not sufficient to conclude that microglia-mediated inflammation is critical for activating cxcl18b expression. Indeed, data in Figure 1 indicate that cxcl18b expression occurs prior to microglia migration to the ONL.

We thank the reviewer for this thoughtful and important comment. We fully acknowledge that dexamethasone is a broad-spectrum anti-inflammatory agent that acts via glucocorticoid receptor signaling and may influence multiple immune and non-immune pathways beyond microglia.

In our study, dexamethasone treatment led to a reduction in both microglial recruitment and the number of cxcl18b⁺ MG at 72 hpi, suggesting a potential association between inflammation and cxcl18b activation. However, we agree that this observation remains correlative and is not sufficient to establish a direct link between microglia activity and cxcl18b induction. Our time-course analysis indicates that cxcl18b expression peaks at 24 hpi, preceding robust microglial accumulation in the ONL, further highlighting the need to clarify the temporal dynamics and cellular sources of inflammatory cues.

To address this question more conclusively, selective ablation of microglia during cone injury would be necessary. However, implementing such an approach would require a complex intersection of three transgenic lines—Tg(mpeg1: nfsB-mCherry) for microglia ablation, Tg(lws2: nfsB-mCherry) for cone ablation, and Tg(cxcl18b: GFP) for reporting—posing substantial genetic and experimental challenges.

We have revised the Results section accordingly to state: “These findings suggest that microglia-mediated inflammation may contribute to the activation of cxcl18b-defined transitional states that precede MG proliferation, although a causal relationship remains to be established.” (revised lines 251–253). We also added a new paragraph in the “Result: Clonal analysis reveals injury-induced MG proliferation via cxcl18b-defined transitional states associated with inflammation” as “While dexamethasone suppressed both microglial recruitment and cxcl18b⁺ MG generation, its broad anti-inflammatory action precludes definitive conclusions about microglial causality. Dissecting this relationship would require concurrent ablation of microglia and cone photoreceptors using a triple-transgenic strategy, which is beyond the scope of the current study. Targeted approaches will be necessary to resolve the specific role of microglia in initiating cxcl18b expression.” (revised lines 251–258) to explicitly acknowledge this limitation and the need for future studies using microglia-specific ablation models to resolve the mechanism.

(8) Could the authors clarify the basis of investigating NO signaling, given the relative expression of the genes by either cxcl18b+ MG or uninjured MG? Based on the expression illustrated in Supplemental Figure 4A, there is almost no expression of nos1 or nos2b in any MG. The authors are encouraged to revisit the earlier single-cell data sets to identify those cells that express components of NO signaling to determine the source(s) of NO that could be impacting the Muller glia.

We thank the reviewer for raising these important points.

Nitric oxide (NO) signaling has been implicated in the regeneration of multiple zebrafish tissues, including the heart (Rochon et al., 2020; Yu et al., 2024), spinal cord (Bradley et al., 2010), and fin (Matrone et al., 2021). Based on these findings, we hypothesized that NO signaling might also contribute to retinal regeneration.

As described in the manuscript, we compiled a redox-related gene list and systematically screened their roles in injury-induced MG proliferation using CRISPR-Cas9-mediated gene disruption. Among the candidates, disruption of *nos* genes significantly reduced the number of PCNA⁺ MG cells following G/R cone ablation (Figure 4), prompting us to further investigate the role of NO signaling.

(9) Line 319-320: this sentence appears to be missing text as "while no influenced across the *nos* mutants and *gsnor* mutants" does not make sense.

We appreciate the reviewer's observation and agree that the original sentence was unclear. We have revised the sentence in the manuscript as follows:

"In contrast, no significant change in MG proliferation was observed in *nos1*, *nos2a*, or *gsnor* mutants compared to wild type (Figures 4F–4I)" (revised lines 326-328).

(10) Line 326-328: The text should be rewritten as the current meaning would suggest there was no significant loss of photoreceptors in the *nos2b* mutants. That is incorrect. Rather, there was no significant difference between WT and the *nos2b* mutants in the number of photoreceptors lost at 72 hpi following MTZ treatment. Both groups lost photoreceptors, but the number lost in *nos2b* hets and homozygotes was the same as WT.

We agree with the suggestion and have revised our manuscript. We have revised the sentence in the manuscript as follows:

"We observed no significant difference in the loss of cone photoreceptor at 72 hpi between *nos2b* mutants and WT, indicating that the reduced MG proliferation observed in *nos2b* mutants is independent of the injury (WT: 45 ± 8 remaining cones, n = 24; *nos2b*^{+/−}: 49 ± 12, n = 20; *nos2b*^{−/−}: 46 ± 9, n = 20; mean ± SEM) (Figure 4K)." (revised lines 331-335).

(11) There is concern over the inconsistencies with some of the data. In Figure 4, Supplement 1A, the single-cell data found virtually no expression of *nos2b* in either uninjured MG or *cxcl18b*⁺ MG. In contrast, the authors find *nos2b* expression by RT-PCR in the *cxcl18b*:GFP⁺ MG. The *in situ* expression of *nos2b* in Figure 5 - Supplement 1 is not persuasive. The red puncta are seen in a single *cxcl18b*:GFP⁺ cell but also in the plexiform layer and is other non *cxcl18b*:GFP⁺ cells.

We appreciate the concern regarding the apparent inconsistencies in *nos2b* expression across different datasets. We provide the following explanations:

(1) Low expression of *nos2b* in scRNA-seq data:

We propose a potential explanation: Nitric oxide (NO) signaling is known to exert its biological functions in a dose-dependent manner and is tightly regulated post-transcriptionally, especially in inducible nitric oxide synthase (iNOS) (Bogdan, 2001; Nathan and Xie, 1994; Thomas et al., 2008). Thus, even modest changes in *nos2b* expression may exert meaningful biological effects without producing strong transcriptional signals detectable by scRNA-seq, which could fall below the detection threshold of scRNA-seq methods. Supporting this idea, our functional assay (Figure 4J) reveals a clear concentration-dependent effect of NO on MG proliferation, consistent with the biological relevance of *Nos2b* activity despite its low transcript abundance.

(2) Regarding the in situ hybridization data:

We used both commercially available in situ hybridization probes from (HCRTM) and RNAscopeTM (data not shown) to detect *nos2b* transcripts. While the *nos2b* signal was observed in other retinal cell types, including cells in the plexiform layer, our primary study was focused on examining its expression within the *cxcl18b*⁺ MG lineage.

(3) Regarding RT-PCR detection of *nos2b* in *cxcl18b*: GFP⁺ MG:

To enhance detection sensitivity, we enriched *cxcl18b*: GFP⁺ MG by FACS at 72 hpi and performed cDNA amplification before RT-PCR. This approach allowed the detection of low-abundance transcripts such as *nos2b*. It is also important to note that RT-PCR reflects fold changes in expression compared to MG to other retina cell type. The subtle but biologically upregulated of *nos2b* expression may not be readily captured by in situ hybridization or scRNA-seq.

(12) Line 356 - there is a disagreement over the interpretation of the current data. The statement that nos2b was specifically expressed in cxcl18b+ transitional MG states is not entirely accurate. This conclusion is based on expression of GFP from a cxcl18b promoter, which may reflect persistence of the GFP protein and not evidence of cxcl18b expression. Even assuming that the nos2b in situ hybridization and RT-PCR data are correct, the data would indicate that nos2b is expressed in proliferating MG that are derived from the cxcl18b+ transitional states. The single-cell trajectory analysis in Figure 2 indicates that cxcl18b is not co-expressed with PCNA. Furthermore, the single-cell data in Figure 4, Supplement 1, indicates no expression of nos2b in cxcl18b+ MG. The authors need to reconcile these seemingly contradictory pieces of data.

We thank the reviewer for this thoughtful and important comment. We agree that clarification is needed to accurately interpret the relationship between *cxcl18b*, *nos2b*, and MG proliferation, particularly considering the different temporal and technical contexts of our datasets.

(1) Lineage labeling and interpretation of GFP expression:

We acknowledge that in the Tg(*cxcl18b*: Cre-*vhmc*: *mcherry::ef1a*: *loxP-dsRed-loxP-EGFP::lws2*: *nfsb-mCherry*) line, GFP expression reflects historical activity of the *cxcl18b* promoter, rather than ongoing transcription. This GFP signal, due to its prolonged stay, may persist beyond the time window of endogenous *cxcl18b* expression. Accordingly, we have revised the manuscript to replace “*cxcl18b*⁺ MG” with “*cxcl18b*⁺ lineage-traced MG” throughout the relevant sections to prevent potential misinterpretation.

(2) Functional experiments support a lineage relationship between *cxcl18b*⁺ states and *nos2b* activity:

To further investigate the regulatory relationship between *cxcl18b* and *nos2b*, we conducted NO scavenging experiments using C-PTIO in the Tg(*cxcl18b*: GFP) background. We observed that the generation of *cxcl18b*: GFP⁺ MG after injury was not affected by NO depletion, indicating that *cxcl18b* activation precedes NO signaling (data not shown). However, PCNA⁺ MG was significantly reduced under the same treatment, suggesting that NO signaling is not required for *cxcl18b*⁺ transitional state formation, but is necessary for proliferation. Together with our MG-specific *nos2b* knockout data, these results support a model in which *nos2b*-derived NO acts downstream of the *cxcl18b*⁺ transitional state to promote MG cell-cycle re-entry.

(3) The scRNA-seq data with *nos2b* expression:

We agree with the reviewer that our scRNA-seq dataset shows minimal overlap between *cxcl18b* and *pcna* expression, which is consistent with our interpretation that *cxcl18b* expression marks a transitional phase before cell-cycle entry. Furthermore, *nos2b* transcripts were not robustly detected in *cxcl18b*⁺ MG clusters in our scRNA dataset. This discrepancy may be caused by technical limitations of scRNA-seq in capturing low-abundance or transient transcripts such as *nos2b*, as discussed in response to comment #11.

(13) The data in Figure 7 are interesting and suggest a link between NO signaling and notch activity. The use of the C-PTIO NO scavenger is not specific to MG, which limits the conclusions related to autocrine NO signaling in cxcl18b+ MG.

We acknowledge that the use of C-PTIO cannot distinguish between NO signaling within MG and paracrine effects from other retinal cells. Currently, technical limitations prevent MG-specific NO depletion. We have discussed this limitation accordingly in our revised “Limitations of this study” section (revised lines 540-545: “2. While our data suggest that injury-induced NO suppresses Notch signaling activation and promotes MG proliferation, the use of a general NO scavenger (C-PTIO) does not allow us to determine whether this regulation occurs in an autocrine or paracrine manner. The specific role of NO signaling within *cxcl18b*⁺ MG requires further validation using MG-specific NO depletion.”)

(14) Line 446-448. As mentioned before, the data do not support a causative link between microglia recruitment and cxcl18b induction. More specifically, dexamethasone is a broad-spectrum anti-inflammatory drug that blocks microglia activation and recruitment. Critically, the authors demonstrate that expression of cxcl18b occurs prior to microglia recruitment (see Figure 1, Supplement 1). Thus, the statement that cxcl18b induction depends on microglia recruitment is not accurate.

We thank the reviewer for reiterating this important point. We fully agree that the current data do not support a direct causal relationship between microglia recruitment and *cxcl18b* induction. As also addressed in our response to Comment 7, dexamethasone, as a broad-spectrum anti-inflammatory agent, cannot distinguish microglia-specific effects from those of other immune components. We have revised the text in revised lines 251–258 to clarify that microglia-mediated inflammation is associated with—but not required for—activation of *cxcl18b*-defined transitional MG states.

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- <https://doi.org/10.7554/eLife.106274.2.sa0>