Successful regeneration of the adult zebrafish retina is dependent on inflammatory signaling

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Abstract

Inflammation can lead to persistent and irreversible loss of retinal neurons and photoreceptors in mammalian vertebrates. In contrast, in the adult zebrafish brain, acute neural inflammation is both necessary and sufficient to stimulate regeneration of neurons. Here, we report on the critical, positive role of the immune system to support retina regeneration in adult zebrafish. After sterile, ablation of photoreceptors by phototoxicity, we find rapid response of tissue-resident microglia and neutrophils, which returns to homeostatic levels within 14 days post lesion. Pharmacological or genetic impairment of immune cell reactivity results in a reduced Müller glia stem cell response, seen as decreased reactive proliferation, and a strikingly reduced number of regenerated cells from them, including photoreceptors. Conversely, injection of the immune stimulators flagellin, zymosan, or M-CSF into the vitreous of the eye, in spite of the absence of a retinal lesion, leads to a robust proliferation response and the up-regulation of regeneration-associated marker genes in Müller glia. Our results suggest that neuroinflammation is a necessary and sufficient driver for retinal regeneration in the adult zebrafish retina.

Introduction

Immune system activation is one of the first responses to tissue damage, e.g. by infection, disease or injury. Cells of the immune system (leukocytes) can recognize invading pathogens or factors that are secreted by damaged or dying cells (Ferrero-Miliani et al., 2007). Subsequently, leukocytes accumulate at the affected area, removing pathogens and clearing cellular debris, thus supporting the reestablishment of a physiological balance (Nathan & Ding, 2010). Conversely, if inflammation cannot be resolved, a detrimental chronic inflammation can occur, causing progressive tissue damage and pathology (Zhou et al., 2016).

In the lesioned mammalian central nervous system (CNS), accumulation of reactive astrocytes often results in a glial scar that acts as a barrier for successful regeneration (Buffo et al., 2008; Fitch & Silver, 2008; Sofroniew, 2009). Similarly, neurodegenerative diseases like Parkinson's or Alzheimer's show characteristics of chronic inflammation, causing subsequent neuronal death (Amor et al., 2010; Herrero et al., 2015; Kinney et al., 2018).

In contrast to mammals, lesioning of the zebrafish CNS results in a strong regenerative response despite the initial manifestation of inflammation (Kroehne et al., 2011; Kyritsis et al., 2012; Kizil et al., 2015; Bosak et al., 2018; Mitchell et al., 2018; Tsarouchas et al., 2018; White et al., 2017; Silva et al., 2020; Zhang et al., 2020). Whereas immune system activation in the mammalian CNS is typically detrimental for regeneration, studies in zebrafish demonstrated a strong beneficial link between an immune response and neural stem cell reactivity (Kyritsis et al., 2012; Aurora & Olson, 2014; Bosak et al., 2018; Tsarouchas et al., 2018). Remarkably, in the zebrafish adult telencephalon, inflammation is required to initiate

61 a successful regenerative response, and a lipid inflammatory cue, leukotriene-C4, is
62 sufficient to stimulate proliferation of radial glia-type stem cells (Kyritsis et al., 2012;
63 Kizil et al., 2015).

64 In the retina, zebrafish Müller glial cells similarly act as stem cells, and generate
65 neuronal precursor cells (NPCs) in response to retinal lesion (Lenkowski & Raymond,
66 2014; Goldman, 2014; Gorsuch & Hyde, 2014). These cells then amplify, migrate to
67 the lesion site, and differentiate into the lost neuronal subtypes and thus gradually
68 restore vision (Raymond et al., 2006; Hammer et al., 2022). Dying neurons release
69 the proinflammatory cytokine TNF-α, triggering the regenerative response of Müller
70 glia (Conner et al., 2014; Nelson et al., 2013). Likewise, the inflammation-associated
71 factors Interleukin-11 and TGF-ß stimulate Müller glia cell cycle re-entry and NPC
72 generation (Lenkowski et al., 2013; Zhao et al., 2014). Furthermore, microglia – the
73 CNS tissue resident macrophages – support this initial regenerative response by
74 secreting proinflammatory factors (Kizil et al., 2015; Conedera et al., 2019; Iribarne &
75 Hyde, 2022; Zhang et al., 2020). However, to date the molecular pathways involved
76 in damage recognition, stem cell proliferation, neuronal precursor cell amplification
77 and differentiation during retinal regeneration are poorly understood; in particular, the
78 role of inflammation is unclear (Lahne et al., 2020; Lenkowski & Raymond, 2014;
79 Mitchell et al., 2018, 2019; Iribarne & Hyde, 2022).

80 Here, we analyze the contribution of inflammation to regeneration using a non-
81 invasive, sterile phototoxic ablation model of photoreceptor cells, as the key cell type
82 affected by retinal disease, in the adult zebrafish retina (Mitchell et al., 2018; Silva et
83 al., 2020; Weber et al., 2013; Zhang et al., 2020). Following light lesion, we observe
84 strong convergence of tissue resident microglia to the lesion site. Blocking
85 inflammation pharmacologically caused reduced reactive proliferation of Müller glia

86 stem cells and impaired photoreceptor regeneration. Similarly, in a genetic model of
87 microglia deficiency, we find that microglia are required to support Müller cell
88 proliferation. Conversely, when the immune stimulators flagellin, zymosan, or M-CSF
89 are injected into the vitreous of the eye, notably in the absence of retinal lesion,
90 Müller glial cells are triggered to undergo reactive proliferation and regeneration-
91 associated marker gene expression. Taken together, our results show that in the
92 regeneration-competent adult zebrafish retina, acute inflammation is an important
93 positive regulator of retina regeneration.

94

95 # Results

96 ## Leukocytes react to sterile ablation of photoreceptor cells

97 The immune system of vertebrates rapidly responds to retinal damage. In larval
98 zebrafish, *mpeg1*:mCherry positive monocytes react to photoreceptor ablation by
99 rapid migration towards the site of lesion (White et al., 2017). Similarly, microglia
100 respond by accumulation and phagocytosis of debris in response to neurotoxic
101 ablation of inner retinal cells in adult zebrafish (Mitchell et al., 2018). To investigate if
102 leukocytes are recruited in an injury model at adult stages, we used intense diffuse
103 light that causes sterile ablation of all photoreceptor subtypes by phototoxicity (Weber
104 et al., 2013). In this model, due to the refractive properties of the adult zebrafish
105 visual system, the photoreceptors in a central stripe of the retina are ablated,
106 whereas ventral and dorsal retina is much less affected and conveniently serves as
107 an internal control area (Weber et al., 2013; Fig. 1 D). We analyzed the accumulation
108 and appearance of leukocytes in the central retina on sections by
109 immunohistochemistry for L-Plastin, a pan-leukocyte marker (Redd et al., 2006;
110 Kroehne et al. 2011) in *Tg(mpeg1:mCherry)* reporter animals, labelling the monocyte
111 lineage, including macrophages and microglia (Figure 1A). In comparison to
112 unlesioned (sham) controls, L-Plastin positive cells accumulated in the central lesion
113 zone already at 2 day post lesion (dpl; Figure 1A). Whereas leukocytes showed a
114 ramified morphology in sham control retinae, their appearance changed to an
115 amoeboid and swollen shape at 2 dpl (insets in Figure 1A, D), as described by
116 Mitchell et al., 2018 upon neurotoxic lesion. At 2 dpl an increased number of L-
117 Plastin positive leukocytes at the outer nuclear layer (ONL) in comparison to sham
118 controls is revealed (Figure 1A, B). In flatmounts of *Tg(mpeg1:mCherry) x*
119 *Tg(opn1sw1:GFP)* double transgenic zebrafish that express GFP as a marker in the

120 entire UV cone population, displaying the central lesion by the absence of GFP, the
121 accumulation of monocytes can nicely be observed (Figure 1D). While in sham,
122 microglia display an equal distribution and a ramified structure they accumulate in the
123 central lesion area. Interestingly they disappear from the unlesioned peripheral
124 regions. In a time course, the number of L-Plastin positive cells peaked at 2 dpl and
125 subsequently declined to sham levels at 14 dpl (Figure 1B). To further determine the
126 identity of retinal leukocytes, we analyzed immunoreactivity of L-Plastin in transgenic
127 *Tg(mpeg1:mCherry)* animals.In sham control retinae, *mpeg1*:mCherry positive cells
128 always co-expressed L-Plastin, indicating that all tissue resident homeostatic
129 leukocytes were of the monocyte lineage, namely microglia (Figure 1A, S1A). In
130 contrast, additional L-Plastin positive, but *mpeg1*:mCherry negative cells could be
131 detected upon injury at 2 dpl (insets in Figure 1A). Subsequent quantification of the
132 number of L-Plastin and *mpeg1*:mCherry double positive cells revealed a decrease
133 from 99% to approximately 83%, indicating the presence of another cell type of the
134 leukocyte lineage at the lesion site (Figure S1A). Neutrophils are known to rapidly
135 respond to tissue damage and are labeled by transgenic *Tg(mpo:GFP)* (Renshaw et
136 al., 2016; Kurimoto et al., 2013; Figure 1D, S1B). In contrast to sham controls, which
137 never showed any *mpo*:GFP positive cells in the homeostatic retina, we observed
138 rapid accumulation of neutrophils after light lesion. *mpo*:GFP positive cells first
139 appeared in the retina by 12 hpl, and a diffuse GFP positive pattern (referred to as
140 matrix), in the outer segment layer and the inner nuclear layer (INL) was present from
141 15 hpl onwards (Figure 1C), presumably reflecting neutrophil NETosis (Zhu et al.,
142 2021). Analyses of *Tg(mpeg1:mCherry)* and *Tg(mpo:GFP)* double transgenic animals
143 revealed that *mpeg1*:mCherry positive cells co-localized with the GFP positive matrix,
144 suggesting an uptake of the matrix material by monocytes (Figure S1B). T cells of the
145 lymphoid lineage were reported to stimulate Müller glia proliferation, and to augment

146 retina regeneration in a stab wound assay (Hui et al., 2017). To examine if T cells are
147 also recruited to the lesion site, we used transgenic *Tg(lck:NLS-DsRed)* animals, but
148 could not detect any *lck* positive T cells, neither in the homeostatic nor in the
149 regenerating retina up to 7 days post lesion (Figure S2).

150 Taken together, our data show a strong accumulation and activation of innate
151 immune cells following a sterile light lesion that is resolved by 14 dpl, consistent with
152 the mounting of an acute inflammatory response after retinal injury.

153

154 **Müller glia activate NF-κB signaling in response to injury**

155 In zebrafish, Müller glia have key functions in the regulation of retinal homeostasis as
156 well as during regeneration (Lenkowski & Raymond, 2014). To study how Müller glia
157 react to injury during regeneration and inflammation, we analyzed the activation of
158 the proinflammatory signaling pathway NF-κB using *Tg(NF-κB:GFP)* and
159 *Tg(gfap:NLS-mCherry)* transgenic animals. The *Tg(NF-κB:GFP)* line reports
160 canonical NF-κB activity via the expression of GFP under the control of six human
161 NF-κB binding motifs driving a c-fos minimal promoter (Kanther et al., 2011). In sham
162 control retinae, *NF-κB*:GFP expression was only found in the retinal vasculature,
163 microglia and, occasionally in Müller glia residing in the inner nuclear layer (Figure
164 2A). In sharp contrast, robust activation of *NF-κB*:GFP was present in numerous
165 *gfap*:NLS-mCherry positive Müller glia already at 1 dpl (Figure 2A). Moreover, *NF-*
166 *κB*:GFP expression was present in cells located in the ONL showing the
167 characteristic morphology of photoreceptor cells. Both cell types continued to express
168 *NF-κB*:GFP at 2 dpl. At 4 dpl, *NF-κB*:GFP expression was absent from the ONL but
169 remained detectable in Müller glia. To determine if NF-κB activation might be

170 functionally relevant, we investigated the expression of *matrix metallopeptidase 9*
171 (*mmp9*), a known downstream target of NF-κB with important functions in degrading
172 extracellular matrix and chemokines (Cheng et al., 2012; LeBert et al., 2015; Xu et
173 al., 2018; Yang et al., 2017). Thus, we performed *mmp9 in situ* hybridization in
174 combination with immunohistochemistry against glial fibrillary acidic protein
175 (GFAP/Zrf-1) labeling Müller glia and proliferating cell nuclear antigen (PCNA)
176 labeling cells in S-phase and shortly after. In contrast to sham controls that never
177 showed *mmp9* expression in the homeostatic retina, *mmp9* was strongly expressed
178 by Zrf-1 positive Müller glia at 1 dpl, indicating *de novo mmp9* expression in these
179 cells (Figure 2B). Moreover, numerous *mmp9* and Zrf-1 expressing cells were also
180 positive for PCNA, identifying them as reactively proliferating Müller glia in response
181 to injury. Expression of *mmp9* remained strong at 2 dpl, but dropped below detection
182 levels at 4 dpl, consistent with transcriptome data for *mmp9* (Kramer et al., 2021;
183 Silva 2020; Celotto et al., 2023). Taken together, our results showed that the NF-κB
184 signaling pathway is transiently activated in Müller glia in response to a sterile
185 ablation of photoreceptor cells, indicative of an inflammatory response by Müller glia.

186

187 **Dexamethasone-mediated immunosuppression reduces retinal regeneration**

188 To study the role of the immune system during retinal regeneration, we used
189 Dexamethasone (Dex), a potent immunosuppressant (Coutinho & Chapman, 2011;
190 Donika Gallina et al., 2015; Kyritsis et al., 2012; Silva et al., 2020; Zhang et al.,
191 2020). Experimental zebrafish were treated with Dex from 10 days prior to lesion until
192 the time point of analysis (Figure 3A). Vehicle control experiments were carried out
193 with the respective amount of the solvent methanol (MeOH). In contrast to MeOH-
194 treated controls, Dex-treatment for 10 consecutive days resulted in an overall

195 reduction of retinal microglia (Figure 3B). Similarly, leukocyte recruitment upon light
196 lesion was significantly reduced in Dex-treated animals at 2 dpl, compared to control
197 (Figure 3B). Quantification of L-Plastin positive cells corroborated a significant
198 reduction at all indicated time points (Figure 3C). In addition, Dex-treatment reduced
199 the number of resident retinal microglia during homeostasis, and no increase in
200 leukocyte number was noted after lesion. To address if the observed changes might
201 be caused indirectly by the known neuroprotective properties of Dex (Gallina et al.,
202 2015), we analyzed cell death using the TdT-mediated dUTP-digoxigenin nick end
203 labeling (TUNEL) assay; however, we did not observe any difference in the number
204 of TUNEL positive cells after light lesion in control MeOH- or Dex-treated animals
205 (Figure S3).

206 Reactive proliferation of Müller glia is a hallmark of retinal regeneration; we therefore
207 further analyzed if Dex-treatment influences proliferation after sterile light lesion using
208 immunolabeling of the proliferation marker PCNA (Figure 3D). In contrast to MeOH-
209 treated controls, Dex-treated animals showed a significant reduction in PCNA
210 positive cells at 4 dpl. Quantification of PCNA positive cells during the course of
211 regeneration revealed that reactive proliferation, driven by Müller cells and neuronal
212 progenitors, is significantly reduced in Dex-treated animals at all time-points
213 examined in comparison to MeOH controls. Furthermore, homeostatic proliferation
214 (most likely by cells of the ciliary margin) in unlesioned retinae is not affected (Figure
215 3E). To investigate if Dex-treatment specifically affects Müller glia proliferation, we
216 analyzed the number of PCNA positive cells in transgenic *Tg(gfap:NLS-GFP)*
217 animals, which express strong nuclear GFP in all Müller glia (with some leakage of
218 GFP to the cytoplasm, identifying the characteristic Müller glia cell shape, Figure 3F).
219 Indeed, in comparison to MeOH-treated animals, the number of PCNA and *gfap*:NLS-

220 GFP double positive cells was significantly reduced after Dex-treatment.
221 Quantification showed that the percentage of Müller glia co-localizing with PCNA is
222 reduced to less than 50% at all analyzed time points (Figure 3G). This suggests that
223 Müller glia reactivity is impaired upon Dex-treatment. Consistent with this possibility,
224 NF-κB activation is reduced in Müller glia after Dex-treatment, as seen in the *Tg(NF-*
225 *κB:GFP)* activation reporter line (Figure 3H). As expected, cells in the ONL and
226 Müller glia show a robust *NF-κB*:GFP expression in MeOH-treated control animals at
227 2 dpl, whereas a reduced number of cells in the ONL activated the *NF-κB*:GFP
228 transgene and no GFP positive Müller glia could be detected at 2 dpl after Dex-
229 treatment. Consistently, activation of the NF-κB downstream target *mmp9* was almost
230 absent in Dex-treated animals at 2 dpl, compared to MeOH controls (Figure 3I).
231 Taken together, these results show that Dex-mediated immunosuppression efficiently
232 reduces the accumulation of leukocytes and impairs Müller glia reactivity, as well as
233 reactive proliferation and regeneration-associated marker gene expression, during
234 retinal regeneration.

235

236 **Immunosuppression reduces regeneration of photoreceptors**

237 To examine if the reduced cell proliferation has consequences for the regenerative
238 outcome, we probed the effect of Dex-immunosuppression on restoration of
239 photoreceptors after light lesion. Thus, we performed Dex-treatment and light lesions
240 on *Tg(opn1sw1:GFP)* animals expressing GFP in all UV cones, followed by repeated
241 EdU injections to label newborn cells, and analyzed the animals at 28 dpl when
242 cellular regeneration is completed (Figure 4A). Consistent with the afore-mentioned
243 decrease of retinal leukocytes after Dex-treatment, we observed an almost complete
244 loss of retinal leukocytes after 28 days of continuous immune suppression (Figure

245 S4). In comparison to MeOH-treated control animals, the overall number of EdU
246 positive cells was significantly lower after Dex-treatment at 28 dpl (Figure 4B).
247 Similarly, the number of EdU positive UV-cones (Figure 4C) was severely decreased
248 in Dex-treated animals compared to MeOH-treated controls. Moreover, in addition to
249 a reduction in number, EdU positive UV cones appeared malformed with improperly
250 shaped outer segments in Dex-treated animals (insets Figure 4B). Quantifications
251 showed that the number of EdU positive nuclei was similarly reduced in all retinal
252 layers (Figure 4C). In conclusion, Dex-mediated immunosuppression interferes with
253 reactive proliferation and reactive neurogenesis, indicating that the injury-induced
254 immune response is essential for proper retina regeneration.

255

256 **Retinal microglia support reactive Müller glia proliferation**

257 Activated microglia and macrophages clear debris from dead cells, and they interact
258 with Müller glia and influence their cellular response (Aslanidis et al., 2015; Keightley
259 et al., 2014; Palazzo et al., 2020; Wang et al., 2011). Consistently, our results show
260 that Dex-mediated immunosuppression interferes with reactive proliferation and
261 neurogenesis, indicating that the injury-induced immune response is essential for
262 successful retina regeneration. To independently test this notion, we investigated
263 whether microglia positively contribute to reactive proliferation during retina
264 regeneration, using Interferon regulatory factor 8 (*irf8*) myeloid-defective mutants to
265 genetically deplete microglia in embryonic and juvenile fish (Shiau et al., 2015). To
266 verify leukocyte deficiency at adult stages, we analyzed L-Plastin immunoreactivity in
267 homozygous adult *irf8* mutants and heterozygous control siblings, and found that the
268 number of leukocytes in the homeostatic retina was significantly reduced in *irf8*
269 mutants (Figure 5A, B). Next, we asked whether the microglia deficiency also affects

270 the proliferation of Müller cells and NPCs during regeneration. We therefore
271 combined the light lesion paradigm with EdU-labeling at 3 dpl prior to analysis at 4
272 dpl (Figure 5C). Compared to heterozygous control siblings, the number of
273 accumulated L-Plastin positive cells was clearly reduced in *irf8* mutant animals at 4
274 dpl (Figure 5D). Similarly, the number of EdU positive cells is lower in *irf8* mutants at
275 4 dpl compared to heterozygous controls (Figure 5D). Quantification of both the
276 number of L-Plastin positive cells as well as the number of EdU positive cells
277 confirmed a statistically significant reduction (Figure 5E). Thus, consistent with our
278 above findings using Dex-mediated immunosuppression, these results show that
279 genetic depletion of leukocytes correlates with decreased reactive proliferation upon
280 injury, supporting the notion of an important role of immune cells during zebrafish
281 retina regeneration.

282

283 **Inflammatory stimuli trigger a regeneration response in Müller glia in the**
284 **absence of lesion**

285 We hypothesized that inflammation might not only be necessary, but also sufficient,
286 to trigger reactive proliferation of Müller glia and the generation of NPCs. To address
287 this notion, we injected different immune activators into the vitreous of the eye - thus
288 leaving the retina unlesioned (Fig 6A) - and analyzed the response of Müller glia at 2
289 and 4 days post injections (dpi; Figure 6). The toll-like receptor (TLR) agonist
290 zymosan has previously been shown to stimulate an inflammatory response both in
291 the zebrafish and mouse eye, and in the zebrafish telencephalon (Kyritsis et al.,
292 2012; Kurimoto et al., 2013; Zhang et al., 2020). Next, we tested injections of
293 flagellin, the principal structural protein of bacterial flagella, which is known to cause
294 sepsis in zebrafish larvae (Barber et al., 2016). As a control, to ensure that injection

295 of both zymosan and flagellin did not cause retinal cell death and thereby trigger a
296 regenerative response indirectly, we performed TUNEL assays at 2 dpi. Neither
297 zymosan nor flagellin injection resulted in a significant increase of TUNEL positive
298 cells in comparison to control PBS-injected eyes (Figure 6B). Interestingly, the
299 number of L-Plastin positive cells also did not significantly increase after injecting
300 zymosan or flagellin (Figure 6C). In contrast, cell proliferation using PCNA
301 immunohistochemistry is clearly induced in both zymosan as well as flagellin-injected
302 animals in comparison to PBS-injected controls (Figure 6D). Quantification of PCNA
303 positive nuclei confirmed a significant increase in zymosan and flagellin-injected
304 animals at 4 dpi, but not at 2 dpi (Figure 6E). Furthermore, we analyzed activity of
305 NF-κB after injections of PBS, zymosan and flagellin using the *NF-κB*:GFP reporter
306 line. Consistent with the afore-mentioned expression in non-injected retinae, PBS-
307 injected controls show *NF-κB*:GFP expression in blood vessels, microglia and few
308 Müller glia residing in the INL (Figure 6F). In contrast, *NF-κB*:GFP is strongly
309 activated in additional cells of the inner and outer nuclear layer in zymosan and
310 flagellin-injected animals at 2 dpi. Again, GFP positive cells in the ONL showed
311 mostly the characteristic morphology of photoreceptor cells (see also above, Fig. 2B
312 & 3H), in particular in the zymosan-injected animals. Consistent with the activation of
313 the *NF-κB*:GFP reporter, retinal expression of *mmp9* is also found in zymosan and
314 flagellin-injected animals, but not in PBS-injected controls at 2 dpi (Figure 6G).
315 Finally, we analyzed the expression of the transcription factor *her4.1*, a downstream
316 target of the Notch signaling pathway, which is upregulated in NPCs generated in
317 response to photoreceptor damage (Wan et al., 2012). No expression of *her4.1* is
318 detected in PBS-injected controls, but *her4.1* expression is activated in zymosan and
319 flagellin-injected samples at 4 dpi (Figure 6H). These results show that activators of

320 the immune system can strongly stimulate Müller glia reactivity and proliferation,
321 even in the absence of tissue damage.

322

323 **M-CSF injection triggers leukocyte accumulation and Müller glia reactivity in**
324 **the absence of a lesion**

325 In order to identify the stimulatory potential of specific individual inflammatory
326 mediators, we focused on macrophage colony-stimulating-factor (M-CSF/CSF-1),
327 based on preliminary data from our recent single-cell RNAseq dataset of
328 regenerating retina (Celotto et al., 2023), and using the same injection paradigm as
329 before (Figure 7A). In mammals, M-CSF stimulate CSF-1 receptor signaling, which is
330 involved in monocyte colonization and stimulation (Chitu et al., 2016; Wu et al.,
331 2018). Injection of human M-CSF induced an increased cell proliferation at 4 dpi
332 compared to PBS controls (Figure 7B, C), despite the absence of unspecific damage
333 to the retina, as seen by lack of an increase in TUNEL positive cells at 2 dpi.
334 Quantification corroborated a significant increase of proliferating cells, scored as
335 PCNA positive nuclei, in M-CSF-injected retinae, compared to PBS-injected controls
336 (Figure 7D). To address if M-CSF causes an increase in the number of retinal
337 leukocytes, we performed immunolabeling of L-Plastin in M-CSF- and PBS-injected
338 specimens. We observed a significant accumulation of L-Plastin positive cells in M-
339 CSF-, but not in PBS-injected, animals at 4 dpi (Figure 7E & F). Further, we found
340 augmented NF-κB activity after M-CSF injection in *NF-κB*:GFP reporter animals.
341 PBS-injected controls showed the homeostatic *NF-κB*:GFP expression described
342 above (Figure 7G). In sharp contrast, additional *NF-κB*:GFP positive cells were found
343 in the M-CSF-injected animals at 2 dpi, located in the inner nuclear layer and
344 showing the characteristic morphology of Müller glia. Photoreceptor cells in the ONL

345 did not activate the *NF-κB*:GFP reporter following M-CSF injection whereas they do
346 so upon lesion (see above, Fig. 2B & 3H), which we tentatively suggest to reflect a
347 more selective role of M-CSF signaling for Müller glia. Consistent with the activation
348 of *NF-κB*:GFP, *mmp9* was also found to be expressed in M-CSF injected animals,
349 but not in PBS-injected controls at 2 dpi (Figure 7H). Taken together, these data
350 demonstrate that human recombinant M-CSF can induce leukocyte accumulation in
351 the neuronal retina of zebrafish, and can stimulate Müller glia reactive proliferation
352 and alter gene expression even in the absence of a lesion.

353

354

355 **Discussion**

356 In contrast to mammals, the zebrafish retina readily regenerates photoreceptor cells
357 that are lost following a phototoxic lesion. Several signaling pathways have been
358 implicated in the restoration of lost neurons (Lahne et al., 2020; Lenkowski &
359 Raymond, 2014; Goldman, 2014; Hochmann et al., 2012). A role for the immune
360 system in regeneration of the adult zebrafish retina has previously been suggested
361 (Mitchell et al., 2018; Sifuentes et al., 2016; Silva et al., 2020; White et al., 2017;
362 Zhang et al., 2020; Iribarne et al., 2022), but it is not understood in detail. Here, we
363 confirm and extend these previous studies on the role of inflammation, and its impact
364 on Müller glia reactivity, by modulating immune activity during regeneration of the
365 adult zebrafish retina after a sterile phototoxic ablation of photoreceptor cells. We find
366 that microglia and neutrophils infiltrate and accumulate at damaged sites of the
367 retina. Importantly, Müller glia themselves react by activating the proinflammatory
368 NF-κB signaling pathway in response to retinal damage. Our functional studies show
369 that Dexamethasone-mediated immunosuppression reduces (i) leukocyte
370 accumulation, and (ii) the reactive Müller glia response at the proliferative and gene
371 expression level, and thus reduces the regeneration of photoreceptors. (iii)
372 Conversely, in gain-of-function assays, injection of flagellin, zymosan or M-CSF as
373 inflammatory factors that are thought to facilitate the immune system, some Müller
374 glia are stimulated and reactive proliferation is induced (Gorsuch & Hyde, 2014;
375 Lenkowski et al., 2013, Nelson et al., 2013; Figure 8). Overall, we suggest that cells
376 of the innate immune system act as necessary and sufficient positive regulators for
377 successful regeneration of the adult zebrafish retina. Regulators of the inflammatory
378 state, such as Dexamethasone and the M-CSF that stimulates Müller glia-based
379 photoreceptor regeneration in our injection assays, might therefore provide advanced

380 options for clinical treatments of retinal disease, as recently also suggested for
381 corticosteroid management of early phases of spinal cord injury (Nelson et al.,
382 2019).

383

384

385 **Sterile ablation of photoreceptors recruits innate immune cells**

386 Consistent with previous studies, we find that retinal *mpeg1*:mCherry positive tissue-
387 resident microglia display a ramified morphology in homeostasis, but change to an
388 activated amoeboid cell shape upon a sterile light lesion, similar to what is observed
389 after CNS lesion (Karlstetter et al., 2010; Kroehne et al., 2011; Kyritsis et al., 2012;
390 Mitchell et al., 2018). Along with activation, we find a strong accumulation of microglia
391 at the lesion site, which is resolved over a period of 14 days. The underlying dynamic
392 events indicate an acute inflammatory response of leukocytes that is tightly controlled
393 in a spatiotemporal manner. We assume that it is mostly tissue-resident microglia
394 that mediate the observed immune response; however, we cannot exclude that
395 additional peripheral macrophages are also recruited from the bloodstream and
396 contribute to the observed reaction, in particular since we also observe neutrophils
397 entering the retina. Consistent with our assumption, live imaging in larval zebrafish
398 revealed that peripheral macrophages do not enter the developing retina during drug-
399 mediated ablation of rod photoreceptors (Oosterhof et al., 2017; White et al., 2017).
400 In contrast, neurotoxic ablation of the ganglion cells and neurons of the inner nuclear
401 layer using ouabain triggers proliferation of adult retinal microglia, and it has been
402 suggested that the observed increase in cell number might also involve peripheral
403 macrophages entering the retina from circulation (Mitchell et al., 2018). The

404 observation of *mpo*:GFP positive neutrophils in the vasculature, as well as neutrophil
405 accumulation in the photoreceptor outer segment layer upon light lesion, indicates a
406 certain permeabilization of the blood-retina-barrier, which may also allow additional
407 leukocytes to invade the damaged retina (Eshaq et al., 2017; McMenamin et al.,
408 2019; Sim et al., 2015). Further studies of the cell-permeability of the blood-retina-
409 barrier during regeneration, e.g. by *in vivo* imaging approaches, and specific labeling
410 of either peripheral macrophage or microglia populations, will likely be interesting in
411 this regard.

412 Our observation that neutrophils arrive in the retina following non-invasive light lesion
413 suggests an interesting aspect of retina regeneration, with parallels to observations
414 after neurotoxic ablation of retinal ganglion cells (Mitchell et al., 2018). Neutrophils
415 have different functions at the site of injury, and they are among the first cells to react
416 to injury. It has been proposed that they participate in clearing cellular debris, by
417 secretion of factors influencing angiogenesis and regeneration, but they may also
418 support resolution of inflammation (Wang, 2018). In mice, neutrophils promote the
419 regeneration of the optic nerve by expression of oncomodulin, and can influence the
420 response of glial cells and axonal outgrowth after spinal cord injury (Kurimoto et al.,
421 2013; Stirling et al., 2009; Tsarouchas et al., 2018). Furthermore, neutrophils are
422 indicated to resolve inflammation by a localized O_2 depletion (Campbell et al., 2014).
423 Thus, neutrophils could be involved in the mediation of the immune response in adult
424 retina regeneration in zebrafish upon lesion. Further investigation of the observed
425 GFP positive matrices may therefore be interesting. Here, we speculate that these
426 structures might act as 'extracellular-traps' that could prevent cellular debris from
427 spreading further into the surrounding tissues, protecting the latter from secondary
428 cell death (Branzk & Papayannopoulos, 2013; Walker et al., 2007), which might help

429 suppress a systemic inflammatory/regenerative reaction in the retina. The
430 observation of GFP positive inclusions in mpeg1 positive macrophages furthermore
431 suggests degradation of the trap-like structures by macrophage uptake (Farrera &
432 Fadeel, 2013).

433 While microglia and neutrophils seem to play important roles in retina regeneration,
434 we did not observe any infiltration of *lck*:NLS-dsRed positive T cells into the retina
435 after lesion, arguing that T cells may be dispensable at least for the initial
436 regeneration response. A recent report suggests that T cells can positively influence
437 the regenerative response after an invasive mechanical lesion of the retina (Hui et al.,
438 2017); this difference might be due to differences in the lesion paradigm employed,
439 and/or timing of the regeneration events. As mentioned above, the non-invasive light
440 lesion that we employed results in a specific ablation of photoreceptors without
441 physically disrupting the blood-retina barrier. In contrast, the needle stab employed
442 by Hui et al., 2017 disrupts the blood-retina barrier, thereby allowing T cells to access
443 the lesion.

444 **Müller glia react to photoreceptor cell death in an inflammatory manner**

445 Upon retinal injury and progressive dystrophies, mammalian Müller glia undergo
446 physiological changes and show transcriptional alterations as a hallmark of their
447 gliotic response (Bringmann et al., 2009; Bringmann & Wiedemann, 2011). Despite
448 the regenerative response of zebrafish Müller glia, they exhibit initial signs of reactive
449 gliosis following retinal damage (Thomas et al., 2016). Here, we report that ablation
450 of photoreceptors induces an acute inflammatory response at the molecular level.
451 Importantly, we observed an inflammatory-like response directly in the lesion-
452 responding Müller glia. Using *NF-κB*:GFP reporter fish (Kanther et al., 2011), we find

453 that canonical NF-κB becomes activated in Müller glia upon damage, in keeping with
454 transcriptomic changes of Müller glia upon light lesion (Sifuentes et al., 2016; Celotto
455 et al., 2023). In the regenerating chick retina, NF-κB is active in Müller glia and acts
456 as an important pathway controlled by factors that are secreted by microglia (Palazzo
457 et al., 2020). A potential activator of NF-κB may be TNFα; indeed, several studies
458 suggested that dying neurons secrete this factor and thus trigger Müller glia
459 proliferation and the generation of NPCs (Kumar & Shamsuddin, 2012; Nelson et al.,
460 2013).

461 Consistent with previous reports, we also found the NF-κB target gene, matrix
462 metallopeptidase *mmp9,* to be expressed in reactive Müller glia, displaying a highly
463 dynamic and strictly spatiotemporally controlled activation profile (Kaur et al., 2018;
464 Kim et al., 2007; Silva et al., 2020; Takada et al., 2004). In *mmp9* knockout zebrafish,
465 Müller glia-derived progenitors show a hyperproliferative response after injury, but
466 reduced survival of regenerated photoreceptors (Silva et al., 2020). The exact
467 function of *mmp9* is not completely clear yet; we speculate that *mmp9,* as a
468 metalloproteinase, might be involved in the degradation of extracellular matrix (ECM),
469 thus 'opening' the retina for incoming immune or neuronal precursor cells, and/or
470 modulating ECM interacting cytokines (Gong et al., 2008; LeBert et al., 2015; Xu et
471 al., 2018; Yoong et al., 2007). Interestingly *mmp9* is a key marker gene known to be
472 differentially expressed between wet and dry forms of age-related macular
473 degeneration, providing further evidence for a possibly critical role during
474 inflammatory processes (Fritsche et al., 2016). Understanding the contribution and
475 molecular control of *mmp9* in zebrafish retina regeneration may therefore provide
476 further insights into the mechanisms and molecules that are required to restore lost
477 neural tissues.

478 **Immunosuppression alters the regenerative response of the zebrafish retina**

479 Immunosuppression with the glucocorticoid dexamethasone drastically decreases the
480 amount of retinal microglia and blocks accumulation of reactive leukocytes in the
481 retina after lesion (Gallina et al., 2014; White et al., 2017). In addition, activation of
482 NF-κB signaling was also successfully blocked in Müller glia, as shown by the loss of
483 *NF-κB*:GFP and expression of its target gene. NF-κB activation has been described
484 in a plethora of cellular functions such as cell survival, cell death and mediation of
485 inflammation by the regulation of cytokine expression, as well as cell proliferation and
486 migration (Liu et al., 2017). In Müller glia, NF-κB signaling could mediate an
487 inflammatory-like response, consistent with their expression of pro- and anti-
488 inflammatory cytokines (Nelson et al., 2013; Polager & Ginsberg, 2002; Zhao et al.,
489 2014). Consistently, following Dex-treatment, we find that the reactive proliferation of
490 Müller glia was strongly reduced, indicating that inflammatory-like signaling promotes
491 Müller glia cell cycle reentry. Interestingly, homeostatic proliferation, which mainly
492 occurs in the ciliary marginal zone and only sporadically in the central retina, remains
493 unaffected, similar to our findings in the zebrafish telencephalon (Kyritsis et al.,
494 2012). Moreover, Dex-treatment is not only affecting reactive proliferation, as is also
495 confirmed by other research groups (Iribarne & Hyde, 2022; Silva et al., 2020; White
496 et al., 2017). Regenerated UV-cones show dysmorphic outer segments, indicating
497 that Dex-treatment also impairs the proper differentiation of photoreceptor cells. On
498 the other hand, in mmp9 mutants, initial Dex-treatment supports the restoration of the
499 photoreceptors and thus can act positively (Silva et al., 2020). Collectively, we
500 propose that inflammatory signaling is required for cell cycle reentry of Müller glia
501 during regeneration, but might also play additional roles during maturation of
502 regenerated photoreceptors (Figure 8). An incomplete regenerative outcome was

503 also detected in larval retinal pigment epithelium (RPE) regeneration in *irf8*-mutants
504 deficient in microglia. Upon ablation of RPE, the neural retina showed an increased
505 reactive proliferation in mutants, but the amount of regenerated RPE cells is lower
506 than in wild type siblings (Hanovice et al., 2019).

507 To further understand the impact of resident microglia on the regeneration process in
508 adult retina, we addressed the proliferative potential of retinae in response to injury in
509 *irf8* mutant fish, as a genetic model for myeloid deficiency in larval and juvenile
510 zebrafish (Shiau et al., 2015). We confirm microglia deficiency at adult stages (>6
511 month), indicating that *irf8* is required throughout lifetime in primitive as well as a
512 definitive wave of monocyte populations of the eye (Ferrero et al., 2018; Shiau et al.,
513 2015). However, a reduced number of leukocytes is still found to populate the adult
514 retina. Upon lesion, we observed that the number of cells that are in cell cycle is
515 significantly reduced in *irf8* mutants in comparison to heterozygous siblings in
516 response to lesion. Interestingly, the number of L-Plastin+ leukocytes in *irf8* mutants
517 increases upon injury, but never reaches levels comparable to heterozygous control
518 siblings. In line with other reports, these findings indicate that microglia are
519 supportive for Müller cell proliferation and NPC amplification and thereby support
520 initial steps of regeneration (Conedera et al., 2019; Zhang et al., 2020; Iribarne &
521 Hyde, 2022). The necessity of (tissue-resident) macrophages is also observed in
522 other tissues, e.g. telencephalon, spinal cord, caudal fin and heart, showing the
523 contribution of the innate immune system to tissue regeneration (Kyritsis et al., 2012;
524 De Preux Charles et al., 2016; Petrie et al., 2015; Portillo et al., 2017; Roche et al.,
525 2018; Tsarouchas et al., 2018). This supportive role of macrophages in zebrafish is
526 unlike that of the rodent retina, where microglia appear to inhibit Müller-glia-mediated
527 tissue regeneration by suppression of *ascl1* expression. This interesting species

528 difference might be explained by distinct expression profiles of zebrafish vs. mouse
529 microglia during the time course of regeneration (Issaka Salia & Mitchell, 2020;
530 Mitchell et al., 2019; Todd et al., 2020).

531 **Immune stimulation triggers Müller glia reactivity and proliferation**

532 Retinal pathology is often coupled to neuronal loss, activation of the immune system
533 and Müller glia reactivity (Iribarne & Hyde, 2022). In particular, mammalian Müller glia
534 react to injury mostly by (proliferative) gliosis, which contributes to glial scarring, thus
535 enhancing loss of vision (Bringmann et al., 2006). In zebrafish, retinal injury also
536 results in an acute immune reaction; however, in contrast to mammals, this is
537 followed by a regenerative response.

538 Importantly, inflammatory signals can indeed initiate reactive proliferation, even in the
539 absence of injury, as seen by our vitreous injections of toll-like receptor (TLR)
540 agonists zymosan and flagellin to trigger an inflammatory response (Akira & Takeda,
541 2004; Barber et al., 2016; Zhang et al., 2020). Leukocyte number was not increased
542 following these injections, which might be explained by three possible scenarios: i)
543 Zymosan and flagellin do not stimulate leukocyte proliferation ii) recruitment from the
544 bloodstream is not induced; while in both scenarios a local leukocyte activation can
545 occur. iii) The accumulation could already be resolved within 48h, since the response
546 of immune cells is rapid and highly dynamic. Furthermore, the injected concentration
547 of zymosan in a rodent model, where leukocytes are seen to accumulate, is ten-fold
548 higher than the concentration used in our injections, and could reflect a dose
549 dependency (Kurimoto et al., 2013). In contrast to the more generic inflammatory
550 stimuli, M-CSF-injected retinae show an increased number of leukocytes. This may
551 be the consequence of both proliferation and recruitment from the bloodstream, since

552 M-CSF receptor (CSF-1R) is thought to regulate the population of microglia in neural
553 tissue during development (Wu et al., 2018). Following M-CSF injection, we find that
554 Müller glia activate NF-κB signaling, which fits well with studies reporting TLR and
555 CSF-1 receptor expression in Müller glia and microglia (Bsibsi et al., 2002; Kumar &
556 Shamsuddin, 2012; Olson & Miller, 2004; Oosterhof et al., 2017; Rocío Nieto-
557 Arellano, 2019). Most importantly, we found that in the absence of injury, stimulation
558 of the immune system activates the regenerative response program of Müller glia, as
559 shown by upregulation of *mmp9* and *her4.1,* as well as an increase in proliferation;
560 consistently, expression of *her4.1* also indicates the generation of NPCs from Müller
561 glia. The expression of the CSF-1 receptor in neural progenitor cells is also
562 associated with proliferation, survival and differentiation, supporting the theory that
563 M-CSF stimulates a proliferative/regenerative response in Müller glia (Chitu et al.,
564 2016). Nevertheless, whether Müller glia reactivity is a direct consequence of
565 CSF/TLR-agonist treatment, or an indirect consequence of potentially altered
566 microglia signaling, is so far not clear, because receptors for CSF/TLR signaling
567 appear to be expressed in both cell populations (Kochan et al., 2012; Kumar &
568 Shamsuddin, 2012; Letiembre et al., 2007; Lin et al., 2013; Mitchell et al., 2019;
569 Tsarouchas et al., 2018; Wu et al., 2018). Hence, it will be interesting to test whether
570 CSF-1R or TLR agonists alone are sufficient to drive increased Müller glia
571 proliferation, using a model that completely lacks microglial cells in the retina.
572 Moreover, it will be interesting to determine if the observed effects of the
573 CSF1R/TLR-agonists are mediated via NF-κB signaling, or via other pathways, such
574 as the MAP kinase pathway, or combinations thereof (Chen et al., 2018; Kawasaki &
575 Kawai, 2014; Wan et al., 2012).

576

577 ## Conclusion

578 Our work strongly supports the notion that acute inflammatory signaling provides a
579 beneficial contribution during adult zebrafish retina regeneration, akin to findings in
580 the telencephalon (Kyritsis et al., 2012; Kizil et al., 2015). Reactivity of retinal
581 leukocytes appears to be tightly controlled after injury, and the presence of
582 leukocytes positively influences reactive proliferation of Müller glial cells, as well as
583 the downstream differentiation of Müller glia progeny into mature photoreceptor cells.
584 Importantly, immune stimulation through TLRs, or via M-CSF injection, was sufficient,
585 in the absence of injury, to trigger Müller glia proliferation and NPC formation. Hence,
586 we conclude that inflammatory signaling plays a critical role during zebrafish retina
587 regeneration.

588 Further investigations on possible interactions between leukocytes and Müller glia will
589 help to understand the balance between the inflammatory reaction and its resolution
590 during regeneration of neural tissue in zebrafish. In addition, it will be important to
591 identify critical factors secreted by leukocytes in an acute reaction to lesion that are -
592 directly or indirectly- involved in triggering Müller glia reactivity, and thus,
593 regeneration. Finally, this study provides a framework for investigating potentially
594 beneficial effects of an acute immune response, also in comparison with failed
595 regeneration and chronic inflammatory outcome in mammalian models.

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598

599 # Materials and Methods

600 ## Ethics statement

601 The animal experiments were performed in strict accordance with the European
602 Union and German law (Tierschutzgesetz). Experimental procedures were approved
603 by the animal ethics committee of the TU Dresden and the Landesdirektion Sachsen
604 (permits AZ: 24-9168.11-1/2013-5; AZ: TV A 1/2017; TVV 21/2018; AZ: TVV
605 55/2018).

606 ## Zebrafish Maintenance

607 Zebrafish (*Danio rerio*) were kept under standard housing conditions as previously
608 described (Brand et al., 2002). All fish used in this study were adults, both males and
609 females, and 6 to 12 months of age. Transgenic fish used are:
610 *Tg(−5.5opn1sw1:EGFP)*^{kj9} (Takechi et al., 2003); *Tg(mpeg1:mCherry)* (Ellett et al.,
611 2011); *Tg(mpo:GFP)* (Renshaw et al., 2016); *Tg(gfap:NLS-GFP)* (described here);
612 *Tg(gfap:NLS-mCherry)* (Lange et al., 2020), *Tg(lck:NLS-mCherry)*^{sd31} (Butko et al.,
613 2015), *Tg(NF-kB:GFP)* (Kanther et al., 2011), Δirf8^{st95} (Shiau et al., 2015).

614 ## Generation of *Tg(gfap:nls-GFP)*

615 The *Tg(gfap:NLS-GFP)* reporter line with its NLS-tag shows highly nuclear
616 localization of GFP, but also some leaky cytoplasmic expression in Müller glial cells,
617 and thus serves as an ideal marker for MGCs in our experiments. To generate this
618 line, the GFP reporter was PCR amplified and flanked by restriction sites using GFP-
619 for (atatGGCCGGCCgccaccatggctccaaagaagaagcgtaaggt) and GFP-rev
620 (ggtgtgcatgttttgacgttgatggc) primers. By PCR, the nuclear localization sequence
621 (NLS) was added as a 5' overhang to the GFP. The PCR product was subcloned into

622 the p2.1 Topo Vector. Next, Topo vector with the reporters and the
623 *pTol(gfap:mcherry-T2A-CreERT2)* construct (SH and MB, unpublished) were
624 digested using the enzymes Asc and FseI and ligated to replace the mCherry-
625 CreERT2 cassette with the NLS-reporter. For germline transformation, either
626 linearized plasmid DNA or plasmid DNA with transposase mRNA were injected into
627 fertilized eggs (F0) in E3 medium (Brand et al., 2002), raised to adulthood and
628 crossed to AB wild-type fish as previously described (Kawakami et al., 2004).

629 **Diffuse light lesion**

630 To ablate photoreceptor cells, diffuse light lesion was performed as described in
631 Weber et al., 2013. Briefly, dark-adapted fish were transferred to a beaker containing
632 250 ml system water and exposed to the light of an EXFO X-Cite 120 W metal halide
633 lamp (~200,000 lux) for 30'. For recovery, fish were connected to the system and
634 kept under standard light conditions.

635 **Drug treatment**

636 Dexamethasone (Dex, Sigma-Aldrich, Germany) was diluted from a 25 mg/ml stock
637 solution in methanol to 15 mg/l in sterilized system water. For Dex treatment, adult
638 fish were kept individually in sterilized tanks. Control groups with the corresponding
639 amount of methanol (MeOH) without Dex, both groups did not show retinal cell death
640 (supplement S3). Solutions were renewed daily, and tanks were exchanged every 5
641 days. Fish were fed with brine shrimp 1h prior to solution exchange. Animals were
642 pretreated for 10 days and respective conditions were maintained until sacrificed.
643 Light lesions were performed in corresponding solutions.

644 **Tissue preparation and sectioning**

645 For retinal flat mounts, eyes were removed and a small incision in the cornea was
646 made using a sapphire blade scalpel (ZT215 W0.5mm A60°). Eyes were prefixed in
647 4% PFA in calcium-free ringer solution (PFAR) for 30' with slow agitation. Cornea,
648 sclera and the lens were removed in calcium-free ringer solution and the retinae were
649 cut at four sides. Flat mounts were fixed in 4% PFAR at 4 C overnight with gentle
650 agitation. For storage, samples were transferred to 100% methanol and stored at -
651 20°C.

652 For sections, the lens was removed and fish heads were fixed at 4 C in 4% PFA in
653 0.1 M phosphate buffer (PB). Following that, samples were decalcified and cryo-
654 protected with 20% sucrose/20% EDTA in 0.1 M PB. Tissues were embedded in
655 7.5% gelatine/20% sucrose in 0,1% PB, stored at -80 °C and sectioned into 14 µm
656 cryo-sections using a Microm HM560. Sections were stored at -20°C.

657 ***In situ* Hybridization**

658 *In situ* hybridization on sections was performed as described in Ganz et al., 2015.
659 Hybridization was carried out overnight at 62 °C. Probes were generated using
660 *mmp9* (primer forward: CTTGGAGTCCTGGCGTTTCT; primer reverse:
661 GCCCGTCCTTGAAGAAGTGA) and *her4.1* (Takke et al., 1999) as targets. For
662 detection, NBT/BCIP or SIGMA*FAST*™ Fast Red TR/Naphtanol AS-MX was used.
663 For combination with immunohistochemistry, the primary antibody was incubated with
664 anti-digoxigenin-AP. Secondary antibody was applied after staining development for
665 2h and washed with PBS with 0.3% Triton-X100 (PBSTx). Sections were mounted in
666 glycerol.

667 **Immunohistochemistry & TUNEL**

668 Sections were dried for 2 h at 50 °C and rehydrated with PBSTx. Following this,
669 sections were incubated with primary antibody: anti-Zrf-1 ZIRC mouse IgG_1 1:200
670 (Fausett & Goldman, 2006), anti-Zpr-3 ZIRC IgG_1 1:200 (Zou et al., 2008), anti-GFP
671 Abcam chicken 1:3000 (Leinninger et al., 2009), anti-DsRed Clontech rabbit 1:500
672 (Glass et al., 2005), anti-PCNA mouse IgG_{2a} DACO PC10 1:500 (Grandel et al.,
673 2006), anti-L-Plastin rabbit 1:5000, (Redd et al., 2006) and incubated overnight at
674 4 °C. Excess antibody was washed off using PBSTx followed by incubation with
675 secondary antibody against the respective primary antibody host (Molecular Probes;
676 Alexa 488, Alexa 555, Alexa 635; 1:750) containing 1 µg/ml 4′,6-Diamidin-2-
677 phenylindol (DAPI). Antibody-solution was washed off and slides were mounted with
678 glycerol.

679 To retrieve the PCNA antigen, sections were incubated for 8’ in 50 mM Tris buffer pH
680 8.0 at 99 °C followed by 10 min PBS prior to primary antibody incubation.

681 Modifications for retinal flat mounts are prolonged incubation times for primary and
682 secondary antibody solution for 48 h respectively at 4 °C with slow agitation and
683 extensive washing 10x 30’ after each antibody incubation. Flat mounts were mounted
684 on slides in glycerol.

685 TUNEL (TdT-mediated dUTP-biotin nick end labeling) assays were performed on
686 sections using ApopTag® Red in Situ Apoptosis Detection Kit (Merk) according to
687 manufacturer’s instructions.

688 **EdU labeling & detection**

689 To trace proliferating cells, EdU (5-ethynyl-2′-deoxyuridine) pulses were given
690 intraperitoneally. Fish were anesthetized in 0.024% Tricaine and EdU was injected
691 intraperitoneally (20 µl of 2.5 mg/ml EdU in PBS per pulse). Detection was performed

692 on sections using “Click-iT® Plus EdU Alexa Fluor® 555 Imaging Kit” (Thermo
693 Fischer Scientific) according to manufacturer’s instructions.

694 **Intravitreal injections**

695 Fish were anesthetized in 0.024 % tricaine and covered with tricaine-moistened
696 paper towel, leaving the eye exposed. The outer cornea was removed.
697 Subsequently, a small incision in the cornea was made using a sapphire blade
698 scalpel (ZT215 W0.5mm A60°). A Hamilton syringe equipped with a 33 gauge, blunt-
699 end needle was inserted and 0,5 µl of solution (flagellin from *S. typhimurium*, 0,1
700 µg/µl, Sigma; zymosan from *S. cerevisiae*, 0,2 µg/µl BioParticles; Human M-CSF, 50
701 ng/µl, PreproTech; Human Il-34, 50 ng/µl, PreproTech; PBS) was injected into the
702 vitreous of the eye without touching the retina. The incision was covered with
703 Histoacryl® (Braun) to avoid leakage.

704 **Image acquisition**

705 Images were acquired using a Zeiss Axio Imager, equipped with ApoTome. EC Plan-
706 Neofluar 5x/0.16, Plan-Apochromat 20x/0.8 and LD C-Apochromat 40x/1.1 W Korr
707 UV VIS IR objectives were used for magnification. For detection either a
708 monochromatic Axiocam HR (1388x1040 pixels, 6.45*6.45) for fluorescence or a
709 polychromatic Axiocam MR Rev3 (1388x1040 pixels, 6.45*6.45) for bright-field
710 imaging was used. Sequential image acquisition was used in co-stained samples with
711 multiple fluorophores. Images were acquired in AxioVision Rel. 4.8 and processed
712 using Fiji (Schindelin et al., 2012). Figure panels were assembled in Adobe
713 Photoshop CS6.

714 **Cell counting and statistical analysis**

715 For cell quantification, retinal sections with a thickness of 14 µm were used. For
716 single fluorophore cell quantification, the first 3-5 consecutive retinal sections rostral
717 to the optic nerve were selected and manually counted. For normalization, the length
718 of the retina was determined for every section by measuring a snapshot of the retina
719 at the level of the inner nuclear layer. For quantification of multiple fluorophores, 20x
720 images were acquired. Cells were quantified using the cell counter tool in Fiji. Length
721 measurements were applied on the same images for normalization. For statistical
722 analyses, ≥3 fish were used for calculations. Counted cell numbers were normalized
723 to the respective parameter (reference cell population or length) and the counts per
724 fish were averaged. The average number for every fish was used as an analytical
725 unit. For statistical analysis, Graph Pad Prism was used to determine p-values with a
726 one-way ANOVA test with Tukey's post hoc analysis. For unpaired two-tailed
727 student's T-Test, Excel was used. Significance levels are displayed as: not significant
728 = $p>0.05$, * = $p\leq0.5$, ** = $p\leq0.01$, *** = $p\leq0.001$. Graphs shown in bar charts were
729 created using Excel, error bars represent standard error of the mean.

730

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Competing interests

The authors declare that they have no conflict of interest.

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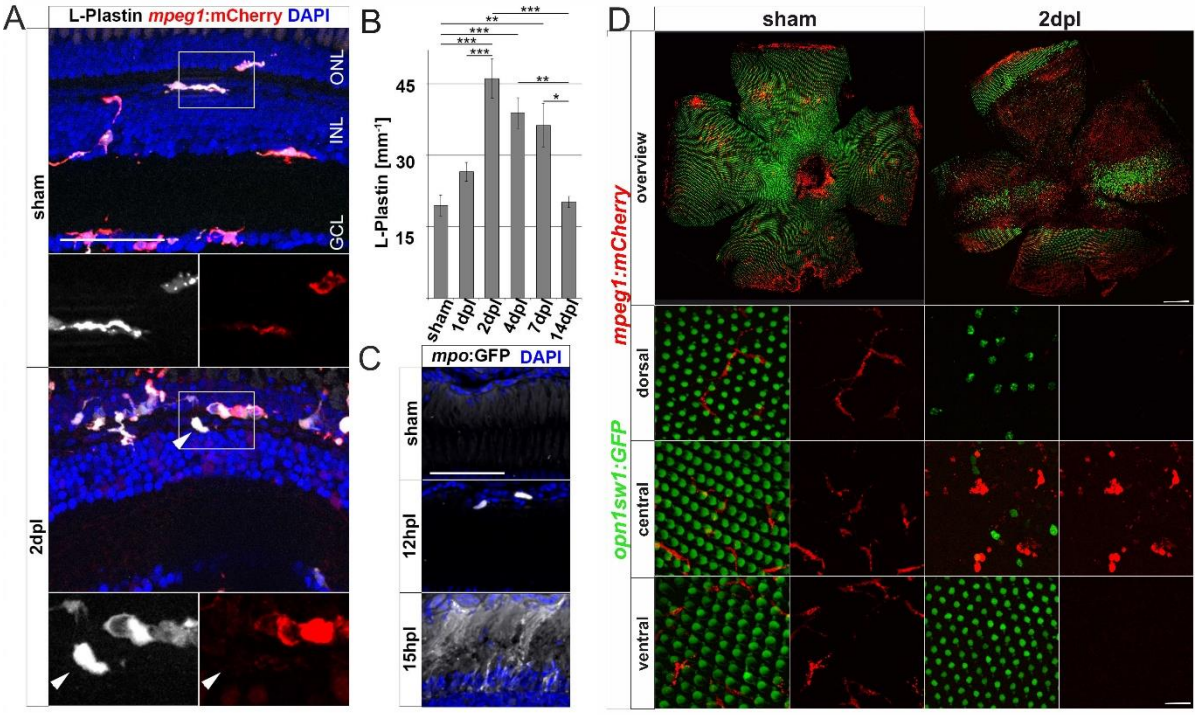

Figure 1: Sterile phototoxic ablation of photoreceptors triggers leukocyte accumulation. (A) Retinal sections show that the majority of L-Plastin$^+$ cells are *Tg(mpeg1:mCherry)*$^+$, display morphological changes after lesion, and accumulate at the outer nuclear layer (ONL) in response to lesion. However, L-Plastin$^+$ *Tg(mpeg1:mCherry)* negative cells were also observed upon injury (arrowhead). (B) Quantification on sections of L-Plastin$^+$ cells in sham and regenerating retinae at 1, 2, 4, 7 and 14 days post lesion (dpl) shows an increase of cells within 2dpl that is released within 14 dpl. (C) In contrast to sham, *Tg(mpo:GFP)*$^+$ neutrophils are detected upon lesion. They gather at the lesion site (12 hpl) and a diffuse GFP positive pattern (matrix) is formed from 15 hpl onwards. (D) Retinal flat mounts of *Tg(opn1sw1:GFP)* x *Tg(mpeg1:mCherry)* show the ramified structure of leukocytes in a UV-cone-specific reporter line and their reaction to lesion. Upon light lesion, leukocytes increase in number at the lesion site and display an amoeboid, activated morphology and accumulate in the central part of the lesion while unharmed areas appear devoid of *mpeg1:*mCherry positive cells. Scale bars in A & C: 50 µm Scale Bars in D: 200µm, inlets 20µm, error bars indicate standard error; * = p≤0,05; ** = p≤0,01; *** = p<0,001; n≥4; ANOVA Tukey's post hoc test; INL= inner nuclear layer; GCL=ganglion cell layer.

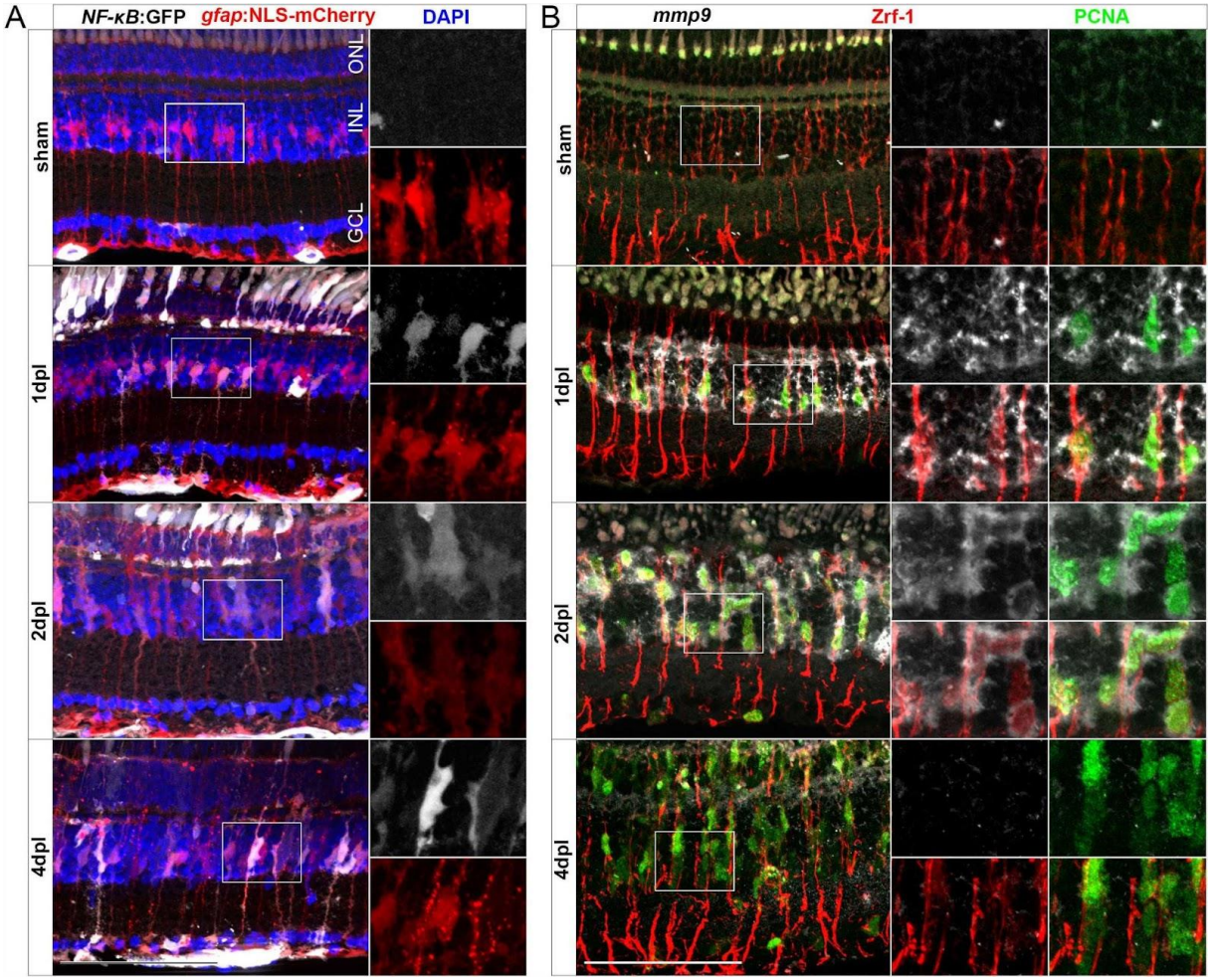

Figure 2: Müller glia transiently activate the *NF-κB:*GFP reporter and express the *NF-κB* target metalloproteinase *mmp9* in response to injury. (A) In comparison to sham, the *NF-κB:*GFP reporter is activated at 1 and 2 days post lesion (dpl) in *gfap*:NLS-mCherry labeled Müller glia localized in the inner nuclear layer (INL) and photoreceptors of the outer nuclear layer (ONL) and is restricted to the INL at 4 dpl. (B) *In situ* hybridization of *mmp9* in combination with immunohistochemistry for proliferating cell nuclear antigen (PCNA) and glial fibrillary acidic protein (GFAP/Zrf-1) labelling Müller glia, shows that *mmp9* is not expressed in sham retinae. In contrast, *mmp9* is transiently expressed at 1 and 2 dpl and returns to undetectable levels at 4 dpl. Scale bar: 50µm. GCL=ganglion cell layer.

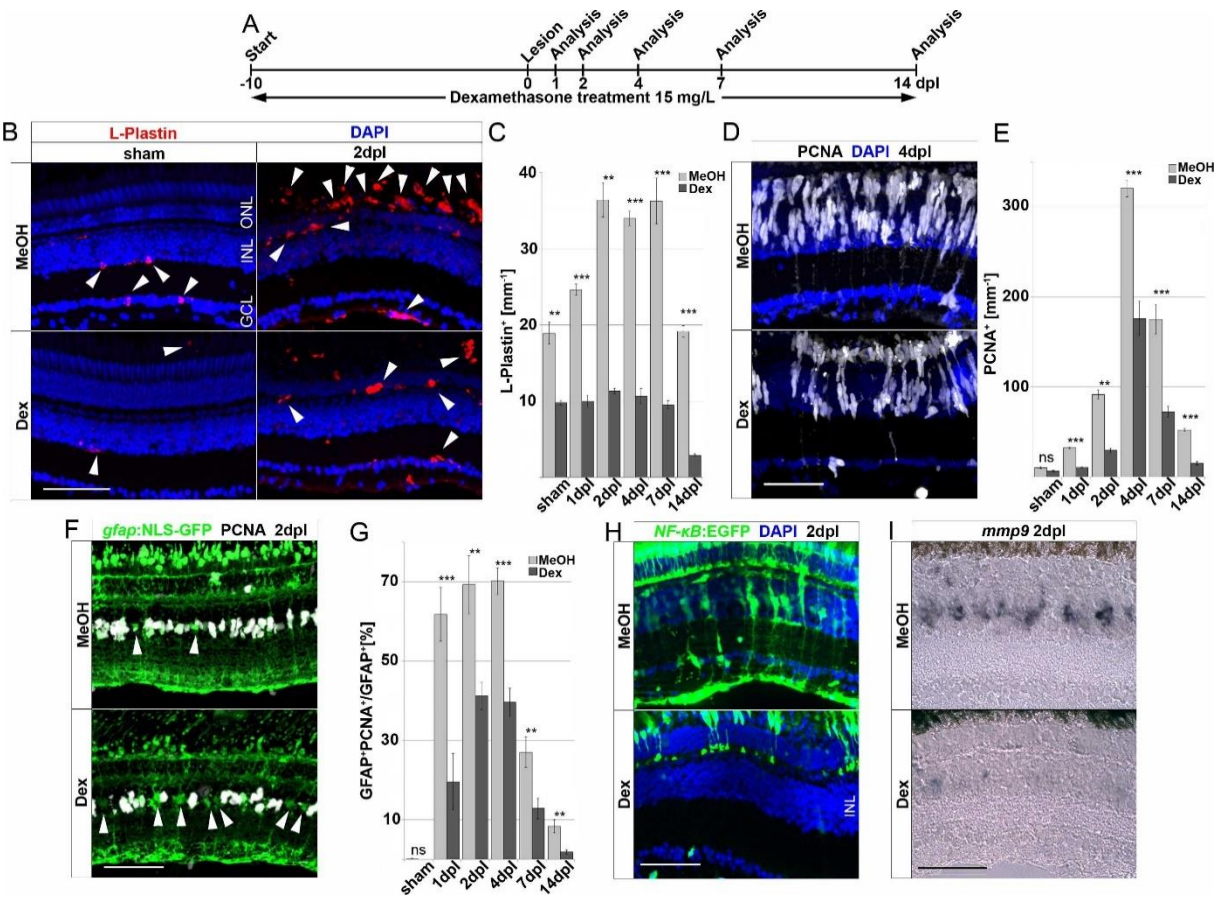

Figure 3: Immunosuppression interferes with leukocyte accumulation and Müller glia reactivity. (A) Scheme of experimental outline. Fish were treated with Dexamethasone (Dex) or vehicle (Methanol; MeOH) from 10 days prior to injury until the day of analysis (sham, 1, 2, 4, 7 and 14 days post lesion; dpl). (B) Dex-treatment reduces the number of L-Plastin$^+$ leukocytes (arrowheads) in sham (left panel) and regenerating retinae at 2 dpl (right panel). (C) Quantification of L-Plastin$^+$ cells in MeOH- and Dex-treated sham or lesioned animals at 1, 2, 4, 7 and 14 dpl. (D) Immunohistochemistry for proliferating cell nuclear antigen (PCNA) reveals impaired proliferation in Dex-treated animals at 4 dpl. (E) Quantification of PCNA$^+$ cells in MeOH- and Dex-treated sham and regenerating retinae at 1, 2, 4, 7 and 14 dpl shows impaired proliferative response in the Dex-treated group. (F) Immunohistochemistry for PCNA in *gfap*:NLS-GFP labeled Müller glia shows reduced numbers of proliferating Müller glia in the Dex treated group at 2 dpl compared to MeOH controls (arrowheads indicating non proliferative Müller cells). (G) Quantification of proliferating Müller glia in vehicle and Dex-treated sham and regenerating retinae at 1, 2, 4, 7 and 14 dpl, indicating that Dex hinders Müller glia proliferation. (H) In comparison to vehicle, the *NFκB*:GFP reporter is not activated in the inner nuclear layer (INL) of Dex-treated retinae at 2 dpl. (I) Injury-induced expression of *mmp9* is strongly reduced in Dex-

treated retinae at 2 dpl. Scale bar: 50 µm, Error bars indicate standard error; ns = $p>0,05$, * = $p\leq0,05$; ** = $p\leq0,01$; *** = $p<0,001$; $n\geq6$; two-tailed student's T-Test, GCL=ganglion cell layer, ONL=outer nuclear layer.

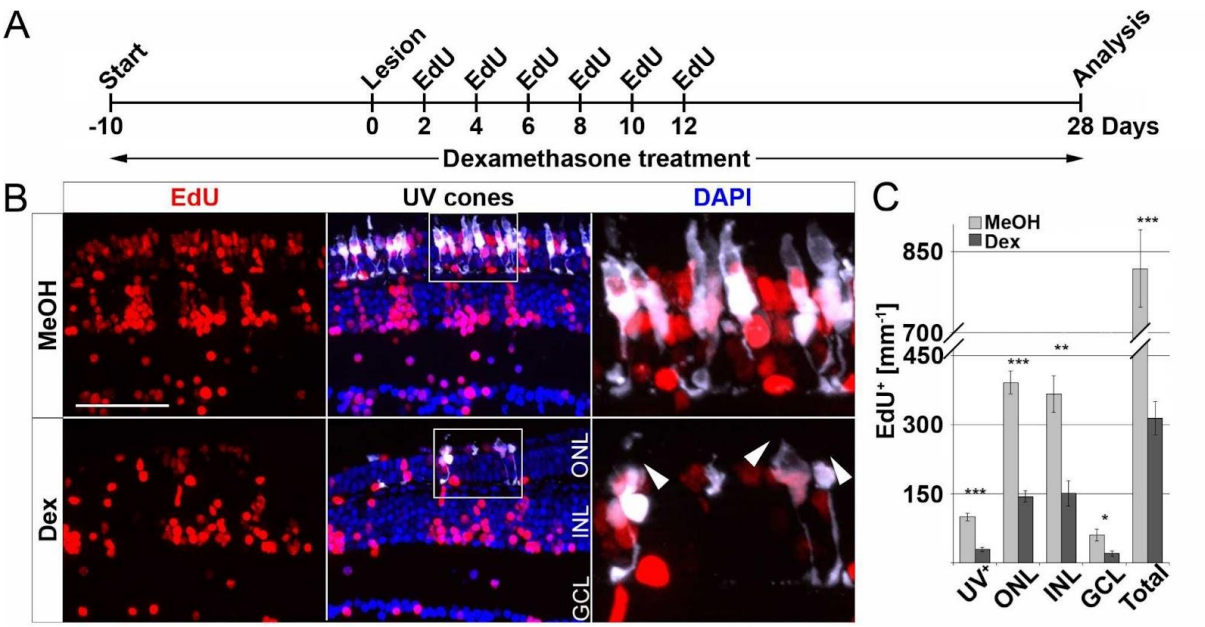

Figure 4: Long-term immune suppression impairs photoreceptor regeneration. (A) Scheme of experimental outline. *opn1sw1*:GFP, labeling UV-cones, transgenic animals were treated with Dexamethasone (Dex) or vehicle (Methanol; MeOH) from 10 days prior to lesion until the day of analysis (28 days post lesion; dpl 38 days post treatment; dpt). EdU pulses were applied at 2, 4, 6, 8 and 10 dpl. (B) In comparison to MeOH-treated animals, the number of regenerated EdU^+ UV-cones is significantly reduced after Dex-treatment (compare upper panel with lower panel). Furthermore, the morphology of photoreceptor cells is disrupted in comparison to MeOH controls (arrowheads). (C) Quantification of EdU^+ cells with respect to retinal layers showing significant reduction of EdU positive nuclei in the Dex-treated group. Scale bars: 50 µm. Error bars indicate standard error; * = p≤0,05; ** = p≤0,01; *** = p<0,001; n≥6; two-tailed student's T-Test. ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer.

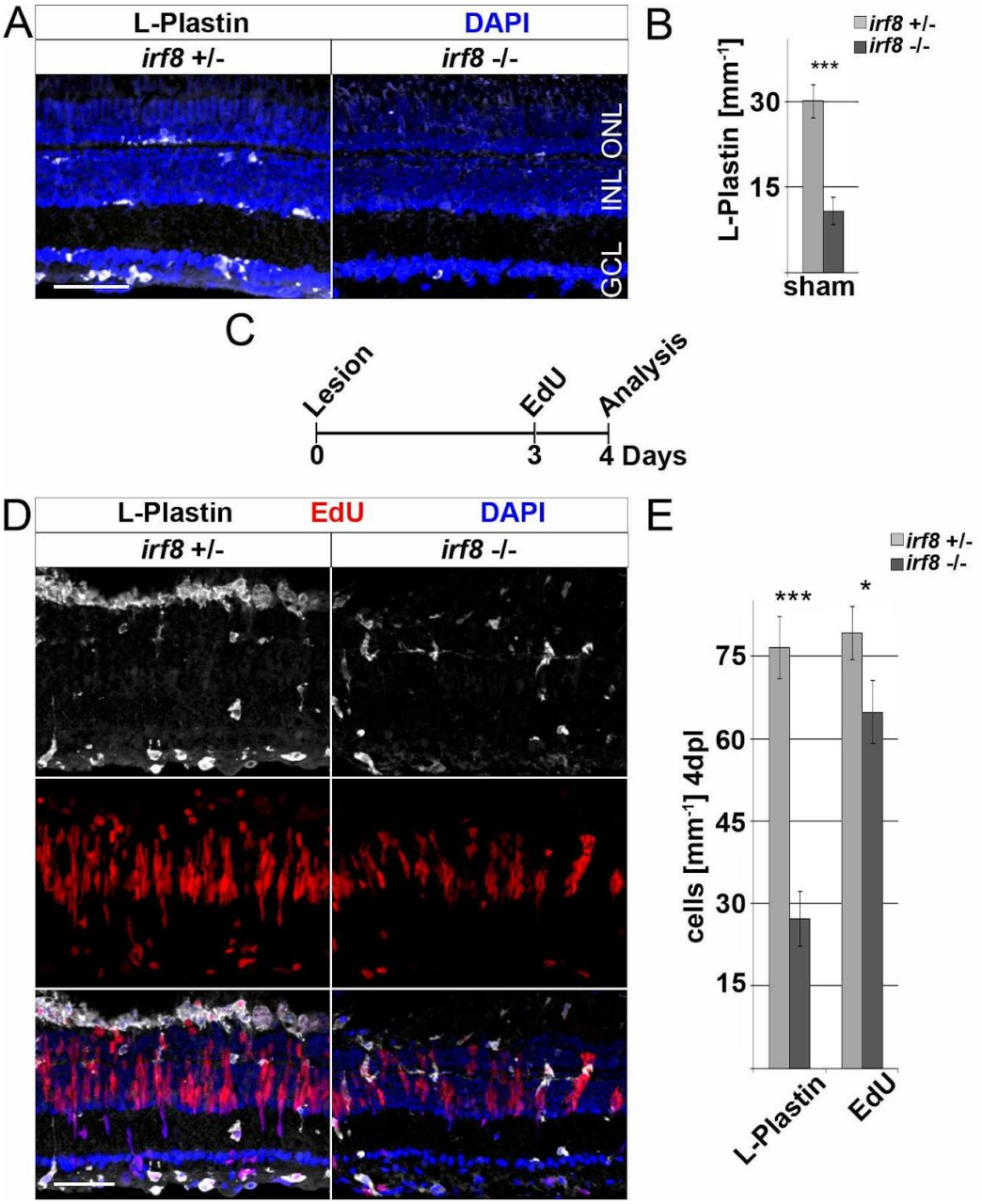

Figure 5: Genetic reduction of microglia inhibits retinal proliferation in response to injury. (A) In contrast to heterozygous control siblings (*irf8*$^{+/-}$), interferon regulatory factor 8 (*irf8)* homozygous myeloid-deficient mutant retinae (*irf8*$^{-/-}$) display a decreased number of L-Plastin^{+} leukocytes in homeostasis. (B) Quantifications of L-Plastin^{+} cells in *irf8*$^{-/-}$ and control heterozygous siblings *irf8*$^{+/-}$. (C) Scheme of experimental outline. *irf8*$^{-/-}$ and *irf8*$^{+/-}$ animals received an EdU pulse at 3 days post lesion (dpl) and were analyzed at 4 dpl. (D) During regeneration, the accumulation of L-Plastin^{+} cells at the outer nuclear layer (ONL) is impaired in *irf8*$^{-/-}$ but not in control *irf8*$^{+/-}$ siblings. Moreover, the number of EdU^{+} cells is significantly decreased in *irf8*$^{-/-}$ retinae. (E) Quantification of L-Plastin^{+} cells and EdU^{+} nuclei in *irf8*$^{-/-}$ and control *irf8*$^{+/-}$ animals at 4 dpl, show

reduced amounts of positive cells respectively. Scale bar: 50 µm. Error bars indicate standard error; * = p≤0,05; *** = p<0,001; n=5; two-tailed student's T-Test; INL=inner nuclear layer; GCL ganglion cell layer.

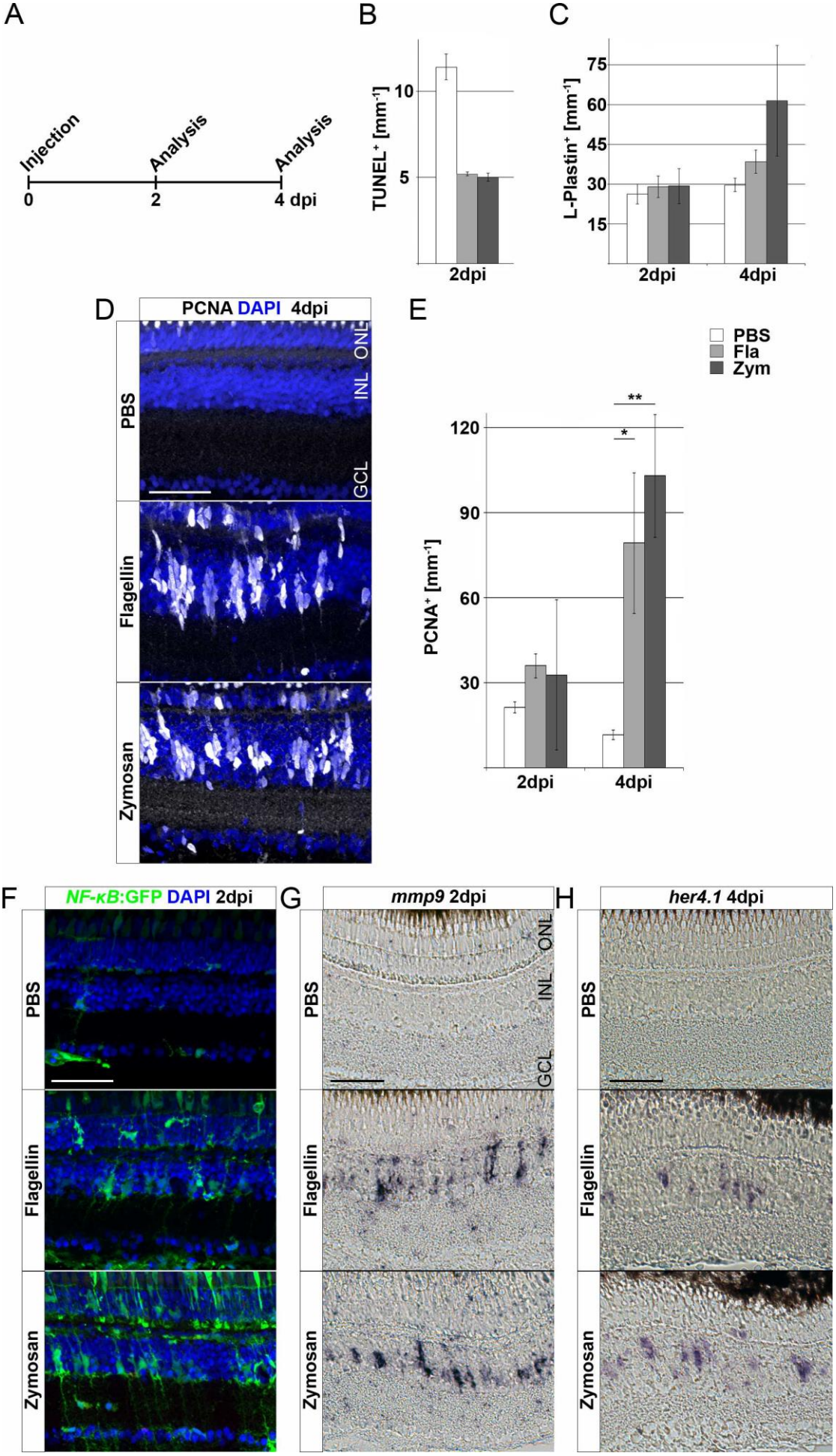
A
Injection
Analysis
Analysis
0
2
4 dpi
B
TUNEL+ [mm-1]
10
5
2dpi
C
L-Plastin+ [mm-1]
75
60
45
30
15
2dpi
4dpi
D
PCNA DAPI 4dpi
PBS
Flagellin
Zymosan
ONL
INL
GCL
E
PBS
Fla
Zym
PCNA+ [mm-1]
120
90
60
30
**
*
2dpi
4dpi
F
NF-κB:GFP DAPI 2dpi
PBS
Flagellin
Zymosan
G
mmp9 2dpi
PBS
Flagellin
Zymosan
ONL
INL
GCL
H
her4.1 4dpi
PBS
Flagellin
Zymosan

Figure 6: Inflammatory stimuli trigger Müller glia reactivity. (A) Scheme of experimental outline. Wild type or *NF-κB*:GFP reporter animals were injected with flagellin or zymosan into the vitreous of the eye and analyzed at 2 and 4 days post injection (dpi). (B) Quantification of TUNEL$^+$ nuclei in control and injected retinae at 2 dpi revealing no increase in cell death. (C) Quantification of L-Plastin$^+$ cells in control (PBS) and flagellin or zymosan injected retinae at 2 and 4 dpi display no significant increase in leukocytes due to the injection of factors. (D) Immunohistochemistry for proliferating cell nuclear antigen (PCNA) reveals proliferation in flagellin or zymosan injected but not in control animals at 4 dpi. (E) Quantification of PCNA$^+$ nuclei in injected retinae at 2 and 4 dpi. (F) In comparison to PBS injected animals, injection of flagellin or zymosan results in strong activation of the *NFκB*:GFP reporter at 2 dpi. (G & H) *In situ* hybridization of *mmp9* and *her4.1* show expression of both genes in flagellin or zymosan injected but not in PBS injected eyes at 2 and 4 dpi, respectively. Scale bar: 50 µm. Error bars indicate standard error; * = p≤0,05; ** = p≤0,01; *** = p<0,001; n=6; two-tailed student's T-Test; ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer.

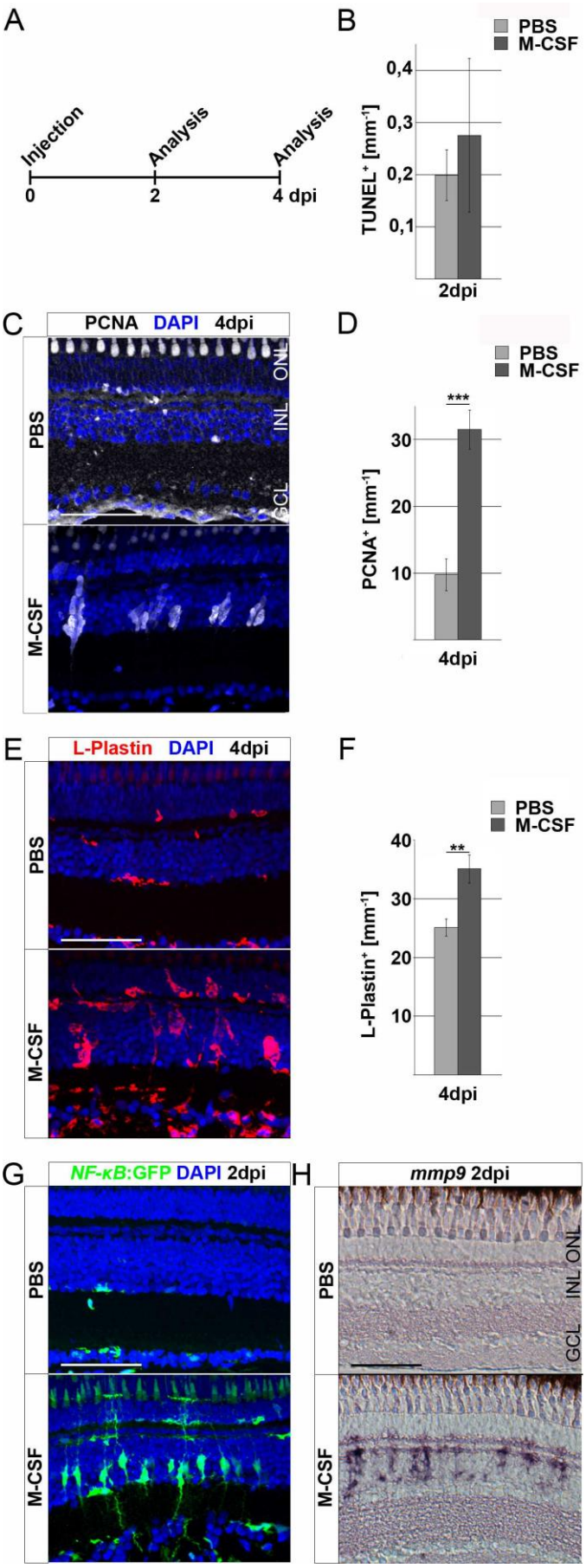
A
Injection
Analysis
Analysis
0
2
4 dpi
B
PBS
M-CSF
TUNEL+ [mm-1]
0,4
0,3
0,2
0,1
2dpi
C
PCNA DAPI 4dpi
PBS
M-CSF
ONL
INL
GCL
D
PBS
M-CSF
***
PCNA+ [mm-1]
30
20
10
4dpi
E
L-Plastin DAPI 4dpi
PBS
M-CSF
F
PBS
M-CSF
**
L-Plastin+ [mm-1]
40
30
20
10
4dpi
G
NF-κB:GFP DAPI 2dpi
PBS
M-CSF
H
mmp9 2dpi
PBS
M-CSF
INL ONL
GCL

Figure 7: M-CSF stimulates an inflammatory response and initiates Müller glia cell cycle re-entry in the absence of lesion. (A) Scheme of experimental outline. Wild type or *NFκB*:GFP reporter animals were injected with M-CSF or PBS into the vitreous of the eye and analyzed at 2 and 4 days post injection (dpi). (B) Quantification of TUNEL$^+$ nuclei in control and M-CSF-injected retinae at 2 dpi reveals no chance in cell death. (C) Immunohistochemistry for proliferating cell nuclear antigen (PCNA) reveals proliferation in M-CSF injected but not in control injected animals at 4 dpi. (D) Quantification of PCNA$^+$ cells in control (PBS) and M-CSF injected retinae at 4 dpi. (E) L-Plastin staining shows more positive cells in M-CSF injected retinae at 4 dpi compared to controls. (F) Quantification of L-Plastin$^+$ cells in control and M-CSF injected retinae at 4 dpi, show accumulation of L-Plastin cells in M-CSF injected specimen. (G) In comparison to PBS, injection of M-CSF results in strong activation of the *NFκB:*GFP reporter at 2 dpi. (H) *In situ* hybridization of *mmp9* shows transcriptional activation of this gene in M-CSF injected but not in control-injected animals at 2 dpi. Scale bars = 50µm, Error bars indicate standard error; ** = p≤0,01; *** = p<0,001; n>5; two-tailed student's T-Test. ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer.

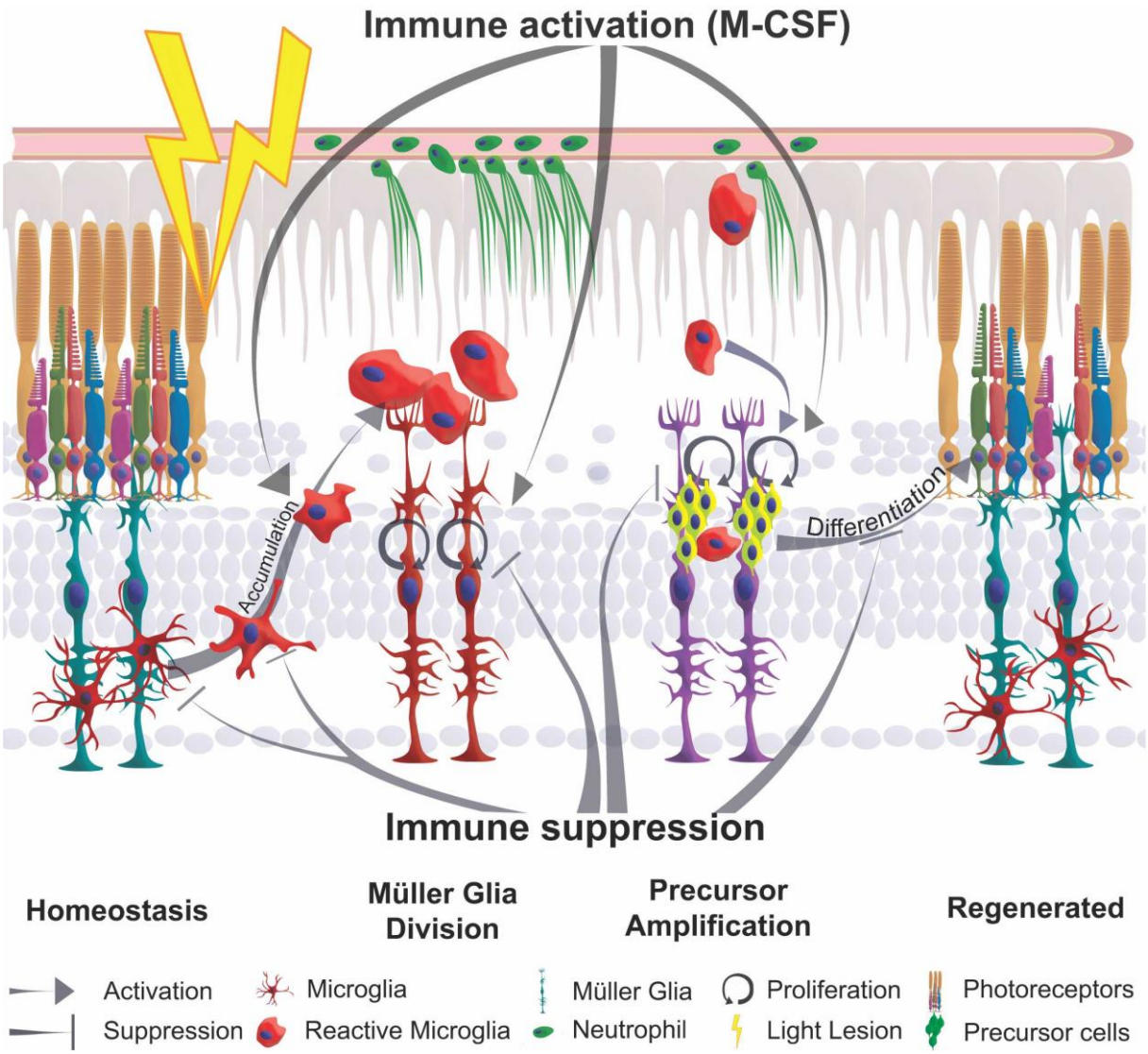

Figure 8: Schematic summary of the impact of inflammation in retinal regeneration. In response to injury, microglia undergo a phenotypic change from amoeboid to ramified shape and accumulate at the lesion site. This process is inhibited by immune suppression. The reactive proliferation of Müller glia during regeneration is also impaired upon immunosuppression. Leukocytes stimulate the proliferation of neuronal precursor cells. M-CSF as inflammatory mediator triggers Müller glia proliferation, leukocyte accumulation and the generation of neuronal precursor cells. This process may also act indirectly via the stimulation of immune cells. Overall, the innate immune system, via mediators such as M-CSF, supports the differentiation into photoreceptor cells and thereby acts as a regenerative cue for retina regeneration.