Retinal ganglion cell survival after severe optic nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune responses in the zebrafish retina

Si Chen, Kira Lathrop, Takaaki Kuwajima and Jeffrey Gross
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First decision letter

MS ID#: DEVELOP/2021/199694

MS TITLE: Retinal ganglion cell survival after severe optic nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune responses in the zebrafish retina

AUTHORS: Si Chen, Kira Lathrop, Takaaki Kuwajima, and Jeffrey Gross

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this report by Chen, et al. the authors aim to investigate the mechanisms underlying the resiliency of zebrafish retinal ganglion cells (RGCs) to optic nerve transection. Understanding how zebrafish RGCs survive axotomy while mammalian RGCs do not is of high interest to identify targets to help with RGC-based diseases such as glaucoma. In the manuscript, the authors perform RNA-seq on sorted RGCs after optic nerve transection to identify targets underlying RGC-survival. This analysis reveals that genes associated with the Jak/stat pathway genes and inflammatory processes are induced in RGCs after injury. Follow up experiments show that Jak/Stat signaling is induced in injured RGCs and inhibition of this signaling pathway is detrimental to RGC survival. Finally the authors investigate the retinal microglial response to axotomy and find that ablation of microglia or inhibition of inflammation via dexamethasone has a significant impact on the survival or RGCs.

Comments for the author

Major points
1. RNA-seq from sorted Isl2b-GFP cells is used to identify genes induced in RGCs. However, I think additional steps can be taken to convince readers that this approach mostly captures RGCs. I also have concerns that contaminating microglia/macrophages may be contributing to this dataset. In Figure 1A, to demonstrate the Isl2b-GFP is restricted to the RGC layer a merged image is shown of half of a top half of a retinal section and then insets of each individual channel is shown from the RGC-layer. I think it would be useful to show all the retinal nuclear layers with the Isl2b-GFP channel only. This can convince readers that you don't have contributions from photoreceptors or bipolar cells, etc. scRNA-seq from Hoang, et al. 2020 (PMID: 33004675), do seem to show Isl2b is primary expressed in RGCs so I don't think this is a huge concern but it could be demonstrated more clearly. What is more concerning is the genes that are attributed to RGCs such as Il1b, cxcr4b and other immune-associated genes. Using the data from scRNA-seq data mentioned above these genes are almost exclusively found in microglia/macrophages and not detected in RGCs. This is also consistent with the literature in mouse retina where genes such as Il1b are not found in neuronal populations but rather microglia. Therefore, I am concerned that in addition to RGCs this dataset has contributions from contaminating immune cells. I would suggest either validating expression of cxcr4b, Il1b, etc. in RGCs, cross validating RGC expression of genes with scRNA-seq generated from the Blackshaw lab, Baier lab, or others.

This type of analysis will substantially change the interpretation of the results as they are presented here.
These datasets can be queried online here:
https://proteinpaint.stjude.org/F/2019.retina.scRNA.html

2. In figure 1 it is argued that Isl2b-GFP can be downregulated without RGC death (7dpi), yet in Figures 3 and 4 Isl2b-GFP+ cell counts are used as a proxy for RGC survival at 7dpi. I think an orthogonal marker should be used to quantify RGC survival one that does not have evidence for transient downregulation to injury stimulus.

3. For the experiments with the Jak/Stat pathway inhibitor P6 or dexamethasone, I think the interpretation could be expanded. Because these compounds will not work cell autonomously on RGCs and surely effect Muller glia and microglia, which have been reported to respond to both of these pathways and significantly contribute to RGC-survival, it is hard to piece apart the contributions. For example, the authors show that P6 significantly decreases RGC survival after axotomy. However, when microglia are ablated P6 no longer has an effect. This is interpreted as "Jak/Stat activity is dispensable in the absence of microglia recruitment".
However, both P6 and Dexamethasone can act directly on microglia and the effects observed here could be due directly to this. In this interpretation P6 does nothing in the absence of microglia because the effect of Jak/Stat inhibition was due to the microglial response to this drug. This same problem applies to the dexamethasone experiments and it would be interesting to see if Dex still has an effect with microglia ablation. Overall these effects are large and interesting, but I think the interpretations are not strongly supported by the data, additional experiments, caveats or re-interpretations should be provided.

Minor Points
1. In figure 3a the “zoom” panels should show the merged stain. Or both channels independently with arrows.
It is hard to determine the co-labeling of pStat3 with RGCs as the image is presented now.
2. Sex should be used instead of “gender” when describing animals in line 96.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Chen and colleagues entitled “Retinal ganglion cell survival after severe optic nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune responses in the zebrafish retina” uses RNA sequencing to identify genes and pathways that modulate retinal ganglion cell (RGC) survival following optic nerve transection (ONT) and pharmacological perturbation to demonstrate that both pro-survival and pro-death signals are interacting to influence RGC survival following ONT. The major findings include:
1. The majority of isl2b:GFP+ RGCs survive following ONT, although the isl2b:GFP RGC cell number is reduced to about 75% at 7 dpi.

2. Differentially expressed genes in the isl2b:GFP+ cell population at 24 hpi (as well as 12 hpi) include components of the JAK/STAT signaling pathway, which was the most highly enriched pathway in the upregulated genes, and components of the innate immune response, which was also upregulated.

3. JAK/STAT pathway is required to maintain the number of isl2b:GFP+ RGCs following ONT. Phospho-Stat3 is expressed at 1 and 7 dpi, and pharmacological inhibition of Jak reduces the number of isl2b:GFP+ cells at 7 dpi compared to vehicle control.

4. Macrophages/microglia are recruited to the ganglion cell layer following ONT and pharmacological inhibition of microglia/macrophages increases isl2b:GFP+ RGC cell number at 7 dpi.

5. And finally, JAK/STAT signaling is dispensable for maintaining isl2bI:GFP+ RGC cell numbers when microglia/macrophages are inhibited.

Comments for the author

This is a well-written research report that contributes to the fields of retinal degeneration and regeneration with the advance that crosstalk between the innate immune response and RGC-based JAK/STAT signaling modulates RGC cell survival. At this time, however, there are major issues to be addressed before the data reported in this paper justify the conclusions drawn.

Major Issues
1. On its own, isl2b:GFP is not sufficient for assessing RGC survival due to the decrease in GFP fluorescence intensity at 7 dpi (Fig. 1C), which is the time point used for the survival assessments in later figures. Furthermore, isl2b decreases by 4.4-fold in gene expression at 24 hpi according to the RNAseq results in Table S2 and depending on the dynamics of protein translation and GFP half-life, this may be reflected at 7 dpi. The authors acknowledge the decrease in fluorescence intensity in lines 260-268 and immunostain for cleaved caspase 3 to detect an increase in apoptotic cells at 7 dpi (Supp. Figure 1). However, in the pharmacological inhibition experiments, particularly with P6 treatment (Fig. 3F,G and Fig. 4K with Supp Fig. 2), it is unclear whether the quantification of isl2b:GFP+ cells is an assessment of RGC survival or simply a further reduction in isl2b:GFP expression. In order to fully assess RGC survival, an additional metric is needed, such as the cleaved caspase 3 immunostain, to indicate that Jak inhibition increases cell death, Dex/PLX3397 decreases cell death, and the combination of P6 and PLX3397 also decreases cell death.

2. One major conclusion is that JAK/STAT inhibition is dispensable for RGC cell survival when microglia/macrophages are inhibited. However, there is no confirmation that JAK/STAT signaling is inhibited with the combined PLX3397/P6 drug treatment. Immunostaining for phospho-Stat3 would confirm that, indeed JAK/STAT signaling is still inhibited in the combined drug treatment.

3. The statement at lines 399-400 that "these data strongly support a model in which crosstalk between neurotoxic signals emanating from macrophages/microglia…" is too strongly worded for the data presented in the figures. With the Dex treatment, isl2b:GFP+ cell number increased, but Dex did not change the percent area of mCherry+ microglia/macrophages and although PLX3397 drastically decreased the percent area of mCherry+ microglia/macrophages, no data was presented to show that neurotoxic signals emanating from macrophages/microglia were affected. In order to support this claim, data such as sphericity with the Dex treatment and qPCR for inflammatory signals, needs to be presented.

Minor Issues
1. The Larison and Bremiller, 1990 citation on lines 246-247 is not the correct citation for the zn-8 antibody. Larison and Bremiller describe an antibody that labels double cone photoreceptors.

2. Clarification is needed in the Materials & Methods about the treatment conditions on each fish. On lines 198-201, RGC survival quantification is described as being calculated as the ratio of isl2b:GFP+ RGCs in the left (ONT+) eye divided by the isl2b:GFP+ RGCs in the right (ONT- control) eye of the same fish. However, in Figures 3 and 4 there are quantifications of RGC survival for ONT- eyes with Ctr or drug treatment. Does this mean that, throughout the entire study, the right eye was always ONT- and untreated while the left eye was often ONT+ (with or without treatment) but also sometimes ONT- with treatment?

3. In the figure legends and in the Materials & Methods at line 99, the biological replicates are sometimes referred to as N and sometimes as n. They should all be referred to as n.

4. In lines 152-156 it is unclear how the drug concentrations were determined. Were they determined by assessing experimentally at several different concentrations or were they determined based on the two references cited? Perhaps the authors intend on line 155 to say something like, "Similar to (Elsaeidi et al. 2014; Bollaerts et al. 2019)…"

5. In the "Pharmacological experiments" section of Materials & Methods (lines 151-161), it is not indicated what the vehicle control is for the PLX3397 treatment.

6. At line 198, "40X magnification" should instead read "40X objective."

7. The figure legends should include a statement of how many times the experiment shown was replicated in the laboratory as requested by the "Checklist of key methodological and analytical information."

First revision

Author response to reviewers' comments

We thank the Reviewers for their thoughtful critiques to the manuscript and suggestions for improvement. We have addressed all of the concerns and the revised manuscript is much stronger, so we're grateful for the constructive comments. Below, we outline changes to the manuscript (e.g. new data, text changes, etc.). We have colored these responses blue here as well as in the manuscript, to make it easier for reviewers to track revisions. We remain confident that the manuscript will be of interest to the broader readership of *Development* and especially this special issue on "The Immune System in Development and Regeneration". We hope that in this revised form, the manuscript is now acceptable for publication.

Reviewer 1 Comments for the Author:

Major points

1. RNA-seq from sorted Isl2b-GFP cells is used to identify genes induced in RGCs. However, I think additional steps can be taken to convince readers that this approach mostly captures RGCs. I also have concerns that contaminating microglia/macrophages may be contributing to this dataset. In Figure 1A, to demonstrate the Isl2b-GFP is restricted to the RGC layer a merged image is shown of half of a top half of a retinal section and then insets of each individual channel is shown from the RGC-layer. I think it would be useful to show all the retinal nuclear layers with the Isl2b-GFP channel only.

This can convince readers that you don't have contributions from photoreceptors or bipolar cells, etc. scRNA-seq from Hoang, et al. 2020 (PMID: 33004675), do seem to show Isl2b is primary expressed in RGCs so I don't think this is a huge concern but it could be demonstrated more clearly. What is more concerning is the genes that are attributed to RGCs such as Il1b, cxcr4b and other immune-associated genes. Using the data from scRNA-seq data mentioned above these genes are almost exclusively found in microglia/macrophages and not detected in RGCs. This is also consistent with the literature in mouse retina where genes such as Il1b are not found in neuronal populations but rather microglia. Therefore, I am concerned that in addition to RGCs this dataset has contributions from contaminating immune cells. I would suggest either validating expression of cxcr4b, Il1b, etc. in RGCs, cross validating RGC expression of genes with scRNA-seq generated from the Blackshaw lab, Baier lab, or others.

This type of analysis will substantially change the interpretation of the results as they are presented here.
These datasets can be queried online here:
https://proteinpaint.stjude.org/F/2019.retina.scRNA.html

A) We have added a new image of a transverse section of an adult *isl2b*:eGFP retina that shows transgene expression only in the RGC layer. These new data are included in Fig. 1A. As noted in the text, our data detected *isl2b*:eGFP in ~65% of all RGCs. This is probably a conservative estimate as Kolsch et al. 2021 recently noted that an *isl2b*:tagRFP transgene was found in nearly all RGCs.
B) While we cannot rule out the possibility that a macrophage/microglia cell that ingested a GFP+ cell was collected during our sort, we think it more likely that these genes are in fact expressed in RGCs after injury. This is consistent with published reports. For example, Il1b is expressed in mouse RGCs and is upregulated after ischemic injury (Dvoriantchikova et al., 2012). Similarly, Cxcr4b is also expressed in rat RGCs (Heskamp et al., 2013) and mouse RGCs, possibly enriched in the resilient alpha-RGC subtype (Hilla et al., 2021). Thus, our data showing upregulation in injured zebrafish RGCs are entirely consistent with published studies. We did search the zebrafish scRNA-Seq databases for adult RGCs subjected to either light or NMDA injury, and these genes were only detected in only a small subset of RGCs, but strongly in microglia, as the reviewer notes. However, these two injuries do not affect the RGCs so it is possible that a direct RGC injury in zebrafish, such as the optic nerve transection presented here, stimulates RGC expression, as it does in rodents.

2. In figure 1 it is argued that Isl2b-GFP can be downregulated without RGC death (7dpi), yet in Figures 3 and 4 Isl2b-GFP+ cell counts are used as a proxy for RGC survival at 7dpi. I think an orthogonal marker should be used to quantify RGC survival one that does not have evidence for transient downregulation to injury Stimulus.

To address this concern, we performed additional cleaved caspase-3 staining on 7dpi A) ONT+; B) ONT+/P6-treated; and C) ONT+/PLX3397-treated animals. Data demonstrate a significant increase in caspase-3+ RGCs in P6-treated animals, which is rescued by PLX3397 treatment. These data support RGC death after ONT, that is increased when the Jak/Stat pathway is inhibited, and prevented when macrophages and microglia are depleted by PLX3397. These new data are included in a new Fig. S3.

3. For the experiments with the Jak/Stat pathway inhibitor P6 or dexamethasone, I think the interpretation could be expanded. Because these compounds will not work cell autonomously on RGCs and surely effect Muller glia and microglia, which have been reported to respond to both of these pathways and significantly contribute to RGC-survival, it is hard to piece apart the contributions. For example, the authors show that P6 significantly decreases RGC survival after axotomy. However, when microglia are ablated P6 no longer has an effect. This is interpreted as "Jak/Stat activity is dispensable in the absence of microglia recruitment".
However, both P6 and Dexamethasone can act directly on microglia and the effects observed here could be due directly to this. In this interpretation P6 does nothing in the absence of microglia because the effect of Jak/Stat inhibition was due to the microglial response to this drug. This same problem applies to the dexamethasone experiments and it would be interesting to see if Dex still has an effect with microglia ablation. Overall these effects are large and interesting, but I think the interpretations are not strongly supported by the data, additional experiments, caveats or re-interpretations should be provided.

To address this comment we performed additional pSTAT3 immunostaining on 1dpi ONT+ *isl2b*:GFP+;*mpeg1*:mCherry fish. pSTAT3 staining was located in RGCs, as expected, but also within macrophages/microglia. These new data are included in Fig. S5. Per the Reviewer's suggestion, we have expanded our interpretations to include this possibility. Future studies targeting loss- and/or gain-of-function assays to RGCs and/or leukocytes will be needed to parse specific roles within each cell type, but we feel that these are beyond the scope of this initial study.

Minor Points

1. In figure 3a the "zoom" panels should show the merged stain. Or both channels independently with arrows.
It is hard to determine the co-labeling of pStat3 with RGCs as the image is presented now.

We have added the merged panel of the zoom adjacent to DAPI/pSTAT3 images in revised Fig. 3A.

2. Sex should be used instead of "gender" when describing animals in line 96.

This has been corrected.

Reviewer 2 Comments for the Author:
This is a well-written research report that contributes to the fields of retinal degeneration and regeneration with the advance that crosstalk between the innate immune response and RGC-based JAK/STAT signaling modulates RGC cell survival. At this time, however, there are major issues to be addressed before the data reported in this paper justify the conclusions drawn.

Major Issues
1. On its own, isl2b:GFP is not sufficient for assessing RGC survival due to the decrease in GFP fluorescence intensity at 7 dpi (Fig. 1C), which is the time point used for the survival assessments in later figures. Furthermore, isl2b decreases by 4.4-fold in gene expression at 24 hpi according to the RNAseq results in Table S2 and depending on the dynamics of protein translation and GFP half-life, this may be reflected at 7 dpi. The authors acknowledge the decrease in fluorescence intensity in lines 260-268 and immunostain for cleaved caspase 3 to detect an increase in apoptotic cells at 7 dpi (Supp. Figure 1). However, in the pharmacological inhibition experiments, particularly with P6 treatment (Fig. 3F,G and Fig. 4K with Supp Fig. 2), it is unclear whether the quantification of isl2b:GFP+ cells is an assessment of RGC survival or simply a further reduction in isl2b:GFP expression. In order to fully assess RGC survival, an additional metric is needed, such as the cleaved caspase 3 immunostain, to indicate that Jak inhibition increases cell death, Dex/PLX3397 decreases cell death, and the combination of P6 and PLX3397 also decreases cell death.

As above for Reviewer 1 - Comment 2, to address this concern, we performed additional cleaved caspase-3 staining on 7dpi A) ONT+; B) ONT+/P6-treated; and C) ONT+/PLX3397-treated animals. Data demonstrate a significant increase in caspase-3+RGCs in P6-treated animals, which is rescued by PLX3397 treatment. These data support RGC death after ONT, that is increased when the Jak/Stat pathway is inhibited, and prevented when macrophages and microglia are depleted by

PLX3397. These new data are included in Fig. S3.

2. One major conclusion is that JAK/STAT inhibition is dispensable for RGC cell survival when microglia/macrophages are inhibited. However, there is no confirmation that JAK/STAT signaling is inhibited with the combined PLX3397/P6 drug treatment. Immunostaining for phospho-Stat3 would confirm that, indeed, JAK/STAT signaling is still inhibited in the combined drug treatment.

To address this concern, we performed pSTAT3 immunostaining on 7dpi A) ONT+; B) ONT+/P6 treated/PLX3397 treated animals. pSTAT3 staining was eliminated by PLX3397 and IV injection of P6. These new data are included in Fig. S5.

3. The statement at lines 399-400 that "these data strongly support a model in which crosstalk between neurotoxic signals emanating from macrophages/microglia…" is too strongly worded for the data presented in the figures. With the Dex treatment, isl2b:GFP+ cell number increased, but Dex did not change the percent area of mCherry+ microglia/macrophages and although PLX3397 drastically decreased the percent area of mCherry+ microglia/macrophages, no data was presented to show that neurotoxic signals emanating from macrophages/microglia were affected. In order to support this claim, data such as sphericity with the Dex treatment and qPCR for inflammatory signals, needs to be presented.

We have addressed this comment in several ways:

1) We have toned down the text, as discussed above Reviewer 1 - Comment 3.
2) We have added two new pieces of data, per the Reviewer's comment.
    a) We quantified sphericity of macrophages/microglia in dexamethasone- treated retinae. No significant differences were observed. However, these data are consistent with our previous data showing that Dex had no effect on macrophage/microglia recruitment and literature showing that Dex treatment has no effect on macrophage/microglia recruitment in various contexts (e.g. Chatzopoulou et al., 2016; Warchol, 1999; Xie et al., 2019; Leach et al., 2021) and can actually enhance phagocytic activity by leukocytes (Giles et al. 2001; Desgeorges et al. 2019). These new data are included in Fig. S4B.
    b) We performed qPCR for the inflammatory markers *il1-B*, *il-6* and *tnf-a* after ONT. These markers were all elevated after ONT, showing inflammation after optic nerve injury, but suppressed by dexamethasone treatment. These new data are included in Fig. S4A.

Minor Issues

1. The Larison and Bremiller, 1990 citation on lines 246-247 is not the correct citation for the zn-8 antibody. Larison and Bremiller describe an antibody that labels double cone photoreceptors.

This was corrected to Trevarrow et al., 1990.

2. Clarification is needed in the Materials & Methods about the treatment conditions on each fish. On lines 198-201, RGC survival quantification is described as being calculated as the ratio of isl2b:GFP+ RGCs in the left (ONT+) eye divided by the isl2b:GFP+ RGCs in the right (ONT- control) eye of the same fish. However, in Figures 3 and 4 there are quantifications of RGC survival for ONT- eyes with Ctr or drug treatment. Does this mean that, throughout the entire study, the right eye was always ONT- and untreated while the left eye was often ONT+ (with or without treatment) but also sometimes ONT- with treatment?

We are sorry for the possible confusion here. In all experiments, the right eye was always ONT- (+/- DMSO) and used as the control, while the left eye was always the treated eye (ONT+ and/or with drug) eye. We have clarified the Material & Methods.

3. In the figure legends and in the Materials & Methods at line 99, the biological replicates are sometimes referred to as N and sometimes as n. They should all be referred to as n.

These have been corrected.

4. In lines 152-156 it is unclear how the drug concentrations were determined. Were they determined by assessing experimentally at several different concentrations or were they determined based on the two references cited? Perhaps the authors intend on line 155 to say something like, “Similar to (Elsaeidi et al. 2014; Bollaerts et al. 2019)…”

This has been clarified.

5. In the “Pharmacological experiments” section of Materials & Methods (lines 151-161), it is not indicated what the vehicle control is for the PLX3397 Treatment.

This has been corrected.

6. At line 198, “40X magnification” should instead read “40X objective.”

This has been corrected.

7. The figure legends should include a statement of how many times the experiment shown was replicated in the laboratory as requested by the “Checklist of key methodological and analytical information.”

This has been clarified.

Second decision letter

MS ID#: DEVELOP/2021/199694

MS TITLE: Retinal ganglion cell survival after severe optic nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune responses in the zebrafish retina

AUTHORS: Si Chen, Kira Lathrop, Takaaki Kuwajima, and Jeffrey Gross

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

As an important point raised by the reviewers, the authors need to carefully consider the possibility of contamination by microglia/macrophage in their bulk RNA-seq. Thus, the authors should discuss this point, could re-interpret this data or provide new confirmatory evidence.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

I have filled this out in the original submission.

Comments for the author

This revised manuscript is improved and the majority of my concerns were addressed. I appreciate the changes in the text that allow for broader interpretation of the results with Dexamethasone and Stat inhibition. Overall, I think this study is well done and has interesting results. However, I still disagree with the interpretation that RGCs express the innate immune genes reported by the authors.

- The authors point out the possibility that it is possible an immune cell phagocytosed the reporter gene and was included in the sort. However, I don't think this needs to occur to have microglia in your dataset, particularly in damaged retinas. It is well reported that microglia can contaminate datasets in scRNA-seq studies even after retinal dissociation and flow sorting for particular reporters not expressed in microglia. See: Jorstad, et al.; PMID: 32075759 Boudreau-Pinsonneault, et al: https://www.biorxiv.org/content/10.1101/2021.07.05.451124v1 .

- I do agree that cxcr4 has been convincingly shown to be in RGCs, but this is not the case with Il1b or the interferon factors. The paper cited (Dvortaintchikova, et al) for Il1b expression in RGCs, provides very unconvincing evidence of an immunostain of Il1b (which shows up in all retinal cell types).
This contrasts with the thorough single cell libraries of healthy and damaged RGCs in mouse retina that do not show expression of Il1b (Hoang, et al. 33004674 and Tran, et al. PMID: 31784286). The authors note that Il1b is not found in the zebrafish RGCs scRNA-seq databases in healthy retina but may be upregulated after injury that directly effects RGCS. However, the dataset provided by Hoang, et al is after NMDA injury which does damage zebrafish RGCs (Powell, et al. 27094545 Lahne, et al. 33598455).
- In support of the idea that microglia/macrophages may be contaminating the dataset presented here, both Mafb and Sall1 are in the top upregulated genes from injured RGCs. Mafb is a classical macrophage marker and a critical regulator of microglia (see: Matcovtich-Natan, et al. 2016; PMID 27338705). Similarly, Sall1 is expressed highly in microglia (see; Buttgereit, et al. 2016; PMID: 27776109), and not found in the damaged or healthy zebrafish RGCs.
In the absence of confirmatory evidence that RGCs do in fact express these innate immune genes (i.e. co-localized RNA-scope, scRNA-seq, etc.) , I think the weight of the evidence suggests that there is immune cell contamination in the RNA-seq dataset and the data should be interpreted accordingly.

Minor point:
The text still reads "gender" and should be replaced with "sex".

Reviewer 2

Advance summary and potential significance to field

The revisions to this manuscript by Chen et al. address the major concerns that were raised following the original submission, and now the data more fully justify the conclusions drawn. There are still some minor concerns to address to improve clarity, specifically with the newly added data and text.

Comments for the author

1. The other reviewer previously commented that gender should be changed to "sex" in the Animals subsection of Materials and Methods. However, it still reads "gender" and should be changed to "sex."

2. In the Pharmacological experiments section of Materials and Methods, the drug concentrations are indicated as "5uM P6 and 10uM dexamethasone." The u's should be changed to μ so that is reads "5 μM P6 and 10 μM dexamethasone."

3. The Figure 1 legend indicates that scale bars = 50 μm, and yet, the scale bars in the 20X and 40X images of Figure 1A are the same length. The authors should check the lengths of the bars in the images and/or indicate in the legend that the bars represent different lengths.

4. The new Supplemental Figure 3 would benefit from reorganization. The order of the image panels should match the order of the bars on the graph. Thus either switch the order of the first two image panels in A or switch the first two bars in the bar graph.

5. The right-side image in Supplemental Figure 5B looks simply like the green channel (isl2b:GFP), with both the pStat3 and mpeg1:mCherry dropped out. In contrast right-side of Figure 5A shows the pStat3 and mpeg1:mCherry signals. It would be better to have both panels on the right show the same signals, (matching the right side of Supp Fig 5A) and show a merge of the yellow (pSTAT3) and magenta channels (mpeg1:mCherry) so that the pSTAT3 staining is easier to see. Also, the lower right panel should be labeled as "pStat3 mpeg1:mCherry" like the upper right panel. The legend should then be changed to more accurately describe what is labeled in the left and right columns.

6. The legend for Supplemental Figure 4 does not match the order of panels presented. Sphericity quantification is described for panel A, but the graph is panel B and vice versa for the RT-qPCR presented in panel A (but described as panel B). The description in the manuscript text properly matches the figure.

7. The reference to Fig. S4B in the text of the section titled "Blocking inflammation or depletion of macrophages/microglia protects RGCs after ONT" is confusing. The new text (in blue) that was added to refer to the Fig. 4B sphericity data first states that no change was detected in phagocytic activity (I believe this should say "sphericity") and then says this is consistent with references that Dex can enhance phagocytic activity of leukocytes. But these data are contradictory. Instead, the data in Figure 4F-G demonstrate that Dex treatment increases the isl2b:GFP cell number after ONT as compared to DMSO treatment. In this case, Dex does affect recruitment of microglia/macrophages (no change in percent area of mCherry-expressing microglia/macrophages following ONT as compared to DMSO-treated control in Fig. 4H), yet it does prevent the increase in sphericity (Fig. S4B) that occurs following ONT (Fig. 4E) and it suppresses the expression of inflammatory markers (Fig. S4A), demonstrating that microglia/macrophages can still be present after ONT, but it's the blocking of the inflammation response that promotes RGC survival. The reference to Fig. S4B should be edited to improve clarity.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
I have filled this out in the original submission.

Reviewer 1 Comments for the Author:
This revised manuscript is improved and the majority of my concerns were addressed. I appreciate the changes in the text that allow for broader interpretation of the results with Dexamethasone and Stat inhibition. Overall, I think this study is well done and has interesting results. However, I still disagree with the interpretation that RGCs express the innate immune genes reported by the authors.

- The authors point out the possibility that it is possible an immune cell phagocytosed the reporter gene and was included in the sort. However, I don't think this needs to occur to have microglia in your dataset, particularly in damaged retinas. It is well reported that microglia can contaminate datasets in scRNA-seq studies even after retinal dissociation and flow sorting for particular

reporters not expressed in microglia. See: Jorstad, et al.; PMID: 32075759, Boudreau-Pinsonneault, et al: https://www.biorxiv.org/content/10.1101/2021.07.05.451124v1 .

- I do agree that cxcr4 has been convincingly shown to be in RGCs, but this is not the case with Il1b or the interferon factors. The paper cited (Dvortaintchikova, et al) for Il1b expression in RGCs, provides very unconvincing evidence of an immunostain of Il1b (which shows up in all retinal cell types).
This contrasts with the thorough single cell libraries of healthy and damaged RGCs in mouse retina that do not show expression of Il1b (Hoang, et al. 33004674 and Tran, et al. PMID: 31784286). The authors note that Il1b is not found in the zebrafish RGCs scRNA-seq databases in healthy retina but may be upregulated after injury that directly effects RGCS. However, the dataset provided by Hoang, et al is after NMDA injury which does damage zebrafish RGCs (Powell, et al. 27094545, Lahne, et al. 33598455).

- In support of the idea that microglia/macrophages may be contaminating the dataset presented here, both Mafb and Sall1 are in the top upregulated genes from injured RGCs. Mafb is a classical macrophage marker and a critical regulator of microglia (see: Matcovtich-Natan, et al. 2016; PMID 27338705). Similarly, Sall1 is expressed highly in microglia (see; Buttgereit, et al. 2016; PMID: 27776109), and not found in the damaged or healthy zebrafish RGCs.

In the absence of confirmatory evidence that RGCs do in fact express these innate immune genes (i.e. co-localized RNA-scope, scRNA-seq, etc.) , I think the weight of the evidence suggests that there is immune cell contamination in the RNA-seq dataset and the data should be interpreted accordingly.

We do not disagree with the Reviewer here. We had hoped our previous edit addressed this, showing examples where these genes could be expressed by RGCs. However, in hindsight, we also see the counter argument, that we never explicitly said this could simply be contamination, which is what we should have also done in the last revision. To address this here, we have removed all mention of the innate immune genes from our text presentation of the 12hpi data and we have also removed a transition sentence later in the Results where we refer to these in setting up the Dex and PLX experiments. In addition, at the first (and now only) mention of these genes in the 24hpi RNA-Seq results, we have modified the text as follows:

"Moreover, the interferon regulatory factor genes, irf9 and irf1b (Langevin et al., 2013), and the chemokine receptor, cxcr4b (García-Cuesta et al., 2019), were also upregulated. While these genes have been shown to be expressed in rodent RGCs after injury (Dvoriantchikova et al., 2012; Hilla et al., 2021), it is also possible that a small number of macrophages and microglia contaminated our sorted isl2b:GFP^{+} RGCs."

We hope that these further edits now adequately address this concern.

Minor point:
The text still reads "gender" and should be replaced with "sex".

This has been corrected

Reviewer 2 Advance Summary and Potential Significance to Field:
The revisions to this manuscript by Chen et al. address the major concerns that were raised following the original submission, and now the data more fully justify the conclusions drawn. There are still some minor concerns to address to improve clarity, specifically with the newly added data and text.

Reviewer 2 Comments for the Author:
1. The other reviewer previously commented that gender should be changed to "sex" in the Animals subsection of Materials and Methods. However, it still reads "gender" and should be changed to "sex."'

This has been corrected

2. In the Pharmacological experiments section of Materials and Methods, the drug concentrations are indicated as “5uM P6 and 10uM dexamethasone.” The u’s should be changed to μ so that is reads “5 μM P6 and 10 μM dexamethasone.”

This has been corrected

3. The Figure 1 legend indicates that scale bars = 50 μm, and yet, the scale bars in the 20X and 40X images of Figure 1A are the same length. The authors should check the lengths of the bars in the images and/or indicate in the legend that the bars represent different lengths.

This has been corrected

4. The new Supplemental Figure 3 would benefit from reorganization. The order of the image panels should match the order of the bars on the graph. Thus, either switch the order of the first two image panels in A or switch the first two bars in the bar graph.

This has been corrected

5. The right-side image in Supplemental Figure 5B looks simply like the green channel (isl2b:GFP), with both the pStat3 and mpeg1:mCherry dropped out. In contrast right-side of Figure 5A shows the pStat3 and mpeg1:mCherry signals. It would be better to have both panels on the right show the same signals, (matching the right side of Supp Fig 5A) and show a merge of the yellow (pSTAT3) and magenta channels (mpeg1:mCherry) so that the pSTAT3 staining is easier to see. Also, the lower right panel should be labeled as “pStat3 mpeg1:mCherry” like the upper right panel. The legend should then be changed to more accurately describe what is labeled in the left and right columns.

This has been corrected

6. The legend for Supplemental Figure 4 does not match the order of panels presented. Sphericity quantification is described for panel A, but the graph is panel B and vice versa for the RT-qPCR presented in panel A (but described as panel B). The description in the manuscript text properly matches the figure.

This has been corrected

7. The reference to Fig. S4B in the text of the section titled “Blocking inflammation or depletion of macrophages/microglia protects RGCs after ONT” is confusing. The new text (in blue) that was added to refer to the Fig. 4B sphericity data first states that no change was detected in phagocytic activity (I believe this should say “sphericity”) and then says this is consistent with references that Dex can enhance phagocytic activity of leukocytes. But these data are contradictory. Instead, the data in Figure 4F-G demonstrate that Dex treatment increases the isl2b:GFP cell number after ONT as compared to DMSO treatment. In this case, Dex does affect recruitment of microglia/macrophages (no change in percent area of mCherry-expressing microglia/macrophages following ONT as compared to DMSO-treated control in Fig. 4H), yet it does prevent the increase in sphericity (Fig. S4B) that occurs following ONT (Fig. 4E) and it suppresses the expression of inflammatory markers (Fig. S4A), demonstrating that microglia/macrophages can still be present after ONT, but it’s the blocking of the inflammation response that promotes RGC survival. The reference to Fig. S4B should be edited to improve clarity.

We have reworded this according to the Reviewer’s suggestion to improve clarity.

Third decision letter

MS ID#: DEVELOP/2021/199694

MS TITLE: Retinal ganglion cell survival after severe optic nerve injury is modulated by crosstalk between JAK/STAT signaling and innate immune responses in the zebrafish retina

AUTHORS: Si Chen, Kira Lathrop, Takaaki Kuwajima, and Jeffrey Gross
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.