nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: A novel neutrophil subset promotes CNS neuron survival and axon regeneration
Corresponding author name(s): Benjamin Segal

Editorial Notes:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29151A

Message: 21st Feb 2020

Dear Ben,

We received back the comments on your manuscript entitled "A novel, alternatively activated neutrophil subset promotes CNS neuron survival and axon regeneration in vivo". It was seen by 3 referees, who are all largely positive and rather consistent. I am pasting their specific comments below.

The referees are requesting some control experiments, longer time course for some experiments and clarifications. Also, several of the referees questioned the use of the human HL-60 cell line in this model, as it is a leukemic myeloid cell type, not an immature neutrophil. Primary cells might be better here.

We therefore invite you to revise your manuscript to address the concerns posed below. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions. When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
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Referee expertise:

Referee #1: Neuroimmunology

Referee #2: Neuroimmunology

Referee #3: Neuropathology

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, Sas and colleagues identify a neutrophil subset characterized by immature features and a N2-like molecular signature that is neuroprotective and axonogenic in vitro and in vivo in two models of neural injury in the eye and the spinal cord. The authors determine that the neuroprotective effects of this neutrophil subset maps mechanistically to production of neural growth factors. Then, the authors extend the neural growth factor-producing potential of neutrophils using the HL-60 human granulocyte-like cell line. Overall, the paper is well-written and the experiments are well-performed. The results of the manuscript are important as they extend the roles of neutrophils for the first time to include neuroprotection, a finding that may have translational implications in humans. Yet, a few areas require the attention of the authors to improve the presentation of the results and findings as outlined below:
1- Have the authors depleted neutrophils using 1A8 mAb in the ocular model to determine directly if neutrophil ablation impairs regeneration of the axons? Such a finding would nicely complement the work of adoptive transfers performed and presented in the manuscript.
2- In two figures, important controls are missing, that is, adoptive transfer of peritoneal neutrophils harvested at 4 hours post-zymosan (N1-like). Instead, bone marrow naive neutrophils are used as control. Given that the authors present a novel N2-like neutrophil subset it would be important to add the control adoptive transfer experiment using 4-hour harvested peritoneal neutrophils in Figures 6A, 6B and 5A (in vitro counterpart).
3- it is most likely that there is a time window after which the neutrophil neuroprotective effects will be lost due to irreversible neuronal damage. Given that the authors point out the potential translational applications of their findings down the road, it would be important to examine the timing of neutrophil-mediated presence with the ability to promote neuroprotection. This could either be done in their in vitro system or, preferably, in vivo in adoptive transfer experiments. Defining the time window after zymosan induced neuronal injury that the neutrophil-derived neuroprotective effects can be achieved will have significant implications for the translational aspects of this work as in humans, any translational approach will not be possible to do as prophylaxis but instead after the injury

has been established.
4- I appreciate the use of HL-60 as a means to extend the findings in human neutrophils, however, the authors should tone down the findings by stating that HL-60 cells are leukemic myeloid cells and therefore it is important to discuss this limitation in the manuscript. It would be ideal if the authors used primary human neutrophils and show whether upon zymosan priming they may acquire some of the features observed in the N2-like mouse counterparts. This would be much more clinically relevant and closer to human neutrophils than the HL-60 line.

Reviewer #2:
Remarks to the Author:
In this paper, the authors identified an immature neutrophil as a novel stimulator of optic nerve (ON) regeneration. They made use of intra-occular zymosan injection that is known to induce neuroprotection and regeneration, in order to find this new player. In an impressive series of experiments, they completely characterize this pro-regenerative immature neutrophil phenotype both at the molecular and functional level. Immature neutrophils (3dNφ), as opposed to their mature counterparts, protect retinal ganglion cells (RGCs) from injury-induced death and stimulate RGC axonal outgrowth both in vitro and in vivo. The authors identify growth factors that are highly likely responsible for these effects, again providing compelling in vitro and in vivo evidence. They extend their findings by showing that these neutrophils also stimulate the regeneration of ascending tracts in the spinal cord. Finally, they show that cells from a human immature neutrophil cell line are able to elicit similar neuroregenerative effects in vivo. Overall, this paper identifies a novel player in CNS nerve regeneration and provides compelling evidence for the importance of neuro-immune crosstalk in neuroprotection and -repair. Data are well presented and statistical analysis is appropriate.

The following points need to be addressed:
- In Fig. 1, the authors describe that i.o. neutrophil accumulation was delayed, but not prevented after anti-CXCR2 treatment. To avoid the misinterpretation that this effect was specific for neutrophils, the authors should also state that infiltration of monocytes and dendritic cells was delayed.

- The histograms for MPO in Fig. 2b seem to be not representative for the corresponding quantification and should be replaced by histograms that are more representative.

- Figure legend 5e,f indicates that neutrophils were added to all analyzed conditions. As this is not the case for the PBS group, the Figure legend should be rephrased.

- In order to translate their results from mouse to man (Fig. 7), the authors made use of a human HL60 cell line, polarized into an immature neutrophil phenotype. However, axon regeneration was assessed in Rag-/- mice. To proof relevance for human neuroregeneration, it would be necessary that the authors analyze co-cultures of HL60 cells with human neuronal cultures. For the cell line part, the authors should implement another immune cell control such as Jurkat T cells. For better translation to the human system, the authors should add human primary cells.

- Extended Data Figure 4: The authors show that the 3dNφ neutrophils promoted axon regeneration in Ccr2-deficient hosts, however, the graph in Extended Data Figure 4d

shows only 100 regenerating axons, which represent the PBS baseline in all other experiments. Although there was no difference between WT and KO, it seems that the experiment as such did not work, i.e. the WT animals did not regenerate either. To make a valid statement, the average of 400-500 regenerating axons after 3dNφ transfer would be required, or alternatively, a PBS control showing even less regeneration is necessary.

- The authors discuss this cell type as a putative target for the treatment of CNS injury. The authors should relate their work to other treatments of ON injury in view of the number of regenerating axons and distance of growth. ON regeneration is not a new field and there are numerous reports of treatments, such as nerve transplantation, combination of growth factors, or manipulation of regeneration inhibitors, that showed similar or more impressive data. Published treatments have led to regeneration of several thousands of axons, rather than the here observed maximum of 500, and growth beyond the optic chiasm (reviewed in Benowitz et al., 2017). It is possible that the 14-day time point used here to analyze regeneration was too short to observe long-distance growth.

- The stimulation of regeneration in the spinal cord dorsal columns does not seem to be very extensive. Are these immature neutrophils really therapeutically relevant or are other treatments more likely to make it to the clinic?

- References to Figures should be removed from the Discussion section.

Reviewer #3:
Remarks to the Author:
The manuscript by Sas et al is an exciting finding of an immature neutrophil subset that has neuroregenerative potential in the central nervous system, named "N2". The strength of the study is that beneficial effects are shown in two different regions of the CNS. Finding a novel population of cells with neuroprotective potential and identifying factors that mediate this protection carries potential for harnessing factors made by this subset of N2 to induce neural repair.

While neuroprotective properties of macrophages and T lymphocytes have been described during optic nerve and spinal cord injury, a neutrophil subset has not been previously described. Indeed, as the authors discuss, mature neutrophils are thought to be deleterious. The finding that an immature neutrophil subset is neuroregenerative is interesting since this is consistent with the notion that pathways leading to repair of neurodegeneration may be found through investigation of pathways of neurodevelopment. It would be interesting to ask whether human pediatric or animal perinatal periods are characterized an increase in relative numbers of immature as compared to mature neutrophils and whether this underlies more robust neuroregeneration at younger ages as compared to adult ages. This is beyond scope here, but the concept is intriguing.

The finding that an immature neutrophil subset may be neuroregenerative also has implications for broad immunosuppressive treatments during not only MS, but also for traumatic CNS injury to reduce edema acutely after injury. This immunosuppression might nonspecifically remove a potentially neuroregenerative "N2" neutrophil subpopulation. Thus, clinical significance is high.

The weakest part of the manuscript is the in vitro model simulating some of the in vivo

effects, here using human tissues to begin translation by using HL-60 cells as a surrogate for immature neutrophils. Indeed, neutralization occurs with NGF but not IGF-1, a major difference. Also, did the injection of the human cell line trigger a graft vs host immune reaction when injected in mice? (see below point about limitations of cell transfers even when syngeneic).

The authors may wish to refrain from implying significance of their findings beyond optic nerve and spinal cord to all axons of the CNS (which would be implicated in MS, AD, and stroke). There are regional differences in the CNS transcriptomes during both health and disease for not only neurons, but also astrocytes, oligodendrocytes, and microglia. This can have clinical significance as shown by neuromyelitis optica which preferentially targets these two CNS regions over other regions. Conversely, use of findings in this manuscript regarding soluble factors (NGF, IGF-1) might be optimally suited for treatment of neuromyelitis optica, a disabling disease in dire need of a regenerative treatment.

Despite major enthusiasm and appreciation of clinical importance, some issues must be addressed:

1. While the authors address work where inflammatory mediated mechanisms of RGC and axonal protection via lens injury or zymosan intraocular injection in the optic nerve crush model were previously attributed to monocytes/macrophages (Yin Y et al., J Neurosci 2003), and they do not address another potential mechanism of neuroprotection. Muller glia / astrocytes have also been shown to contribute to RGC and axonal protection in this system (Lorber B et al, JNR 2009). Indeed, their key neurotrophic factors may be acting on Muller glia / astrocytes instead of , or in addition to, RGCs or axons. Use of blocking antibodies to NGF and IGF-1 does not distinguish between these possibilities. Instead a conditional knockout of receptors for these factors in Muller glia / astrocytes versus RGCs is needed.
2. A major limitation in cell therapy for RGC and axonal protection is the extensive reactive astrocytic gliosis and microglial activation that occurs in the host retina following cell transplantation (Johnson TV et al, Brain 2009; Tassoni A et al, Stem Cells 2015). The authors described the presence of infiltrating monocytes following injection of immature neutrophils, and by using CCR2 deficient mice confirmed the role of immature neutrophils in promoting protection independently on infiltrating monocytes. However, they did not assess the reactive state of Muller glia, astrocytes, and microglia in playing either a beneficial and/or detrimental role in neuronal survival following transplantation. Deleterious effects involve retinal detachment and outpouching of the retina. In Extended Data Figure 4e, the DAPI stain appears to show some retinal folding/detachment, likely due to gliosis. This may be even worse in retina transplanted with HL-60 cell line (human vs mouse). Additional staining for GFAP (reactive Muller/astrocytes), Iba1 (microglia) and F4/80 in WT mice undergoing adoptive cell transfer is needed. If extensive gliosis is observed following cell transplantation, but not following injection of soluble factors, it would reveal that the soluble factor injection is better therapeutic strategy, and indeed that cell transfer is not an option. To this end, visual acuity in cell transfer experiments, as assessed by opticokinetic testing, are indicated. In addition to retinal detachment, a layer of cells on the inner retina is likely to impact visual acuity.
3. Lens injury during injection itself is known to promote inflammation-mediated axonal regeneration in ONC (S Leon, J Neurosci, 2000). This would be a confounding element in the study. To manage this confound, the authors should clarify in the methods whether mice developing cataract after i.o (indicative of lens injury) have been excluded from their study

Author Rebuttal to Initial comments

nature research

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July 9, 2020

Editors
Nature Immunology

Dear Editors,

We are pleased to submit a revised version of our manuscript entitled "A novel, alternatively activated neutrophil subset promotes CNS neuron survival and axon regeneration *in vivo*" (NI-A29151A). Based on the reviewers' suggestions we have revised Figures 5a, 6a, and 6b, added a supplementary figure (the new Extended Data Figure 5), added 2 new references (#33 and #41) and made a number of changes to the text (which are highlighted by underlying). We also added panels to Figure 7, Extended Data Fig. 1, and Extended Data Fig. 4. We believe these revisions have strengthened our manuscript, and we are hopeful that it is now acceptable for publication in the *Nature Immunology*. Point-by-point responses to the reviewer comments follow. Each comment is bulleted and italicized. Responses are in regular font.

Reviewer #1

- *Have the authors depleted neutrophils using 1A8 mAb in the ocular model to determine directly if neutrophil ablation impairs regeneration of the axons? Such a finding would nicely complement the work of adoptive transfers performed and presented in the manuscript.*

We agree with the reviewer that a neutrophil depleting experiment would complement our adoptive transfer experiments. However, we have found that systemic administration of the 1A8 mAb (an anti-Ly6G monoclonal antibody) only depletes circulating neutrophils by approximately 80%, even when used at higher doses and frequencies than those typically described in the literature. Furthermore, 1A8 preferentially depletes conventional mature Ly6G^{hi} neutrophils, as opposed to the alternatively activated, immature Ly6G^{low} subset, which would undermine the goal of the suggested experiments (ie, to deplete pro-regenerative neutrophils). We know of no other depleting antibody that is selective for neutrophils.

- *In two figures, important controls are missing, that is, adoptive transfer of peritoneal neutrophils harvested at 4 hours post-zymosan (N1-like). Instead, bone marrow naive neutrophils are used as control. Given that the authors present a novel N2-like neutrophil subset it would be important to add the control adoptive transfer experiment using 4-hour harvested peritoneal neutrophils in Figures 6A, 6B and 5A (in vitro counterpart).*

We acknowledge that peritoneal neutrophils harvested at 4 hours post-zymosan (4h Nϕ) would be a good additional negative control in the figures referenced by the reviewer. Therefore, we have repeated the

relevant experiments with a 4h Nϕ control group (see revised figures 5a, 6a and 6b). As expected, 3 day Nϕ were more effective than 4h Nϕ in promoting axon regeneration and neurite outgrowth.

- *It is most likely that there is a time window after which the neutrophil neuroprotective effects will be lost due to irreversible neuronal damage. Given that the authors point out the potential translational applications of their findings down the road, it would be important to examine the timing of neutrophil-mediated presence with the ability to promote neuroprotection. This could either be done in their in vitro system or, preferably, in vivo in adoptive transfer experiments. Defining the time window after zymosan induced neuronal injury that the neutrophil-derived neuroprotective effects can be achieved will have significant implications for the translational aspects of this work as in humans, any translational approach will not be possible to do as prophylaxis but instead after the injury has been established.*

We performed new *in vivo* and *in vitro* experiments in which the administration of pro-regenerative neutrophils was delayed following optic nerve crush (ONC) injury or the plating of cultured neurons, respectively. These studies show that pro-regenerative neutrophils are effective at promoting axon regeneration when adoptively transferred into the vitreal fluid after a delay of 6 hours (and to some extent, after 12 hours) from ONC injury (revised Extended Data Fig. 5a). Pro-regenerative neutrophils were also effective in promoting neurite outgrowth when added to retinal ganglion cell (RGC) or dorsal root ganglion neuron (DRG) cultures up to 4-8 hours after plating the neurons (Extended Data Fig. 5b, c). These data demonstrate the therapeutic efficacy of pro-regenerative neutrophils when introduced during the post-injury period, and underscore the translational implications of our findings.

- *I appreciate the use of HL-60 as a means to extend the findings in human neutrophils, however, the authors should tone down the findings by stating that HL-60 cells are leukemic myeloid cells and therefore it is important to discuss this limitation in the manuscript. It would be ideal if the authors used primary human neutrophils and show whether upon zymosan priming they may acquire some of the features observed in the N2-like mouse counterparts. This would be much more clinically relevant and closer to human neutrophils than the HL-60 line.*

We describe the limitations of the HL-60 line in the revised discussion (page 12, lines 252-253). With regard to the use of primary neutrophils, we cannot use circulating neutrophils isolated from healthy controls as a source of a pro-regenerative subset because they are uniformly mature and terminally differentiated. As mentioned in the text, we speculate that an atypical subpopulation of $Arg1^+$ immature human neutrophils, that have been identified in the setting of cancer and pregnancy, may be the counterparts of the zymosan-induced murine "N2-like" neutrophils. These human cells fall in the low density mononuclear layer of Ficoll gradients. In the future, [REDACTED]

Reviewer #2.

- *In Fig. 1, the authors describe that i.o. neutrophil accumulation was delayed, but not prevented after anti-CXCR2 treatment. To avoid the misinterpretation that this effect was specific for neutrophils, the authors should also state that infiltration of monocytes and dendritic cells was delayed.*

We have modified the text to specify that intraocular infiltration of monocytes and dendritic cells was delayed by anti-CXCR2 treatment (page 5, lines 73-4).

- *The histograms for MPO in Fig. 2b seem to be not representative for the corresponding quantification and should be replaced by histograms that are more representative.*

We have replaced the histograms for MPO in Fig. 2b with a more representative example.

- *Figure legend 5e,f indicates that neutrophils were added to all analyzed conditions. As this is not the case for the PBS group, the Figure legend should be rephrased.*

We have rephrased the Figure legend as suggested.

- *In order to translate their results from mouse to man (Fig. 7), the authors made use of a human HL60 cell line, polarized into an immature neutrophil phenotype. However, axon regeneration was assessed in Rag-/- mice. To proof relevance for human neuroregeneration, it would be necessary that the authors analyze co-cultures of HL60 cells with human neuronal cultures. For the cell line part, the authors should implement another immune cell control such as Jurkat T cells. For better translation to the human system, the authors should add human primary cells.*

Based on the reviewer's suggestions, we have tested the pro-regenerative properties of HL-60 cells when co-cultured with primary human cortical neurons. HL60 cells induced significant neurite outgrowth by the primary human neurons, while control cells (DG75 cells) did not (Figure 7d in the revised manuscript). DG75 cells are a human B cell lymphocyte (immune cell) line. We used it in the *in vitro* experiment here, in order to be consistent with our *in vivo* experiments (Figure 7b).

- *Extended Data Figure 4: The authors show that the 3dNφ neutrophils promoted axon regeneration in Ccr2-deficient hosts, however, the graph in Extended Data Figure 4d shows only 100 regenerating axons, which represent the PBS baseline in all other experiments. Although there was no difference between WT and KO, it seems that the experiment as such did not work, i.e. the WT animals did not regenerate either. To make a valid statement, the average of 400-500 regenerating axons after 3dNφ transfer would be required, or alternatively, a PBS control showing even less regeneration is necessary.*

We have repeated the experiment with CCR2-deficient versus WT hosts, adding i.o. PBS control groups. The new experiment reproduces the results of the original, and clearly shows induction of axonal regeneration in

both CCR2-deficient and wildtype mice injected with i.o. zymosan compared with PBS (Extended Data Fig. 4f in the revised manuscript).

- *The authors discuss this cell type as a putative target for the treatment of CNS injury. The authors should relate their work to other treatments of ON injury in view of the number of regenerating axons and distance of growth. ON regeneration is not a new field and there are numerous reports of treatments, such as nerve transplantation, combination of growth factors, or manipulation of regeneration inhibitors, that showed similar or more impressive data. Published treatments have led to regeneration of several thousands of axons, rather than the here observed maximum of 500, and growth beyond the optic chiasm (reviewed in Benowitz et al., 2017). It is possible that the 14-day time point used here to analyze regeneration was too short to observe long-distance growth.*
  The stimulation of regeneration in the spinal cord dorsal columns does not seem to be very extensive. Are these immature neutrophils really therapeutically relevant or are other treatments more likely to make it to the clinic?

We have expanded the *Discussion* section to acknowledge alternative interventions that promote ON axon regeneration, as described in the literature. Of note, the most successful approaches have been multimodal (for example, combining i.o. zymosan injections with a CAMP analog and PTEN deletion). In the article by Benowitz, et al. 2017 cited by the reviewer, it is stated that "combinatorial treatments that include two or more...factors enable some retinal ganglion cells to regenerate axons from the eye through the entire length of the optic nerve..." In the future, [REDACTED]. This is mentioned in the revised Discussion section (pages 13-14, lines 281-287).

The reviewer questions whether it is possible that the 14 day time point used for analysis of regeneration was too short to observe long term growth. In response to that point, we assessed axonal regeneration 28 days following ONC injury and treatment with i.o. zymosan combined with αCXCR2. In that timeframe, a significant number of regenerating axons grew a distance of over 2.0 mm from the crush site. In comparison, there was little detectable axon growth beyond 1.0 mm in the control groups (Extended Data Figure 1c). In the zymosan/ αCXCR2 group there was an average of 407 axons/nerve at 0.8 mm from the crush site on day 28, compared with 243 on day 14. Therefore, there is significant continued growth beyond the time point when we typically harvest optic nerves for counting regenerating axons.

Reviewer #3:

- *Did the injection of the human cell line trigger a graft vs host immune reaction when injected in mice? (see below point about limitations of cell transfers even when syngeneic).*

We found no evidence of a graft vs. host immune reaction in the adoptive transfer recipients of HL60 cells.

- *While the authors address work where inflammatory mediated mechanisms of RGC and axonal protection via lens injury or zymosan intraocular injection in the optic nerve crush model were previously attributed to monocytes/macrophages (Yin Y et al., J Neurosci 2003), and they do not*

  address another potential mechanism of neuroprotection. Muller glia / astrocytes have also been shown to contribute to RGC and axonal protection in this system (Lorber B et al, JNR 2009). Indeed,

their key neurotrophic factors may be acting on Muller glia / astrocytes instead of , or in addition to, RGCs or axons. Use of blocking antibodies to NGF and IGF-1 does not distinguish between these possibilities. Instead a conditional knockout of receptors for these factors in Muller glia / astrocytes versus RGCs is needed.

We agree that astrocytes and Muller glia may play an important role in RGC protection and axon regeneration in this model. In future studies, [REDACTED]. In the revised paper we discuss the potential contribution of astrocytes, Muller glia, and microglia to the repair process (pages 11-12, lines 231-237 and page 13, lines 261-4).

- *(The authors) did not assess the reactive state of Muller glia, astrocytes, and microglia in playing either a beneficial and/or detrimental role in neuronal survival following transplantation... Additional staining for GFAP (reactive Muller/astrocytes), Iba1 (microglia) and F4/80 in WT mice undergoing adoptive cell transfer is needed.*

In response to the reviewer's remarks, we have stained retinal cross-sections with GFAP and found evidence of reactive gliosis in mice 7 days following ONC injury and i.o. injection with either 3 day NΦ or PBS. However, the astrogliosis is attenuated by day 14, and comparable between the 2 groups (Extended Data Fig. 4c; page 8, lines 144-148). Iba1 staining was upregulated in the retinas of mice injected i.o. with either PBS or 3 day NΦ on day 7 post-ONC injury, which also waned by day 14. In a previous publication (PMID 25675510), we showed that microglia are activated in response to ONC injury, and are likely the initial responders to β1,3 glucan (the active ingredient of zymosan in this model). We revisit this finding on page 13, lines 261-4.

- *... visual acuity in cell transfer experiments, as assessed by opticokinetic testing, are indicated. In addition to retinal detachment, a layer of cells on the inner retina is likely to impact visual acuity.*

This is the first study describing $Ly6G^{low}Arg1^{+}$ neuroregenerative neutrophils. We show, as a proof of principal, that this novel subset has the ability to rescue dying RGC neurons and to stimulate axonal regeneration using classic experimental paradigms. An extensive literature has been published that employs the optic nerve crush model to investigate a range of therapeutic interventions, as well as underlying endogenous pathways, involved in CNS axon regeneration. In virtually all of those studies the density of regenerating axons, measured at serial distances from the crush site in longitudinal optic nerve sections, is the primary outcome measure. Visual function testing is not standardly performed. In order to restore visual function, optic nerve fibers would have to reach the lateral geniculate nucleus and form functional synapses, a process that would take at least 3 months. Furthermore, the nerves would have to remyelinate, and we (and others) have yet to investigate whether that occurs efficiently in this model. We did not intend to claim that adoptive transfer of pro-regenerative neutrophils is the ultimate cure of conditions characterized by axonal damage. However, we would argue that the discovery of this novel neutrophil subset may be an initial step in the development of immunomodulatory approaches (perhaps in conjunction with other interventions) that promote neurorepair.

Although we plan to [REDACTED].

- *Lens injury during injection itself is known to promote inflammation-mediated axonal regeneration in ONC (S Leon, J Neurosci, 2000). This would be a confounding element in the study. To manage this confound, the authors should clarify in the methods whether mice developing cataract after i.o (indicative of lens injury) have been excluded from their study*

Mice were inspected for lens injury after each i.o. injection. Those with cataracts were eliminated. This information has been added to the Material and Methods section (page 15, lines 305-306).

Thank you for the opportunity to address to the reviewers' critiques and, consequently, to improve our paper.

Sincerely,

Benjamin M. Segal, M.D.
Chair, Department of Neurology
Director, Neuroscience Research Institute
Co-director, Neurological Institute
The Gilbert and Kathryn Mitchell Endowed Professor of Neurology

Decision Letter, first revision:

Subject: Nature Immunology - NI-A29151B pre-edit

Message: Our ref: NI-A29151B

1st Sep 2020

Dear Ben,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "A novel, alternatively activated neutrophil subset promotes CNS neuron survival and axon regeneration in vivo" (NI-A29151B). I am attaching the edited manuscript. The manuscript is generally well-written, I have only some minor comments, mostly dealing with journal style.

I have made changes marked in tracked-changes, queries in red and comments are embedded throughout the manuscript, so please have the view comments option enabled.

Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the points below. We won't be able to proceed further without this detailed response.

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Please include a separate "Data availability" subsection at the end of your Online Methods. This section should inform our readers about the availability of the data used to support the conclusions of your study and should include references to source data, accession codes to public repositories, URLs to data repository entries, dataset DOIs, and any other statement about data availability. We strongly encourage submission of source data (see below) for all your figures. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, these should be included in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf.

The title should provide a clear and compelling summary of the main findings in fewer than 100 characters including spaces and without punctuation.

As a guideline, Articles allow up to 50 references in the main text. An additional 20 references can be included in the Online Methods. Only papers that have been published or accepted by a named publication or recognized preprint server should be in the numbered list. Published conference abstracts, numbered patents and research data sets that have been assigned a digital object identifier may be included in the reference list.

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consideration (and not formally accepted) may be cited only internally within the text and should not be added to the reference list. Please provide names of all authors of unpublished data. If you cite personal communications or unpublished data of any individuals who are not authors of your manuscript, you must supply copies of written permission from the primary investigator of each group cited. Permission in the form of an email will suit this purpose.

All references must be cited in numerical order. Place Methods-only references after the Methods section and continue the numbering of the main reference list (i.e., do not start at 1).

Genes must be clearly distinguished from gene products (e.g., "gene Abc encodes a kinase," not "gene Abc is a kinase"). For genes, provide database-approved official symbols (e.g., NCBI Gene, http://www.ncbi.nlm.nih.gov/gene) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

All figures and tables, including Extended Data, must be cited in the text in numerical order.

Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

All relevant figures must have scale bars (rather than numerical descriptions of magnification). Add scale bars to figures 1e and 2c.

All relevant figures must have defined error bars.

Graph axes should start at zero and not be altered in scale to exaggerate effects. A 'broken' graph can be used if absolutely necessary due to sizing constraints, but the break must be visually evident and should not impinge on any data points.

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The Methods must include a statistics section where you describe the statistical tests used. For all statistics (including error bars), provide the EXACT n values used to calculate the statistics (reporting individual values rather than a range if n varied among experiments) AND define type of replicates (e.g., cell cultures, technical replicates). Please avoid use of the ambiguous term "biological replicates"; instead state what constituted the replicates (e.g., cell cultures, independent experiments, etc.). For all representative results, indicate number of times experiments were repeated, number of images collected, etc. Indicate statistical tests used, whether the test was one- or two-tailed, exact values for both significant and non-significant P values where relevant, F values and degrees of freedom for all ANOVAs and t-values and degrees of freedom for t-tests.

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Other
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Best regards,

Laurie

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ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
The authors have addressed most of my comments and the comments of the other reviewers satisfactorily. The manuscript provides new insights on the neuroprotective role of neutrophils and neutrophil subsets.

Reviewer #2:
Remarks to the Author:
Overall, my comments and suggestions have been adequately addressed. To mention the most important points, the authors have now included data on neurite outgrowth of primary human cultures, improved the important control experiment in Extended Data Fig. 4f and addressed other treatment strategies of optic nerve injury in the discussion. Thus, I recommend this manuscript for acceptance. However, I would suggest that the authors refrain from mentioning multiple sclerosis and stroke in the first sentence of the abstract, as these diseases were not investigated in this study.

Reviewer #3:
Remarks to the Author:
The resubmitted manuscript addresses reviewers' concerns and is improved.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29151C

Message: In reply please quote: NI-A29151C

Dear Ben,

I am delighted to accept your manuscript entitled "A novel neutrophil subset promotes CNS neuron survival and axon regeneration" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

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Kind regards,

Laurie

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