JCB

# Myelination generates aberrant ultrastructure that is resolved by microglia

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May 18, 2022

Re: JCB manuscript #202204010

Prof. Mikael Simons
German Center for Neurodegenerative Diseases
Feodor Lynen Str 17
Munich 81377
Germany

Dear Prof. Simons,

Thank you for submitting your manuscript entitled "Myelination generates aberrant ultrastructure that is resolved by microglia in a phosphatidylserine-dependent manner". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers appreciate that your study uses multiple independent techniques to address outstanding questions regarding the roles of microglia. However, they have significant concerns that currently your data does not fully support your model. They have provided constructive comments and experimental suggestions, which we hope you agree are helpful. In particular, we agree that the evidence for a role of PS needs to be significantly strengthened. In addition, we hope you will be able to address all of the remaining reviewer concerns in a revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore,

if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Elior Peles
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Djannatian et. al, performs careful characterization of aberrant myelin structures that form during development and describes the various types of abnormal myelin that forms. The manuscript then explores the possibility that microglia uptake aberrant structures while finding that they are not necessary for entire sheath engulfment.
This manuscript has multiple strengths including the use of both mice and zebrafish to address questions regarding aberrant myelin. The 3D reconstructions from mouse optic nerve are beautiful, rigorously performed at various time points, and rich with information. Additionally, the zebrafish provide a good model for studying changes to aberrant myelin and microglia/oligodendrocyte roles in resolving these structures. The experimental data showing oligodendrocytes can retract sheaths and break them down internally also provides intriguing data for the field of myelin biology.
In the current iteration however, the paper makes various claims that are not supported, and multiple experiments could be strengthened by examining additional data. In particular, the title is misleading and the paper indicates a mechanism for microglia removal of myelin is identified, but no necessity or sufficiency experiments show conclusive results. This is important because it is the main advance in the publication, since microglia engulfment of myelin has been observed previously.

Major Comments:
1. Claims that microglia engulf aberrant myelin in a phosphatidylserine dependent manner are not supported.
a. Most importantly, the actual changes in amounts of aberrant myelin are not measured for any of the PS receptor mutations. This needs to be addressed prior to publication.
b. TAM receptors are not specific to PS, they also bind Gas6
c. MFG-E8-EGFP not only contains a PS binding site, but also an integrin binding site which is expressed in oligodendrocytes. Another marker to consider would be annexins.
d. Does MFG-E8-EGFP label all aberrant markers? It is intriguing that PS is present more on fragments, maybe microglia only uptake this debris and not aberrant myelin on the sheath (indicating another mechanism for removing aberrant myelin exists). How does the PS labeling used compare to PS labeling from annexins? Additionally, if a different label were used, the live imaging could be used to directly image and see if microglia remove PS labeled aberrant myelin on the sheath, PS labeled myelin fragments, or broadly remove myelin.
e. Presence of myelin in microglia could be normal myelin, aberrant myelin from the sheath, or aberrant myelin fragments (or a combination). Can be addressed in the text.
f. Loss of microglia or PS receptors on microglia leads to hypomyelination, but this is not explored in the manuscript and leads to difficulties making conclusions about changes to aberrant myelin, because reduced total myelin could then lead to less aberrant myelin.

2. The title claims that microglia resolve aberrant myelin but this is not fully examined
a. In the absence of microglia the aberrant myelin is increased at 10dpf, but it is unclear if the aberrant structures will resolve by other methods at later time points or continue to increase over time.
b. Retractions of myelin by the oligodendrocyte also appear to contribute to this resolution as well and it would be interesting to note whether oligodendrocytes resolve major myelin defects or only specific types and compare this to the type of myelin abnormalities resolved by the microglia.

Minor Comments:
1. Additional data from 3D optic nerve reconstructions could be highly valuable for more accurate interpretations
a. What profiles of myelin are being contacted by microglia? What percent are normal myelin, what percent are each aberrant myelin profile? This could indicate whether microglia are primarily targeting specific types of myelin or not.
b. How many microglia are present at each time point?
2. In line 140, the claim states that microglia show a preference for some myelin sheaths over others, but the experiment looks at regions rather than specific myelin sheaths and the claim should be adjusted to reflect this.

Reviewer #2 (Comments to the Authors (Required)):

This paper uses cutting-edge imaging and animal modelling to examine the question of myelin remodelling during development. The authors demonstrate two distinct processes. First, a microglial dependent process in which excess myelin is removed and taken up by the phagocytic cells. Second, a process independent of microglia in which individual myelin sheaths are retracted by oligodendrocytes. While both processes have been identified previously the discussion of both in a single study and the clarity and power of the experimental approach makes this an important paper

The authors might consider three further analysis to improve the paper

First, the speculation that microglial removal of excess myelin might be a mechanism of removing errors in myelination could be tested by looking at myelin sheath architecture in the mutant fish lacking PS receptors. The prediction would be that these fish would have greater variation in myelin sheath length and/or alterations in morphology

Second, I share the author's view that the hypo myelination observed in these mutants is extremely interesting and agree that interference with developmental myelination by myelin debris is likely explanation. This could be addressed by quantification of oligodendrocyte differentiation in the mutant fish

Third, and more trivial, I assume that the myelin sheath retracted by the oligodendrocyte never exhibit phosphatase are searing exposure. Could this observation be included?

A minor point - a better explanation of the specificity of microglial scanning would be helpful

Reviewer #3 (Comments to the Authors (Required)):

I reviewed this with a senior graduate student in my group. We read and reviewed independently and then created the following consensus.

In this paper, the authors investigate how aberrant myelin structures are resolved by microglia engulfment during development. The Simons lab previously discovered that myelin forms "outfoldings" transiently during development, but how these outfoldings resolve during development remain a mystery. To investigate this, the authors use an impressive combination of serial block face electron microscopy, live imaging of oligodendrocytes and microglia in zebrafish, innovative cell biology tools, and an impressive in vivo Crispr screen-really a technical tour de force. Using electron microscopy, the authors find that aberrant myelin structures such as "whorls" and "outfoldings" are relatively common early in development in the mouse optic nerve but get resolved by the close of developmental myelination. In the same dataset, the authors find evidence for microglia closely interacting with these aberrant myelin features. To further study the role of microglia in regulating these aberrant structures, the authors image microglia and oligodendrocytes in the zebrafish spinal cord and find that while phagocytosis of entire sheaths is very rare, microglia often phagocytose aberrant myelin structures. The authors also use multiple complementary phosphatidylserine receptor knockouts to show that myelin phagocytosis is dependent on multiple PS-receptors in zebrafish. Finally, the authors provide evidence for microglia-independent myelin sheath retractions and suggest that oligodendrocytes can also degrade their own myelin.

Together, these rigorous experiments define a novel cell biology mechanism that controls myelin sheath generation/refinement during development, opening the door for numerous future studies. Overall, the data in this paper are rigorously collected and clearly presented. Another strength is that, when possible, the authors use orthogonal techniques to investigate an experimental question that strengthens their conclusions. I am suggesting the following minor revisions to help readers put the work in context and appreciate a few possible limitations or alternative interpretations of the data:

1. The authors state that they were unable to find any instances of microglia phagocytosis/engulfment of entire sheaths in Figure 3. These results differ from Hughes et al., 2020, where the authors found that approximately 70% of eliminated myelin sheaths at 4dpf had been engulfed by microglia (Figure 2j, Hughes et al., 2020; PMID:32632287). It would be helpful for the authors to discuss this discrepancy at greater length in the discussion of the paper, especially given that the reporter lines and time of

imaging seem to be similar between both experiments. Are there potential technical explanations for this difference? Biological reasons?
2. I was expecting/hoping to see an experiment in the paper to determine whether the ultrastructural myelin defects seen early in development do or do not resolve in the absence of microglia. For example, there are pharmacological or genetic ways to transiently or constitutively deplete microglia from the mouse CNS; it would be very interesting to determine using the same high-resolution EM as in Figure 1 whether microglia are indeed required for clearance of the various types of myelin abnormalities seen in mouse. This is just a suggestion for future work and not a direct ask for this paper, but commenting on this as a limitation of the study would be useful.
3. The experiments using genetic reporters to mark PS are compelling, and it is particularly useful that the authors use two orthogonal tools that show the same phenotype. However, both PS reporters rely on overexpression. Can the authors comment on whether overexpression of these reporters could perturb endogenous PS content?
4. In Figure 4, these are global KOs, not microglia-specific KOs, and these genes are expressed by other cell types (potentially important is that these phagocytic receptors are also used by astrocytes for synapse refinement in the mouse, which, if affected in these mutants, could cause broader effects that indirectly influence myelin). Please acknowledge this caveat to interpreting the results of these global knockouts in the text.
5. In Figure 5I, even at the P14 time point, less than 1 percent of cells are positive for MBP fragments associated with LAMP1 puncta. This may indeed represent an important biological process (i.e. the breakdown of myelin by oligodendrocytes), but the frequency is very low. Can the authors comment in the text on why this may be so infrequent in these micrographs (I assume it is because of how quickly these MBP fragments are completely degraded in lyosomes-exploring this process using oligodendrocyte-specific perturbation of lyosome function or autophagy would be interesting in future studies). Here, since we don't know for sure that oligodendrocytes "degrade" myelin-only that this MBP signal colocalizes with a lysosomal marker, softening the language is advised.

-Brad Zuchero

We would like to thank all three reviewers for the careful assessment and the constructive criticism of our manuscript.
To address the reviewers' comments, we have additionally performed 1) a characterization of myelin aberrations in PS receptor mutants, 2) an ultrastructural analysis of myelin aberrations in *csf1r*$^{DM}$ mutants, 3) a better characterization of the hypomyelination in PS receptor mutants, 4) an analysis of the myelin aberration profiles associated with microglia in mouse optic nerve, 5) an analysis of retracting myelin sheaths regarding PS exposure.
These experiments have helped to improve the quality of the paper. The revision experiments were mainly performed by Swathi Radha and Agata Rhomberg, who have been added as authors of the manuscript.
The specific response to each reviewer is given below:

Reviewer #1:

In the current iteration however, the paper makes various claims that are not supported, and multiple experiments could be strengthened by examining additional data. In particular, the title is misleading and the paper indicates a mechanism for microglia removal of myelin is identified, but no necessity or sufficiency experiments show conclusive results. This is important because it is the main advance in the publication, since microglia engulfment of myelin has been observed previously.

Major Comments:
1. Claims that microglia engulf aberrant myelin in a phosphatidylserine dependent manner are not supported.
a. Most importantly, the actual changes in amounts of aberrant myelin are not measured for any of the PS receptor mutations. This needs to be addressed prior to publication.

We thank the reviewer for this comment. We addressed this issue by quantifying myelin outfoldings in confocal images of the zebrafish spinal cord.
The triple knockout of bai1, axl and tim1 results in significantly more myelin outfoldings as in the wild-type larvae (control: 0.0 (0.0-2.2, median and IQR), bai1;axl;tim: 6.9 (0.0-9.6, median and IQR), P = 0.0362, n = 13). We therefore conclude that the removal of myelin aberrations is at least partially dependent on PS. We have included this new data in Fig. 4I of the revised manuscript.
In addition, we followed the reviewers suggestion and modified the title.

b. TAM receptors are not specific to PS, they also bind Gas6

Yes, this is true. Gas6 serve as bridging molecule and activating ligand for receptor binding. Gas6 binds to PS and mediates its binding to the TAM receptors. Thus, TAM is an indirect PS receptor. We have edited the manuscript to make sure we describe the receptors correctly as indirect PS receptors (p. 10).

c. MFG-E8-EGFP not only contains a PS binding site, but also an integrin binding site which is expressed in oligodendrocytes. Another marker to consider would be annexins.
MFG-E8 binds to PS through its C1C2 domain and to αvβ3/5 integrin receptors through its RGD domain. We used a truncated version of MFG-E8 that contains the C1C2, but not the RGD domain, thereby rendering the construct specific for PS-binding. We clarified this in the manuscript (p. 9).

d. Does MFG-E8-EGFP label all aberrant markers? It is intriguing that PS is present more on fragments, maybe microglia only uptake this debris and not aberrant myelin on the sheath (indicating another mechanism for removing aberrant myelin exists). How does the PS labeling used compare to PS labeling from annexins? Additionally, if a different label were used, the live imaging could be used to directly image and see if microglia remove PS labeled aberrant myelin on the sheath, PS labeled myelin fragments, or broadly remove myelin.

This is an excellent suggestion of the reviewer that microglia take up myelin debris and not aberrant myelin on the sheaths. Our data supports such a model. We have so far not observed MFG-E8-EGFP labeling of myelin outfoldings. However, we cannot rule out that MFG-E8-EGFP binding to PS was incomplete. The abundance of small myelin dystrophies together with myelin fragments within the extracellular space may indeed indicate that microglial uptake of myelin debris is an important mechanism to resolve myelin aberrations. We mention this possibility in the discussion (p. 15).
As suggested by the reviewer we performed experiments using the secA5-YFP reporter described by Ham et al. (FASEB J 2010; 24:4336–4342). However, we obtained inconsistent expression and poor labeling, which may be due to low PS concentrations at the detection limit of annexin V. In fact, MFG-E8-EGFP is known to detect much lower concentrations of PS in a $Ca^{2+}$-independent manner, thus outperforming annexin V in live imaging (Kay & Grinstein *Sensors* 2011, *11*(2), 1744-1755). The figure was modified accordingly.

e. Presence of myelin in microglia could be normal myelin, aberrant myelin from the sheath, or aberrant myelin fragments (or a combination). Can be addressed in the text.

We agree that it would be interesting to know which type of myelin is phagocytosed by microglia. Our ultrastructural analysis suggests that microglia preferentially associate aberrant myelin sheaths (Fig. 2F, see our response to minor comment 1a). However, additional possibilities exist. As suggested, we address these possibilities in the discussion (p. 14-15).

f. Loss of microglia or PS receptors on microglia leads to hypomyelination, but this is not explored in the manuscript and leads to difficulties making conclusions about changes to aberrant myelin, because reduced total myelin could then lead to less aberrant myelin.

To better characterize myelination in the PS receptor mutants, we analyzed the number of OPCs and oligodendrocytes and myelin sheath length. Similar to the *csf1r*$^{DM}$ mutants, oligodendrocyte differentiation and myelin sheath growth was unaffected by the ablation of PS receptors. This new data is included in Figure S4, K-M of the revised manuscript.

The quantification of aberrant myelin was normalized by the myelinated area in Fig. 3M (*csf1r*$^{DM}$ mutants) and Fig. 4I (PS receptor mutants), showing that aberrant myelin is increased despite the hypomyelination.

2. The title claims that microglia resolve aberrant myelin but this is not fully examined
a. In the absence of microglia the aberrant myelin is increased at 10dpf, but it is unclear if the aberrant structures will resolve by other methods at later time points or continue to increase over time.

We additionally analyzed aberrations in the *csf1r*$^{DM}$ mutants at 18 dpf and found that myelin alterations persist. Thus, at least up to 18dpf, myelin alterations are not resolved by other mechanisms. We could not analyze later points because the ossification of the spine impairs optical imaging of whole spinal cord segments. This new data is included in Figure S3I of the revised manuscript.

b. Retractions of myelin by the oligodendrocyte also appear to contribute to this resolution as well and it would be interesting to note whether oligodendrocytes resolve major myelin defects or only specific types and compare this to the type of myelin abnormalities resolved by the microglia.

This is a very good suggestion that we have now looked into. We found that prior to retraction, myelin sheaths look entirely normal, as determined by confocal live imaging (with the limitation of the resolution limit of light microscopy). By time-lapse imaging, we were unable to identify any myelin alteration that predict myelin retractions. Furthermore, we did not find MFG-E8 labeling of retracting sheaths, showing that PS exposure is not a hallmark of retracting myelin sheaths. At this point, we cannot exclude that retracting myelin sheaths bear ultrastructural aberrations that we are unable to visualize by light microscopy. We discuss this issue in the manuscript. Examples of normal-appearing, retracting myelin sheaths can be found in video 5 and Fig. 5H.

Minor Comments:
1. Additional data from 3D optic nerve reconstructions could be highly valuable for more accurate interpretations
a. What profiles of myelin are being contacted by microglia? What percent are normal myelin, what percent are each aberrant myelin profile? This could indicate whether microglia are primarily targeting specific types of myelin or not.

We thank the reviewer for this valuable suggestion. We now provide quantitative data of myelin alterations in contact with microglia. We determined the myelin ultrastructure in contact with myelin at P14, and found that the vast majority exhibited a pathological ultrastructure. Only 9.6 ± 1.9% of these myelin segments exhibited normal myelin ultrastructure. Microglia were in contact with pinched-off myelin, myelin bulgings, myelin outfoldings and other myelin alterations (Fig. 2F). Given that overall 88% of the myelinated axons appeared normal and 24% exhibited myelin aberrations, microglia seem to preferentially associate with aberrant sheaths, regardless of the type of aberration.

b. How many microglia are present at each time point?

We have added a quantification of all the microglia that were present within a volume of 80 x 80 x 160 µm at P10, P14, P21 and P60. With age, the number of microglia increases in a similar manner as the number of myelinated axons (Fig. S1D). Only at P10, a very early time point in optic nerve myelination, the ratio of microglia to myelinated axons seemed temporarily increased (Fig. 1G).

Reviewer #2 (Comments to the Authors (Required)):

This paper uses cutting-edge imaging and animal modelling to examine the question of myelin remodelling during development. The authors demonstrate two distinct processes. First, a microglial dependent process in which excess myelin is removed and taken up by the phagocytic cells. Second, a process independent of microglia in which individual myelin sheaths are retracted by oligodendrocytes. While both processes have been identified previously the discussion of both in a single study and the clarity and power of the experimental approach makes this an important paper
The authors might consider three further analysis to improve the paper

First, the speculation that microglial removal of excess myelin might be a mechanism of removing errors in myelination could be tested by looking at myelin sheath architecture in the mutant fish lacking PS receptors. The prediction would be that these fish would have greater variation in myelin sheath length and/or alterations in morphology

We addressed this by quantifying myelin outfoldings in confocal images of the zebrafish spinal cord, as we have previously done for the *csf1r*$^{DM}$ mutants.
The triple knockout of bai1, axl and tim1 results in significantly more myelin outfoldings as in the wild-type larvae (control: 0.0 (0.0-2.2, median and IQR), bai1;axl;tim: 6.9 (0.0-9.6, median and IQR), P = 0.0362, n = 13). We therefore conclude that the removal of myelin aberrations is at least partially dependent on PS. We have included this new data in Fig. 4I of the revised manuscript.

Second, I share the author's view that the hypo myelination observed in these mutants is extremely interesting and agree that interference with developmental myelination by myelin debris is likely explanation. This could be addressed by quantification of oligodendrocyte differentiation in the mutant fish

To better characterize myelination in the PS receptor mutants, we analyzed the number of OPCs and oligodendrocytes and myelin sheath length. Similar to the *csf1r*$^{DM}$ mutants, oligodendrocyte differentiation and myelin sheath growth was unaffected by the ablation of PS receptors. We conclude that hypomyelination is likely due to a reduced number of myelin sheaths made by individual oligodendrocytes. This new data is included in Figure S4, K-M of the revised manuscript.

Third, and more trivial, I assume that the myelin sheath retracted by the oligodendrocyte never exhibit phosphatase are searing exposure. Could this observation be included?

We did not find MFG-E8 labeling of retracting sheaths, showing that PS exposure is not a hallmark of retracting myelin sheaths.
At this point, we cannot exclude that retracting myelin sheaths bear ultrastructural aberrations that cannot be resolved by light microscopy. We mention this issue in the discussion (p. 16).

A minor point - a better explanation of the specificity of microglial scanning would be helpful

We hypothesize that secreted 'find-me' signals, additionally to 'eat-me' signals like PS, may attract phagocytes over longer distances through chemotactic gradients. Potential candidates could be fractalkine, the nucleotides ATP and UTP, lysophosphatidylcholine (LPC) or sphingosine-1-phosphate (S1P), which are known 'find-me' signals in apoptosis. We mention this issue in the discussion (p. 15).

Reviewer #3 (Comments to the Authors (Required)):

I reviewed this with a senior graduate student in my group. We read and reviewed independently and then created the following consensus.
In this paper, the authors investigate how aberrant myelin structures are resolved by microglia engulfment during development. The Simons lab previously discovered that myelin forms "outfoldings" transiently during development, but how these outfoldings resolve during development remain a mystery. To investigate this, the authors use an impressive combination of serial block face electron microscopy, live imaging of oligodendrocytes and microglia in zebrafish, innovative cell biology tools, and an impressive in vivo Crispr screen-really a technical tour de force. Using electron microscopy, the authors find that aberrant myelin structures such as "whorls" and "outfoldings" are relatively common early in development in the mouse optic nerve but get resolved by the close of developmental myelination. In the same dataset, the authors find evidence for microglia closely interacting with these aberrant myelin features. To further study the role of microglia in regulating these aberrant structures, the authors image microglia and oligodendrocytes in the zebrafish spinal cord and find that while phagocytosis of entire sheaths is very rare, microglia often phagocytose aberrant myelin structures. The authors also use multiple complementary phosphatidylserine receptor knockouts to show that myelin phagocytosis is dependent on multiple PS-receptors in zebrafish. Finally, the authors provide evidence for microglia-independent myelin sheath retractions and suggest that oligodendrocytes can also degrade their own myelin.

Together, these rigorous experiments define a novel cell biology mechanism that controls myelin sheath generation/refinement during development, opening the door for numerous future studies. Overall, the data in this paper are rigorously collected and clearly presented. Another strength is that, when possible, the authors use orthogonal techniques to investigate an experimental question that strengthens their conclusions. I am suggesting the following minor revisions to help readers put the work in context and appreciate a few possible

limitations or alternative interpretations of the data:

1. The authors state that they were unable to find any instances of microglia phagocytosis/engulfment of entire sheaths in Figure 3. These results differ from Hughes et al., 2020, where the authors found that approximately 70% of eliminated myelin sheaths at 4dpf had been engulfed by microglia (Figure 2j, Hughes et al., 2020; PMID:32632287). It would be helpful for the authors to discuss this discrepancy at greater length in the discussion of the paper, especially given that the reporter lines and time of imaging seem to be similar between both experiments. Are there potential technical explanations for this difference? Biological reasons?

We have performed extensive time-lapse imaging of microglia and failed to observe microglia that actively strip off entire myelin sheaths from axons. We occasionally found that they engulfed small pieces of myelin without removing entire sheaths. However, the removal of entire sheaths by oligodendrocytes (i.e., retractions) seemed to largely independent of microglia. Using a PS reporter to identify aberrant myelin, we detected that most myelin reporter-positive, PS-labeled membrane was found within the extracellular space without any connection to myelin sheaths.
We cannot fully explain these discrepancies. Technical explanations are possible. Hughes et al. performed time-lapse imaging over 3h, which is in our experience not sufficient for the analyses of sheath retractions. We therefore think that the number of retractions is hugely underrepresented in their analysis, especially because retractions are a frequent event in the zebrafish spinal cord between 3 and 4 dpf. The main obstacle to visualize myelin sheath removal by time-lapse imaging is the high mobility of spinal cord microglia against the need for adequate timely and spatial resolution. Given the low number of microglia at this time point, we chose field of views that included a microglial cell at the beginning of time-lapse imaging and followed its interactions with myelin. Occasionally, we observed condensed myelin debris in distal processes of the microglia, possibly remnants of previous myelin sheath. We have discussed these apparent differences between our study and Hughes and Appel (p. 14). However, it is important to mention that the absence of evidence in our study does not exclude that microglia are able to remove entire myelin sheaths from axons.

2. I was expecting/hoping to see an experiment in the paper to determine whether the ultrastructural myelin defects seen early in development do or do not resolve in the absence of microglia. For example, there are pharmacological or genetic ways to transiently or constitutively deplete microglia from the mouse CNS; it would be very interesting to determine using the same high-resolution EM as in Figure 1 whether microglia are indeed required for clearance of the various types of myelin abnormalities seen in mouse. This is just a suggestion for future work and not a direct ask for this paper, but commenting on this as a limitation of the study would be useful.

This is an excellent suggestion. We performed scanning electron microscopy in zebrafish using our stable *csf1r*$^{DM}$ zebrafish line. We assessed myelin ultrastructure in single cross-sections of 18 dpf wild-type and *csf1r*$^{DM}$ zebrafish spinal cord. Here, we found more than twice as many myelin aberrations in *csf1r*$^{DM}$ mutants as in wild-type larvae (wt: 4.1 ± 2.3%, *csf1r*$^{DM}$: 10.9 ± 2.1%, P =

0.0048, n = 4 fish, Fig. 3, N and O). Interestingly, we could identify all profiles of myelin aberrations that we had previously described for mouse optic nerves. The increased number of myelin aberrations in *csf1r*$^{DM}$ mutants mainly represented small dystrophies of the myelin sheath or fragments pinching off (designated as 'fragments') (Fig. 3 O).

3. The experiments using genetic reporters to mark PS are compelling, and it is particularly useful that the authors use two orthogonal tools that show the same phenotype. However, both PS reporters rely on overexpression. Can the authors comment on whether overexpression of these reporters could perturb endogenous PS content?

MFG-E8 exhibits high-affinity binding to PS exposed on membranes. However, we are not aware that its presence (and its over-expression) affects PS exposure itself.

4. In Figure 4, these are global KOs, not microglia-specific KOs, and these genes are expressed by other cell types (potentially important is that these phagocytic receptors are also used by astrocytes for synapse refinement in the mouse, which, if affected in these mutants, could cause broader effects that indirectly influence myelin). Please acknowledge this caveat to interpreting the results of these global knockouts in the text.

Thank you for this valuable comment. We modified the text accordingly (p. 16).

5. In Figure 5I, even at the P14 time point, less than 1 percent of cells are positive for MBP fragments associated with LAMP1 puncta. This may indeed represent an important biological process (i.e. the breakdown of myelin by oligodendrocytes), but the frequency is very low. Can the authors comment in the text on why this may be so infrequent in these micrographs (I assume it is because of how quickly these MBP fragments are completely degraded in lyosomes- exploring this process using oligodendrocyte-specific perturbation of lyosome function or autophagy would be interesting in future studies). Here, since we don't know for sure that oligodendrocytes "degrade" myelin-only that this MBP signal colocalizes with a lysosomal marker, softening the language is advised.

MBP is locally translated in myelin sheaths and thereafter integrates into the myelin membrane. Therefore, the presence of MBP in oligodendroglial lysosomes points to the degradation of myelin membrane. As such, it is noteworthy that we find significantly more oligodendrocytes with lysosomal MBP staining at an early time point of myelination. We know from cell culture experiments that lysosomal degradation of MBP occurs fast. We agree that this is a likely explanation why only a minor fraction of oligodendrocytes exhibit lysosomal MBP staining. We value your comment that we have not directly shown myelin degradation by oligodendrocytes and adapted the text accordingly (p. 12).

December 2, 2022

RE: JCB Manuscript #202204010R

Prof. Mikael Simons
German Center for Neurodegenerative Diseases
Feodor Lynen Str 17
Munich 81377
Germany

Dear Mikael:

Thank you for submitting your revised manuscript entitled "Myelination generates aberrant ultrastructure that is resolved by microglia". Your paper has now been seen again by two of the original reviewers, both of whom now recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

**As you'll see, reviewer #1 has raised a few remaining minor points which we'd like for you to address/respond to in the final revision. Please be sure to provide a point-by-point rebuttal to these final reviewer issues.**

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes the abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, materials and methods, figure legends, references, tables, or supplemental legends. You are below this limit at the moment but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Therefore, please add size markers to the blots in Supplementary Figure 4B (not just in the Source data).

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).
**Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. We see that you mention in the statistics section of your methods that the data was tested for normality but you do not indicate what test(s) were run to determine this. Please be sure to add the name(s) of the test used to test the data for normal distribution. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."**

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model

g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page.
**We know that you have provided one already but we ask that the statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.**

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

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Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7-14 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Elior Peles, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

Thank you to the authors for the additional experiments and revisions to the manuscript. The language shift and addition of aberrant myelin quantification with regard to PS acting as a potential 'eat me' signal significantly strengthen the manuscript.

Minor Comments:

1. In line 109 the authors should specify that although microglia associate with all myelin aberrations, the only category they associate with significantly more than normal myelin is the myelin fragments.
2. The MFG-E8 associations quantified in figure 4B only account for approximately 70%. Where does the remaining 30% bind?
3. Line 210 delete 'in' within the parentheses
4. For figures 4F and S4F, a slight shift in wording in figure legends similar to the manuscript text would clarify the differences. Potentially could say, 4F: "Quantification shows the total volume of myelin fragments within all microglia"; S4F: "Average volume of myelin fragments within individual microglia"
5. Figure S3B/C, in the figure legend specify the age analyzed

Reviewer #2 (Comments to the Authors (Required)):

My comments have all been addressed. The extra experiments performed in response to the reviewers comments have clarified and improved the manuscript. The combination of technical strength and biological interest makes this a strong paper.

# 2nd Revision - Authors' Response to Reviewers: December 8, 2022

We would like to thank the reviewers for their efforts to re-revise the manuscript and are happy to hear that they recommended the revised manuscript for acceptance.

In the following, we are addressing the remaining minor comments by Reviewer #1.

1. In line 109 the authors should specify that although microglia associate with all myelin aberrations, the only category they associate with significantly more than normal myelin is the myelin fragments.
Thank you for this comment. We have adapted the text on p.5 as follows: “All of the above-described types of myelin aberrations were associated with microglia, but only the category “myelin fragments” was significantly more associated with microglia compared to normal myelin (Fig. 2F)”.

2. The MFG-E8 associations quantified in figure 4B only account for approximately 70%. Where does the remaining 30% bind?
The numbers indicate the percentage of MFG-E8-EGFP-labeled structures that exhibited mbp:mCherry-CAAX co-staining, categorized by their localization. Because we also saw MFG-E8-EGFP-labeled structures without mbp:mCherry-CAAX co-staining, numbers do not sum up to 100%.

We clarified this in the methods section (p.27) and the legend of figure 4B (p.43).

3. Line 210 delete 'in' within the parentheses
We have corrected this.

4. For figures 4F and S4F, a slight shift in wording in figure legends similar to the manuscript text would clarify the differences. Potentially could say, 4F: "Quantification shows the total volume of myelin fragments within all microglia"; S4F: "Average volume of myelin fragments within individual microglia"
We have corrected this.

5. Figure S3B/C, in the figure legend specify the age analyzed
Figure legend S3, B-D, specifies “…in the dorsal spinal cord of 4 dpf wt and csf1R$^{DM}$ larvae”.