nature portfolio

Peer Review File

Microglia deploy TAM receptors to kill motor neurons in a mouse model of amyotrophic lateral sclerosis

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Editorial Note: Parts of this peer review file have been redacted as indicated to maintain the confidentiality of other journals.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Huang et al was originally submitted to [redacted] but has been transferred to Nature Communications due to the senior author assuming emeritus status which precluded a standard revision. New data demonstrating accumulation of MER and AXL protein in Iba1+ microglia in ALS patient spinal cord was an important addition to support the potential relevance of the study to human disease. If new data cannot be generated, then the manuscript text should be revised to address the remaining issues outlined below.

Major:

Despite acknowledgement in their rebuttal letter that motor neurons are not the only Chat+ neurons in the mouse spinal cord, in addition to their demonstration that many non-motor neurons in SOD1 spinal cord express PtdSer on their cell surface (Fig 1i) and would presumably be subject to TAM mediated phagocytosis, the authors continue to refer Chat+ material as being exclusively from motor neurons. See "~10-fold reduction in ChAT+ motor neuron cytoplasm within the SOD1 G93A Axl -/- Mertk-/- compared to SOD1 G93A microglial lysosomes." And "a large reduction in motor neuron engulfment upon TAM mutation (Supplementary Fig 4a)". The same issue is present in the Abstract, where "we find that microglial lysosomes are filled with the remains of motor neurons in SOD1G93A spinal cord". If the authors truly wanted to prove that motor neurons were being engulfed, they could use an AAV system to selectively label SOD1G93A motor neurons and show preferential uptake by microglia in vivo. Without this functional evidence, it should be acknowledged in the text that Chat+ material could derive from other non-motor neuron cells and does not exclusively represent motor neurons.

The outcome of less Chat+ material in spinal cord SOD1 G93A Axl -/- Mertk-/-microglia relative to SOD1 G93A microglia could be explained by increased lysosomal degradative activity of Axl -/- Mertk -/-microglia rather than impaired uptake of Chat+ material. It is also possible that Axl -/-Mertk-/-microglia are just better than wild type microglia at providing a neuroprotective factor to support motor neuron viability rather than showing impairment of production of a toxic factor as mentioned in the discussion. I appreciate the authors efforts to summarize published in vitro studies demonstrating Axl -/-Mertk-/-microglia are impaired at phagocytosis in other settings, but a formal demonstration that microglia engulf SOD1G93A motor neurons in a dish and impairment of this phagocytosis mediated killing by Axl -/-Mertk-/- microglia would be important to establish this conceptual leap in SOD1 ALS. If experiments to address these possibilities can't be performed, then these points should be acknowledged as a limitations of the study.

The last paragraph of the discussion reads like a review of the author's other work, with little relevance to ALS or the findings of the current project. The authors should make an effort to relate how ScFv or nanobody protein engineering tools could be used in the context of ALS.

They authors should also state and explain in the manuscript their view that "The potential of using TAM-directed agents – e.g., Axl and Mer small-molecule tyrosine kinase inhibitors that are in development for the treatment of various cancers – as potential ALS therapies, without addressing the fundamental underlying cause of ALS that is making motor neurons sick in the first place, does not to us seem tenable."

Minor

Gas6 data and GFAP data from in SOD1 and SOD1 Axl-/-Mertk-/- mice in response to reviewer's previous comments should be included in the revised manuscript.

Supplementary Fig 2e describes a 32kDa form of SOD1 as a misfolded aggregate observed in SOD1 G93A mice and in familial ALS patients with SOD1 mutations. However, this high molecular weight species represents a natural homodimer of

the normally 16 kDA monomer of SOD1 (PMID: 15952898). Evidence suggests that trimers may be the toxic form of this protein (PMID: 29666246).

Reviewer #3

(Remarks to the Author)
In their responses to my questions, the authors have provided very reasonable answers. I have no further questions.

NCOMMS-25-97295-T revisions made in response to points raised by Reviewer 1

We thank Reviewer 1 for their evaluation of our manuscript, transferred and revised from [redacted] to *Nature Communications*. Reviewer 2 was satisfied with the revisions we made, and had no additional requests for further changes. In the point-by-point response below, Reviewer 1's comments are in *Arial 11 point blue*, and our replies are in Arial 12 black.

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Major:

Despite acknowledgement in their rebuttal letter that motor neurons are not the only Chat+ neurons in the mouse spinal cord, in addition to their demonstration that many non-motor neurons in SOD1 spinal cord express PtdSer on their cell surface (Fig 1i) and would presumably be subject to TAM mediated phagocytosis, the authors continue to refer Chat+ material as being exclusively from motor neurons. See "~10-fold reduction in ChAT+ motor neuron cytoplasm within the SOD1 G93A Axl -/-Mertk-/- compared to SOD1 G93A microglial lysosomes."

We have replaced "*motor neuron*" in this sentence with "*neuronal*".

In the introductory sentence of the paragraph that contains this sentence, we have also changed "*…since motor neurons are the most abundant ChAT$^+$ cells in this region"* to "*…since motor neurons account for >90% the ChAT$^+$ cells in this region*", and provided a reference. (The ChAT$^+$ cells in the ventral horn of the lumbar spinal cord that are not skeletal or visceral motor neurons are primarily Pitx2$^+$ V0$_C$ interneurons.)

And "a large reduction in motor neuron engulfment upon TAM mutation (Supplementary Fig 4a)".

We have replaced "*motor*" in this sentence with "*cholinergic*".

The same issue is present in the Abstract, where "we find that microglial lysosomes are filled with the remains of motor neurons in SOD1G93A spinal cord".

We have replaced "*motor*" in this sentence of the Abstract with "*cholinergic*".

If the authors truly wanted to prove that motor neurons were being engulfed, they could use an AAV system to selectively label SOD1G93A motor neurons and show preferential uptake by microglia in vivo. Without this functional evidence, it should be acknowledged in the text that Chat+ material could derive from other non-motor neuron cells and does not exclusively represent motor neurons.

We have added this sentence (lines 350-52): "*ChAT is not a definitive marker for skeletal and visceral motor neurons in the ventral lumbar spinal cord, since select interneurons in this region are also cholinergic*" and provided a reference.

In addition to these corrections, we have changed "*diseased motor neurons*" in the title to the Fig. 5 legend to "*diseased cholinergic neurons*".

The outcome of less Chat+ material in spinal cord SOD1 G93A Axl -/- Mertk-/-microglia relative to SOD1 G93A microglia could be explained by increased lysosomal degradative activity of Axl -/- Mertk -/-microglia rather than impaired uptake of Chat+ material.

There is no evidence of which we are aware for *enhanced* lysosomal degradation in *Axl*$^{-/-}$*Mertk*$^{-/-}$ microglia or other macrophages.

It is also possible that Axl -/-Mertk-/-microglia are just better than wild type microglia at providing a neuroprotective factor to support motor neuron viability rather than showing impairment of production of a toxic factor as mentioned in the discussion.

Indeed, this is a possibility. However, we looked for TAM-dependent regulation of an mRNA encoding a neurotrophic/neurotoxic factor, and couldn't find any. As illustrated in the paper (Supp. Fig. 2d), mRNA encoding TGF, which is neurotrophic in several settings, is actually *reduced* in the *SOD1Axl*$^{-/-}$*Mertk*$^{-/-}$ spinal cord. In our earlier work (PMID 33859405), we used single-cell RNA sequencing to examine the transcriptomes of wild-type versus *Axl*$^{-/-}$*Mertk*$^{-/-}$ microglia, and could not detect significant up-regulation of a neurotrophic factor or down-regulation of a neurotoxic factor in double mutant cells.

I appreciate the authors efforts to summarize published in vitro studies demonstrating Axl -/-Mertk-/-microglia are impaired at phagocytosis in other settings, but a formal demonstration that microglia engulf SOD1G93A motor neurons in a dish and impairment of this phagocytosis mediated killing by Axl -/-Mertk-/- microglia would be important to establish this conceptual leap in SOD1 ALS. If experiments to address these possibilities can't be performed, then these points should be acknowledged as a limitations of the study.

We have now acknowledged (lines 397-400) that real-time visualization of microglial engulfment and/or dismemberment of motor neurons in disease was not part of our study, and is an important avenue for future research.

The last paragraph of the discussion reads like a review of the author's other work, with little relevance to ALS or the findings of the current project. The authors should make an effort to relate how ScFv or nanobody protein engineering tools could be used in the context of ALS.

We did not advocate for the use of these tools in ALS. Although they are based on our work, they were independently developed by other investigators. We highlight the results of their application to cancer and lupus models to illustrate that recombinant engagement of the TAM system can be used to phagocytically kill living cells that are deleterious. This is potentially a *much* bigger therapeutic story.

They authors should also state and explain in the manuscript their view that "The potential of using TAM-directed agents – e.g., Axl and Mer small-molecule tyrosine kinase inhibitors that are in development for the treatment of various cancers – as potential ALS therapies, without addressing the fundamental underlying cause of ALS that is making motor neurons sick in the first place, does not to us seem tenable."

We agree, and have now added this language to the second-to-last paragraph of the discussion (lines 410-16).

Minor
Gas6 data and GFAP data from in SOD1 and SOD1 Axl-/-Mertk-/- mice in response to reviewer's previous comments should be included in the revised manuscript.

We have now added the Gas6 data and the GFAP and Aldh1l1 expression data per cell as new panels g and h in Supplementary Figure 2.

Supplementary Fig 2e describes a 32kDa form of SOD1 as a misfolded aggregate observed in SOD1 G93A mice and in familial ALS patients with SOD1 mutations. However, this high molecular weight species represents a natural homodimer of the normally 16 kDA monomer of SOD1 (PMID: 15952898). Evidence suggests that trimers may be the toxic form of this protein (PMID: 29666246).

Since the paper contains no experiments addressing the role of this dimer in disease, we now only mention its reduced expression in the *SOD1^{G93A}Axl$^{-/-}$Mertk$^{-/-}$* spinal cord in the Results.