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The cationic amino acid exporter Slc7a7 is induced and vital in zebrafish tissue macrophages with sustained efferocytic activity

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MS TITLE: The cationic amino acid exporter Slc7a7 is vital for and induced in tissue macrophages with sustained efferocytic activity

AUTHORS: Doris Lou DEMY, Mireille Carrere, Ramil Noche, Muriel Tauzin, Marion Le Bris, Chooyoung Baek, Malika Yousfi, Ignaty Leshchiner, Wolfram Goessling, and Philippe Herbomel
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

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all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The article entitled "The cationic amino acid exporter Slc7a7 is vital for and induced in tissue macrophages with sustained efferocytic activity" by Demy et al. presented a distinct mechanism underlying the macrophage/microglia defect in the slc7a7-deficient mutant zebrafish, which was previously reported by Casano et al. (Cell Reports 11: 1008-1017) where they suggested that the lack of microglia in slc7a7-deficient mutants is caused by the impaired brain colonization of microglial precursors. In this study, the authors, using DIC microscopy and time-lapse confocal imaging, showed that microglial precursors in the slc7a7 mutants and slc7a7 morphants are capable of colonizing the optic tectum (OT) and retina but died shortly after engulfing apototic neurons in the brain. In parallel, they also observed a similar phenotype in the slc7a7-deficient macrophages in the peripheral tissues where developmental cell death is known to occur. The authors hypothesized that slc7a7 is essential for the survival of microglia/macrophages during and after efferocytosis. To further support this hypothesis, they treated the fish embryos with phenylhydrazine, a potent chemical that can cause death of red blood cells, and showed that excessive red blood cell apoptosis can result in extensive death of macrophages in slc7a7 morphants but not in the control animals. Finally, using whole-mount in situ hybridization, the authors showed that the slc7a7 expression is upregulated by apoptotic cell death. Based on these data, the authors concluded that slc7a7 expression is induced in macrophages upon efferocytosis and the functional Slc7a7 is essential for the survival of the efferocytic macrophages.
Overall, this study reveals an intriguing mechanism regulating the survival of macrophage/microglia during and after efferocytosis. This work will be suitable for publication upon addressing the following concerns.

Comments for the author

Specific comments:
1. In Fig.2 and Fig.4:
In order to prove that the death of macrophages/microglia in slc7a7 mutants is indeed induced by efferocytosis, it would be nice if the authors can calculate the rate of death of microglia/macrophages with and without efferocytosis.
2. In Fig. 5 and Fig. 6:
The authors showed that the slc7a7 expression is induced by apoptotic cell death. It would be nice if the authors can provide additional evidence to show that the cell death induced slc7a7 expression is indeed in the macrophages but not in other cells like neutrophils.
3. In Fig. 2E-F:
At 48 hpf (before the colonization of retina), mutant embryos already showed fewer mfap4+ and L-plastin+ cells than siblings. Given the fact that Casano et al. showed that slc7a7 expression can be detected as early as 22 hpf (Casano et al. 2015), it seems that slc7a7 may also involve in the earlier events, such as differentiation and colonization of microglia/macrophages. The authors should discuss these possibilities in the discussion.
4. There are numerous errors throughout the main text and legends. Further editing is required. Some of them are listed below.
1) Fig 2D legend: The quantification of microglia is in the OT instead of retinas.
2) Fig 4 panel labels: B-C and D-E are mislabeled.
3) Fig S4: No panel labels for B and C. B-F were not explained in the main text.
4) Main text Page 7 line 12: Fig. S6E instead of Fig. S5E should be cited.

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Reviewer 2

Advance summary and potential significance to field

The authors carried out a forward genetic screen and identified a zebrafish mutant affecting microglia development. The mechanism that they identified that underlies the absence of microglia is fascinating and novel: the mutation lies in an amino acid transporter, Slc7a7, causing highly 'efferocytic'/engulfing macrophages, including the microglia, to undergo apoptosis, likely due to a defect in processing/accumulation of excess amino acids. The authors show very convincing evidence using in vivo analyses, that this is the mechanism by looking at multiple phagocytic macrophage populations at different developmental stages.
Additionally, by inducing cell death in the neuromast cells using copper sulfate and in erythrocytes using phenylhydrazine, the authors show they could induce a upregulation of slc7a7 in phagocytic macrophages, and respectively death of erythrocyte engulfing macrophages in slc7a7 deficient zebrafish exposed. The authors thus use a range of experiments to independently verify their hypothesis, making this a robust and complete study. This study and the peculiar phenotype identified gives an important hint of the importance of amino acid metabolism, in particular their export from the cell, for in vivo macrophage function. Also it raises intriguing questions for example regarding the human disorder caused by SLC7A7 mutations.

The authors note that another group previously identified the same gene in this screen and came to a very different conclusions than the current. Based on the evidence shown by the current authors and their arguments explaining how the other group came to a likely mis-interpretation of the data seems entirely valid. This leaves open the question how to address this in the current manuscript. The authors explain in the discussion why they think the data in the other paper was misinterpreted, and this seems a fair way to address this.

Comments for the author

The authors thus use a range of experiments to independently verify their hypotheses, making this a robust and complete study. The quality of the microscopic images throughout the manuscript is excellent.

What is the effect of the mutation? The authors mention a A>T transversion at the splice junction between intron 4 and exon 5 and show a schematic, but for completeness it would be good to know what the mutation is and the predicted effect. Does it cause non-sense mediated RNA decay? Will it cause an inframe deletion?

The quality of the microscopic images throughout the manuscript is excellent, nevertheless information indicating what is shown in the images is almost exclusively shown in the legends, making interpretation of the figures per se difficult. It would in my opinion facilitate reading if the figures were more self-explanatory.

Consistent use of genetic nomenclature (genes are sometimes written in regular font, should be italic) and consistent use of wildtype/WT.

Discussion authors mention LPI: I assume this refers to Lysinuric protein intolerance, this abbreviation should be introduced.

Additional minor comments:
Results: In an F3 i.s.o. In a F3 (as it is "In an [Ef]3")

Movie 6 is very short, so short that it is virtually impossible to understand what is shown. It would be useful to have some kind of title in the movies. It would also be helpful to merge the control/mutant movies side-by-side.

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Reviewer 3

Advance summary and potential significance to field

In this study, Demy, Carrère et al investigate the functions of the Slc7a7 cationic amino acid transporter in tissue macrophages, with a focus on microglia in the brain. To do this, they use a Slc7a7-deficient zebrafish model that was previously identified in an ENU mutagenic screen based on lack of microglia (Rossi et al 2015). Revisiting the phenotype of the slc7a7 mutant using a combination of genetics and imaging approaches, the authors confirm that loss of Slc7a7 in zebrafish embryos is associated with defective microglia ontogeny but reach new conclusions regarding the mechanisms underlying this phenotype. In essence these conclusions can be summarized in the following way: microglia precursors in scl7a7 mutant embryos successfully colonize the brain but die soon after as they are unable to cope with the sustained efferocytic activity triggered by the developmental wave of neuronal apoptosis. These findings differ from that of Rossi and colleagues, who concluded that expression of slca7 is necessary for seeding of the brain parenchyma by microglial precursors.

This is an interesting and elegant study. The topic is relevant, given the increasing interest in microglia studies. The use of zebrafish, where the availability of transgenic marker lines coupled to transparency at early stages of life makes a direct in vivo analysis feasible, is also an original approach to address macrophage/microglia development. The manuscript is well written and challenges the original study by Rossi et al. Collectively, the data are solid and convincing, as well as make a compelling case. However several issues should be addressed before the article is suitable for publication, by either text modifications or additional experiments.

Comments for the author

Major points:

1) Throughout the paper, the authors alternate between using the mutant line and the morpholinos for their functional investigations. Although they convincingly demonstrate that the morpholinos recapitulate the mutant phenotype, hence validating this approach, the back-and-forth is confusing at times. The same can be said regarding the use of multiple macrophage reporter lines (mpeg1:Kaede; mpeg1:mCherry; mfap4:mcherry etc…). While this does not impact the conclusions of the study, the manuscript would benefit from more uniformity.

2) Figure 1 shows discrepancies in the total number of microglia cells between the different panels. While panels B, F and G show almost no microglia in the optic tectum at 4 dpf, panels C, I and K indicate that ~10 cells on average are present at the same developmental stage. The authors should clarify this point.

3) In Fig 2D, the authors report that maximum 10 macrophages are present in the optic tectum of 48 hpf wild-type embryos. I find that 10 macrophages is a surprising low cell count, especially based on what has been reported in the literature. The authors should quantify the cell numbers not just using transgenics but by performing WISH or immuno-staining for L-plastin, as performed in Figure 2E.

4) Figure 4: The authors say that slc7a7 is vital for the survival of highly efferocytic tissue macrophages.
Could they perform rescue analyses to support their conclusion and to fully establish that slc7a7 is required within the macrophage lineage in a cell autonomous way?

5) Figure 7 raises several issues. It speculates that expression of the unknown epitope recognized by the 4C4 antibody is induced in macrophages upon efferocytosis. What is the point the authors want to make? Do they believe that slc7a7 might be the epitope? If so, is there a way they could demonstrate it?
Overall, I am not convinced that these experiments do really add to the story. If the authors want to include it in the manuscript, they should use a much more convincing approach. For example, would it be possible for the authors to control apoptosis using an inducible model?

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Minor points:

1) Regarding Figure 2E, I could not find whether quantification of L-plastin+ cells was performed by WISH or immuno staining.

2) In the legend, Figure 2D refers to the retinas and Figure 2F to the optic tectum. It is the opposite in the Figure.

3) Figure 2G: The legend states that the developmental stage is 54 hpf while in the text it says 48 hpf.

4) Figure 3: Neutrophil infiltration in the brain and retinas of the mutant is an interesting and surprising observation. If the authors have data to document the phenotype in more detail (for example: how long do neutrophils stick around? Is there a continual influx from the periphery?), it would constitute a nice addition.

5) Figure 5L: Axes are labeled in French.

6) Figure S2: based on the gene expression analysis in Table S1, slc3a2a shows a higher expression level than slc7a7 in macrophages. The title of the figure is therefore misleading and should be revisited accordingly.

First revision

Author response to reviewers' comments

Responses to the Reviewers (in blue)

Reviewer 1 Advance Summary and Potential Significance to Field:
The article entitled "The cationic amino acid exporter Slc7a7 is vital for and induced in tissue macrophages with sustained efferocytic activity" by Demy et al. presented a distinct mechanism underlying the macrophage/microglia defect in the slc7a7-deficient mutant zebrafish, which was previously reported by Casano et al. (Cell Reports 11: 1008-1017) where they suggested that the lack of microglia in slc7a7-deficient mutants is caused by the impaired brain colonization of microglial precursors. In this study, the authors, using DIC microscopy and time-lapse confocal imaging, showed that microglial precursors in the slc7a7 mutants and slc7a7 morphants are capable of colonizing the optic tectum (OT) and retina but died shortly after engulfing apoptotic neurons in the brain. In parallel, they also observed a similar phenotype in the slc7a7-deficient macrophages in the peripheral tissues where developmental cell death is known to occur. The authors hypothesized that slc7a7 is essential for the survival of microglia/macrophages during and after efferocytosis. To further support this hypothesis, they treated the fish embryos with phenylhydrazine, a potent chemical that can cause death of red blood cells, and showed that excessive red blood cell apoptosis can result in extensive death of macrophages in slc7a7 morphants but not in the control animals. Finally, using whole-mount in situ hybridization, the authors showed that the slc7a7 expression is upregulated by apoptotic cell death. Based on these data, the authors concluded that slc7a7 expression is induced in macrophages upon efferocytosis and the functional Slc7a7 is essential for the survival of the efferocytic macrophages.
Overall, this study reveals an intriguing mechanism regulating the survival of macrophage/microglia during and after efferocytosis. This work will be suitable for publication upon addressing the following concerns.

Reviewer 1 Comments for the Author:
Specific comments:
1. In Fig.2 and Fig.4:
In order to prove that the death of macrophages/microglia in slc7a7 mutants is indeed induced

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by efferocytosis, it would be nice if the authors can calculate the rate of death of microglia/macrophages with and without efferocytosis.

- Indeed. We did try to block efferocytosis by several means, but with no success. Macrophages/microglia have so many sensors and receptors to engulf apoptotic corpses that it is very difficult to prevent them significantly from doing so!
i) We attempted to inhibit efferocytosis by CNS macrophages/microglia as Mellen et al. (Cell Death & Diff. 15:1279) did successfully in explants of developing chick retina - i.e. by incubating zebrafish embryos in phospho-L-serine (acting as a competitor of the phosphatidylserine presented on apoptotic cells and recognized by macrophages), or 3-methyladenine. None of these inhibited efferocytosis significantly at non-toxic doses in our model.
ii) We used an anti-Elmo-1 morpholino reported to inhibit apoptotic corpse engulfment by macrophages in the CNS of zebrafish embryos (van Ham et al, Curr. Biol 2012). In our hands, it only increased the extent of cell death in the CNS (as more recently reported for cranial ganglia neurons by Mikdache et al. 2020, Cell. Mol. Life Sci. 77:161), but did not inhibit efferocytosis of these corpses by macrophages/microglia.
iii) We specifically inhibited apoptosis in neurons; however, neuronal death is a major attractive signal for macrophages to the CNS (Xu et al., 2016); in absence of this signal, macrophages don't colonize the optic tectum, making it impossible to conclude on a rescue in *cerise*.
Thus altogether, despite substantial efforts, we have not managed to suppress efferocytosis by zebrafish macrophages/microglia.

2. In Fig. 5 and Fig. 6:
The authors showed that the slc7a7 expression is induced by apoptotic cell death. It would be nice if the authors can provide additional evidence to show that the cell death induced slc7a7 expression is indeed in the macrophages but not in other cells like neutrophils.

i) In the post-PHZ ISH images, the numerous slc7a7+ cells in the blood circulation clearly contain (slc7a7-negative) efferophagosomes, which indicates that they are macrophages. We have now included tentatively better close-ups on these cells (Fig. 5I',J',J") to show this.
ii) In slc7a7-deficient zebrafish larvae, there is a massive infiltration of neutrophils in the brain and retina - tissues which by 3-4 dpf are full of apoptotic bodies (Fig. 3, Fig. S5). Yet we have detected no slc7a7 RNA signal by ISH in the mutant CNS by then (although we detected a normal level of slc7a7 signal in their gut and kidney), which indicates that these numerous apoptotic bodies did not induce slc7a7 detectably in the neutrophils.
iii) In our unpublished transcriptome data about WT neutrophils in zebrafish larvae, the only CAA transporter gene that we found to be detectably expressed in neutrophils was slc7a3a (encoding CAT- 3), and still weakly relative to slc7a7 in macrophages in a similarly global assay (our Table S1) . If the proximity of apoptotic corpses induced slc7a7 expression in neutrophils, given that there always is some level of apoptosis going on among the various tissues that neutrophils wander through, we would expect at least some detectable slc7a7 expression in the neutrophil population of WT larvae.

3. In Fig. 2E-F:
At 48 hpf (before the colonization of retina), mutant embryos already showed fewer mfap4+ and L- plastin+ cells than siblings. Given the fact that Casano et al. showed that slc7a7 expression can be detected as early as 22 hpf (Casano et al. 2015), it seems that slc7a7 may also involve in the earlier events, such as differentiation and colonization of microglia/macrophages. The authors should discuss these possibilities in the discussion.

- i) At 48 hpf, the retina is already fully colonized (the retinal microglia number has already reached its plateau; see our manuscript p.5 and refs therein).
ii) Casano/Rossi et al. 2015 detected *slc7a7* expression at 22 hpf in only very few early macrophages (~4 / embryo according to their Fig. 3K).
iii) At 48 hpf, the total number of macrophages in genotyped mutants is only slightly (12 %) lower than in WT (Fig. S1F). This can easily be accounted for also by the importance of *slc7a7* in macrophages with sustained efferocytic activity, because primitive macrophages in the interstitial compartment(which in zebrafish includes the open blood circulation in the yolk

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sac) have to engulf a number of apoptotic cells before 48 hpf, e.g. i) a variable number of apoptotic pro-erythroblasts by the onset of blood circulation; ii) the apoptotic neurons of the cranial ganglia by 30- 35 hpf (Herbomel et al., 2001; Mikdache et al., 2020). In addition, Cole & Ross (Dev. Biol. 240:123, 2001) detected a transient wave of cell death in the still neuroepithelial WT retina at 36 hpf that, while not as dramatic as the later neuronal DCD wave, may reasonably explain the slightly (25%) lower number of macrophages that we have counted in the retina of mutant vs. WT embryos at 42 hpf, and that also impacts on the total number of macrophages in the embryo by then. Thanks to the reviewer's remark, we have now included these likely explanations in our Discussion (p.9).

4. There are numerous errors throughout the main text and legends. Further editing is required. Some of them are listed below.
1) Fig 2D legend: The quantification of microglia is in the OT instead of retinas.
2) Fig 4 panel labels: B-C and D-E are mislabeled.
3) Fig S4: No panel labels for B and C. B-F were not explained in the main text.
4) Main text Page 7 line 12: Fig. S6E instead of Fig. S5E should be cited.

- Thank you for noticing these. We have corrected them.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors carried out a forward genetic screen and identified a zebrafish mutant affecting microglia development. The mechanism that they identified that underlies the absence of microglia is fascinating and novel: the mutation lies in an amino acid transporter, Slc7a7, causing highly 'efferocytic'/engulfing macrophages, including the microglia, to undergo apoptosis, likely due to a defect in processing/accumulation of excess amino acids. The authors show very convincing evidence using in vivo analyses, that this is the mechanism by looking at multiple phagocytic macrophage populations at different developmental stages. Additionally, by inducing cell death in the neuromast cells using copper sulfate and in erythrocytes using phenylhydrazine, the authors show they could induce a upregulation of slc7a7 in phagocytic macrophages, and respectively death of erythrocyte engulfing macrophages in slc7a7 deficient zebrafish exposed. The authors thus use a range of experiments to independently verify their hypothesis, making this a robust and complete study. This study and the peculiar phenotype identified gives an important hint of the importance of amino acid metabolism, in particular their export from the cell, for in vivo macrophage function. Also it raises intriguing questions for example regarding the human disorder caused by SLC7A7 mutations.
The authors note that another group previously identified the same gene in this screen and came to a very different conclusions than the current. Based on the evidence shown by the current authors and their arguments explaining how the other group came to a likely mis-interpretation of the data seems entirely valid. This leaves open the question how to address this in the current manuscript. The authors explain in the discussion why they think the data in the other paper was misinterpreted, and this seems a fair way to address this.

Reviewer 2 Comments for the Author:
The authors thus use a range of experiments to independently verify their hypotheses, making this a robust and complete study. The quality of the microscopic images throughout the manuscript is excellent.
- What is the effect of the mutation? The authors mention a A>T transversion at the splice junction between intron 4 and exon 5 and show a schematic, but for completeness it would be good to know what the mutation is and the predicted effect. Does it cause non-sense mediated RNA decay? Will it cause an inframe deletion?

- The likely predicted effect of the mutation was the skipping of exon 5. Following the Reviewer's question, we have extracted and sequenced the full mutant slc7a7 mRNA, and found indeed a clearcut skipping of exon 5 within an otherwise WT mRNA. This causes a frameshift from the beginning of exon 6, leading to a premature stop codon nine codons downstream. Hence the predicted mutant protein comprises only the N-terminal half of the 501 amino acid WT Slc7a7 protein, followed by 9 new amino acids. We have now included a new passage explaining this in detail (p.3). It does not seem that the mutation causes much non-sense mediated RNA decay, as the *slc7a7* mRNA signal by standard ISH in the kidney and

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gut of mutants is similar to WT, and were able to easily amplify mutant slc7a7 cDNA from total mRNA and establish its nucleotide sequence.
Consistent with this, in humans the status of the mutant Slc7a7 mRNA and protein from LPI patients has been studied in detail by a Finnish LPI lab (e.g.Toivonen et al. 2013, Gen. Physiol. Biophys. 32:479 and refs therein). They found that even frameshift mutations causing a premature nonsense codon and loss of the C-terminal third of the protein did not diminish the level of Slc7a7 mRNA. The truncated protein was made, but remained in the cytoplasm (didn't get inserted in the plasma membrane).

The quality of the microscopic images throughout the manuscript is excellent, nevertheless information indicating what is shown in the images is almost exclusively shown in the legends, making interpretation of the figures per se difficult. It would in my opinion facilitate reading if the figures were more self-explanatory.

- Consistent use of genetic nomenclature (genes are sometimes written in regular font, should be italic) and consistent use of wildtype/WT.
- Discussion authors mention LPI: I assume this refers to Lysinuric protein intolerance, this abbreviation should be introduced.

- Indeed ! We have done so now, and have also added a short introduction to the disease in the final paragraph of the Discussion.

Additional minor comments:
- Results: In an F3 i.s.o. In a F3 (as it is "In an [Ef]3").

- Corrected !

- Movie 6 is very short, so short that it is virtually impossible to understand what is shown. It would be useful to have some kind of title in the movies. It would also be helpful to merge the control/mutant movies side-by-side

- All movies do have titles and legends (provided at the end of the Supplementary Information pdf). We have now removed this pair of movies (ex-movies 6 and 7) from the revised manuscript, as Movie 7 reflected only a single observation.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this study, Demy, Carrère et al investigate the functions of the Slc7a7 cationic amino acid transporter in tissue macrophages, with a focus on microglia in the brain. To do this, they use a Slc7a7-deficient zebrafish model that was previously identified in an ENU mutagenic screen based on lack of microglia (Rossi et al 2015). Revisiting the phenotype of the slc7a7 mutant using a combination of genetics and imaging approaches, the authors confirm that loss of Slc7a7 in zebrafish embryos is associated with defective microglia ontogeny but reach new conclusions regarding the mechanisms underlying this phenotype. In essence these conclusions can be summarized in the following way: microglia precursors in scl7a7 mutant embryos successfully colonize the brain but die soon after as they are unable to cope with the sustained efferocytic activity triggered by the developmental wave of neuronal apoptosis. These findings differ from that of Rossi and colleagues, who concluded that expression of slca7 is necessary for seeding of the brain parenchyma by microglial precursors.
This is an interesting and elegant study. The topic is relevant, given the increasing interest in microglia studies. The use of zebrafish, where the availability of transgenic marker lines coupled to transparency at early stages of life makes a direct in vivo analysis feasible, is also an original approach to address macrophage/microglia development. The manuscript is well written and challenges the original study by Rossi et al. Collectively, the data are solid and convincing, as well as make a compelling case.
However, several issues should be addressed before the article is suitable for publication, by either text modifications or additional experiments.

Comments for the Author: Major points:
1) Throughout the paper, the authors alternate between using the mutant line and the

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morpholinos for their functional investigations. Although they convincingly demonstrate that the morpholinos recapitulate the mutant phenotype, hence validating this approach, the back-and-forth is confusing at times. The same can be said regarding the use of multiple macrophage reporter lines (mpeg1:Kaede; mpeg1:mCherry; mfap4:mcherry etc…). While this does not impact the conclusions of the study, the manuscript would benefit from more uniformity.

- This study has been performed over several years, and we took advantage of the best available transgenic lines for macrophages detection at every moment, up to the recent Tg(mfap4:mCherry) line which is nowadays the best macrophage reporter in zebrafish in terms of signal-to-noise ratio.
While the use of these diverse transgenic lines lacks uniformity, we also feel that having obtained so consistent results over time using different genetic backgrounds actually confirms and strengthens our results.
Concerning the sp22 morpholino, we found useful to verify and show that it fully mimicks all the traits of the cerise mutant phenotype – i.e. not only the absence of microglia in the CNS, but also the influx of neutrophils there, and so on… In addition, considering that the *cerise* mutants represent only 25% of the cerise offspring, and they are not distinguishable from their siblings until their microglia is gone by 3-4 dpf, we have also used the sp22 morpholino for most experiments in which we had to examine slc7a7-deficient embryos before 3-4 dpf (e.g. the long time-lapse confocal imaging sequences in which we imaged one WT and one slc7a7-deficient embryo in parallel).

2) Figure 1 shows discrepancies in the total number of microglia cells between the different panels. While panels B, F and G show almost no microglia in the optic tectum at 4 dpf, panels C, I and K indicate that ~10 cells on average are present at the same developmental stage. The authors should clarify this point.

- Here we need to separately discuss images (panels B, F, I, K) and graphs (panels C, G).

a) Images: panels B, F are neutral red staining images, hence a single focal plane within the OT, showing only macrophages in the focal plane that contain at least a few engulfed apoptotic bodies (=the basis of NR staining), whereas panels I, K are maximum projections of confocal image z-stacks spanning the whole depth of the OT, hence showing macrophages throughout the OT, and regardless of their efferocytic status. It is therefore expected that less macrophages are seen in B, F than in I,K. In addition, these images are only examples; there is anyway a variable number of macrophages left in the OT by 4 dpf; as we have explained in the Results section, while some mutant larvae have virtually no more NR stained macrophages in the OT (as in panel B here), others still have a few remaining ones, usually at the posterior-lateral borders of the OT (as shown in Fig. S3A-C; and this is actually why Muriel Tauzin initially named this mutant “cerise”, the French word for “cherry”, as they evoked to her cherries on either side of a midline "stalk").

b) Graphs: the difference in cell counts in the C and G graphs is indeed a significant one. The experiment shown in graph C was performed 7 years before that in graph G, only a few generations away from the isolation of the mutant and the original ENU mutagenesis. We think that generation after generation, the variability of the phenotype has decreased as we i) outcrossed the fish to fresh WT in order to clean the genome from other potential ENU-generated mutations; ii) selected carriers with a strong and clear phenotype. This is likely why the mutant phenotype is stronger overall in graph G than in graph C. Following the reviewer's remark, we have now added a sentence in Fig.1 legend mentioning the seven years lag between graphs C and G, and the above likely explanation, as this provides an interesting element of perspective on the data.

3) In Fig 2D, the authors report that maximum 10 macrophages are present in the optic tectum of 48 hpf wild-type embryos. I find that 10 macrophages is a surprising low cell count, especially based on what has been reported in the literature. The authors should quantify the cell numbers not just using transgenics but by performing WISH or immuno-staining for L-plastin, as performed in Figure 2E.

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- These low numbers in the OT at 48 hpf are in full agreement with the litterature. Indeed, the forebrain, midbrain (= mostly the OT) and hindbrain each contain only about 5-10 L-plastin positive cells by 48 hpf (Herbomel et al., 2001). As mentioned in our manuscript (p.5), the strong and specific colonisation of the OT occurs between 60 and 96 hpf (Herbomel et al., 2001), in parallel with and dependent on the wave of neuronal DCD there (Xu et a. 2016). The mCherry+ macrophages in the Tg(mfap4:mCherry) line follow the same kinetics, colonizing the OT up to 4 dpf to constitute a proper microglial population (Fig. 2D, Fig.1J).

4) Figure 4: The authors say that slc7a7 is vital for the survival of highly efferocytic tissue macrophages. Could they perform rescue analyses to support their conclusion and to fully establish that slc7a7 is required within the macrophage lineage in a cell autonomous way?

- i) It is highly likely that *slc7a7* is required within the macrophage lineage since this gene is essentially expressed in macrophages (then kidney and gut).
ii) The suggested rescue experiment was already presented by Rossi et al. (2015) in their Fig. 3E.

5) Figure 7 raises several issues. It speculates that expression of the unknown epitope recognized by the 4C4 antibody is induced in macrophages upon efferocytosis. What is the point the authors want to make? Do they believe that slc7a7 might be the epitope? If so, is there a way they could demonstrate it? Overall, I am not convinced that these experiments do really add to the story. If the authors want to include it in the manuscript, they should use a much more convincing approach. For example, would it be possible for the authors to control apoptosis using an inducible model?

- We don't particularly believe that Slc7a7 might be the 4C4 epitope. Fig.7 documented preliminary yet compelling evidence in our view that like slc7a7, expression of the 4C4 epitope may be associated and therefore triggered by sustained efferocytic activity in macrophages, rather than being a specific microglia trait. However, we have now done the experiment suggested by the Reviewer, by co- immunodetecting 4C4 and macrophages following the same phenylhydrazine treatment killing erythrocytes that we had shown to induce slc7a7 in the macrophages that engulfed the apoptotic erythrocytes. We found no induction of 4C4 in the efferocytic macrophages in this setting. Therefore, despite intriguingly common expression features with slc7a7, the case of 4C4 will have to be digged further in a future study. So we have removed this Figure from the revised manuscript.

Minor points:
1) Regarding Figure 2E, I could not find whether quantification of L-plastin+ cells was performed by WISH or immuno staining.
2) In the legend, Figure 2D refers to the retinas and Figure 2F to the optic tectum. It is the opposite in the Figure.
3) Figure 2G: The legend states that the developmental stage is 54 hpf while in the text it says 48 hpf.

- Thanks for noticing these. We have corrected them.

4) Figure 3: Neutrophil infiltration in the brain and retinas of the mutant is an interesting and surprising observation. If the authors have data to document the phenotype in more detail (for example: how long do neutrophils stick around? Is there a continual influx from the periphery?), it would constitute a nice addition.

- We already showed that we first detect their influx into the CNS by 3 dpf, and that they are still there by 4 dpf; we also provided a 6 hr time-lapse movie of their dynamics in and around the mutant retina (Movie 5 of previous Ms, 6 in the revised Ms), and measurements of their interaction times with the sick macrophages/microglia in the CNS (Fig. S5). We have now added three more movies, one showing the global dynamics of neutrophils over 10 hrs in morphant vs. WT CNS by 4 dpf (new Movie 5), and two of a z-scroll through the optic tectum of mutant and sibling by 4 dpf, showing the relative positions of neutrophils and dying microglia in the mutant (new Movies 7 and 8). Following the Reviewer's suggestion, we have

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now also scrutinized the presence of neutrophils in the CNS up to 9 dpf, and have documented that in new panels in Fig. S5. We found that by 6 and 9 dpf, neutrophils were less frequently observed in the mutant CNS, and that whenever present, they always correlated with the presence of dying macrophages/microglia, with which they interacted. We have added a sentence mentionning this in the Results (p.6) and also in the Discussion (p.9).
To study if there is a continual influx from the periphery would involve time-consuming long-term cell tracking experiments of neutrophils that are beyond the focus of this paper.

5) Figure 5L: Axes are labeled in French.
- Corrected

6) Figure S2: based on the gene expression analysis in Table S1, slc3a2a shows a higher expression level than slc7a7 in macrophages. The title of the figure is therefore misleading and should be revisited accordingly.

- As indicated in Table S1, Slc3a2 is not a cationic amino acid transporter, but the glycoprotein with which several non-glycosylated SLCs (including Slc7a7) dimerize so as to be targeted to the plasma membrane. Hence the title of Figure S2 is valid. The reviewer's remark made us realize that our sentence about *slc3a2* expression level in the Results section was unnecessarily distracting the main thread of this paragraph, so we have removed it, to focus on expression of the actual transporters.

Second decision letter

MS ID#: JOCES/2020/249037

MS TITLE: The cationic amino acid exporter Slc7a7 is induced and vital in tissue macrophages with sustained efferocytic activity

AUTHORS: Doris Lou DEMY, Mireille Carrere, Ramil Noche, Muriel Tauzin, Marion LE BRIS, Chooyoung Baek, Ignaty Leshchiner, Wolfram Goessling, and Philippe Herbomel
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Please see original comments.

Comments for the author

The authors have addressed my concerns, and the revised manuscript is suitable for publication in Journal of Cell Science.

Reviewer 2

Advance summary and potential significance to field

In the previous review I've described the advance made and potential significance to the field. To add: I think it remains to be shown that some of the features of the disease caused by SLC7A7 mutations involves macrophage defects. It would be very interesting to test this in the future.

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Comments for the author

I have no further comments, the authors addressed the minor issues I pointed out.

Reviewer 3

Advance summary and potential significance to field

The authors have now addressed the comments raised during the first round of reviews and I thus support publication.

Comments for the author

The authors have now addressed the comments raised during the first round of reviews and I thus support publication.

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