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USP18 lack in microglia causes destructive interferonopathy of the mouse brain

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 January 2015

Thank you for submitting your manuscript to The EMBO journal.

Your manuscript has been reviewed at another journal and submitted to The EMBO Journal with referee comments and a point-by-point response. I involved one new expert to assess the suitability for publication in The EMBO Journal. The referee had access to the previous referee comments and point-by-point response.

As you can see from the comments below, the referee finds the paper interesting and suitable for publication here. However, there are also some issues that need to be addressed in order to strengthen the findings reported:

- a better analysis of both USP18 expression and the affect of loss of USP18 on microglia and oligodendrocyte numbers.

- There is also an issue if some of the defects observed could be due to peripheral macrophages.

- Finally for some of the analysis there need to be a better side-by-side comparison of early vs late time points.

- Regarding the point that you used the KO mouse for the whole genome expression and not the conditional microglia specific KO model. I see that it would have been preferable to use the conditional KO mice, but I will also not insist on this point. Just make sure that you are careful with

your interpretations.

Overall, the referee provides constructive comments and I presume that you should be able to the raised issues within a reasonable timeframe. I am also available to discuss the issues further if that is helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thanks for submitting your interesting paper to us.

REFEREE REPORT

Referee #1:

USP18 is known to be a potent negative regulator of IFN-α/β signaling. The authors translated it to the microglia world and obtained interesting evidence implicating Usp18 in maintaining microglial quiescence in the brain white matter. They also show that USP18 acts by suppressing STAT1 activation pathways and implicitly type 1 interferon activity. They also show that USP18 acts on Jak/STAT binding, but not via its protease activity. Finally, they also investigate the role of microglial USP18 in EAE (but the results are not really convincing...).

Major comments:

- Author should provide more data on USP18 expression and how it affects microglia. Are all microglial cells expressing Usp18 in the white matter? Are all microglial cells activated by the loss of Usp18 or only a minor subset of them in a specific location? Do you have more or less microglial cell numbers in WT vs KO vs Cx3cr1Cre:Usp18fl/fl line? Does this change with time? The authors could address it by flow cytometry (FDG assay that can trace LacZ expression and expression of activation markers for example). In addition, double-staining of Iba1 and X-Gal and Iba1 and Mac-3, respectively, using the brain sections of Usp18-/- mice at postnatal and adult ages should be done. Finally, the authors brush off the case of oligodendrocytes by showing that there is the same numbers of oligodendrocytes in WT versus KO. However, adult oligodendrocytes seems to express USP18 although at a lower level than microglia. Is it the case in P4 or P10 oligodendrocytes? Oligodendrocytes, although present at the same numbers, could also be affected by the loss of USP18 and contribute to the disease in the KO. The authors need to address this issue.

- Another issue in this study is the specificity of deletion in the Cx3cr1Cre:Usp18fl/fl line. One reviewer mentioned "The experimental design raises the question of a function in peripheral macrophages, which have access to perivascular and leptomeningeal CNS compartments in health and stream into inflamed CNS parenchyma". The authors responded "We acknowledge the reviewer´s comment. Indeed, we used the myeloid-restricted Cx3cr1Cre:Usp18fl/fl line that will delete Usp18 not only in microglia but also in other myeloid cells." But the text of the manuscript is not really accurate and did not take into account this most important issue. The authors stated "We next crossed these newly generated Usp18fl/fl mice with a transgenic line expressing the Cre recombinase under the control of the CX3CR1 promoter that drives recombination in myeloid cells in general (Yona et al., 2013) but only in microglia in the brain (Goldmann et al., 2013;Wieghofer et al., 2014)." The deletion will not only affect microglia in the brain if perivascular and leptomeningeal CNS macrophages are also targeted by the excision. The authors need to carefully address this issue at all the time points they investigated the presence of the pathology. In case of the involvement of perivascular and leptomeningeal CNS macrophages, blood brain barrier disturbances should then be tested in pups and adult. Finally, the authors stated "Cx3cr1Cre:Usp18fl/fl mice showed an Usp18 deletion only in microglia within the CNS (Fig 2C)." I presume that this was done in adult? The authors should verify the specificity of excision in all the different brain subpopulations at P4 and P10 to avoid any confounding factors, as the pathology starts at this early stage and not in adult.

- The different time points of analysis at the beginning of the paper are also confusing (it is really difficult for the readers to follow), as also initially highlighted by one of the previous reviewers. Authors should be more systematic and present side-by-side data of the early versus late time points of analysis: Is figure 1C adult stage? Why present in Fig1E histology of brain sections of ten-day old and not adult? Why gene expression levels of chemokines and proinflammatory cytokines in the brains of four-day old pups and not ten-day old pups or adult? Which stage is Fig1H? For Fig2C, is it adult or pups?

- The authors reported a robust induction of chemokines at P4 that may attract monocytes. They also should look in adult. The authors stated that they did not see lymphocytes recruitment. What about monocytes? This should be done by flow cytometry that is more sensitive than immunochemistry and should reveal the presence of any type of infiltrating cells. This should also be done in adult.

- In Figure 3, authors are using the whole KO mouse for whole-genome gene expression and others experiments, while they just introduced a microglia specific USP18 KO model in figure 2. This is not logical. They should have used the conditional model rather than the full KO that can present confounding effects.
- The authors should discuss an important recent Nature paper related to their work (Human intracellular ISG15 prevents interferon-α/β over-amplification and auto-inflammation).

Minor Points:

Figure 1E, expression of iNOS and S100a9: Authors should perform triple staining with Mac-3, iNOS and S100a9 to conclude that the cells expressing these factors are the same.

4) Figure 2D: Authors should show the low magnification view of Mac-3 staining in CX3CR1Cre:Ups18fl/fl mice to compare that in USP18-/- mice (Fig. 1E).

5) Figure Legends: There are many mistyping of Lac/ZlacZ. Correct to LacZ/LacZ.

1st Revision - authors' response 03 March 2015

Point-by-point reply:

Referee #1:

USP18 is known to be a potent negative regulator of IFN-α/β signaling. The authors translated it to the microglia world and obtained interesting evidence implicating Usp18 in maintaining microglial quiescence in the brain white matter. They also show that USP18 acts by suppressing STAT1 activation pathways and implicitly type 1 interferon activity. They also show that USP18 acts on Jak/STAT binding, but not via its protease activity.

We would like to thank for these positive statements.

Major comments:

- Author should provide more data on USP18 expression and how it affects microglia. Are all microglial cells expressing Usp18 in the white matter? Are all microglial cells activated by the loss of Usp18 or only a minor subset of them in a specific location? Do you have more or less microglial cell numbers in WT vs KO vs Cx3cr1Cre:Usp18fl/fl line? Does this change with time? The authors could address it by flow cytometry (FDG assay that can trace LacZ expression and expression of activation markers for example). In addition, double-staining of Iba1 and X-Gal and Iba1 and Mac-3, respectively, using the brain sections of Usp18-/- mice at postnatal and adult ages should be done. Finally, the authors brush off the case of oligodendrocytes by showing that there is the same numbers of oligodendrocytes in WT versus KO. However, adult oligodendrocytes seems to express USP18 although at a lower level than microglia. Is it the case in P4 or P10 oligodendrocytes? Oligodendrocytes, although present at the same numbers, could also be

affected by the loss of USP18 and contribute to the disease in the KO. The authors need to address this issue.

We agree with the reviewer and followed her/his suggestions by adding a more detailed description of the WMMA phenotype seen in *Usp18*$^{-/-}$ and *Cx3cr1*^{Cre}:*Usp18*$^{fl/fl}$ mice. We now included a detailed quantification of Iba-1^{+} microglia in different areas of grey and white matter in the brain (new Fig. 1E-L; Fig 3D-I). These new data firmly establish that microgliosis is mostly restricted to the white matter. For that reason we describe the pathological changes found in the absence of USP18 as WMMA. Moreover, we included double stainings for Iba-1, Mac-3 (Lamp-2) and MHC class II respectively, that depict that only microglia in the white matter are activated (new Fig. 1M). Furthermore, we included in the revised version of the manuscript a new detailed side-by-side comparison of USP18-mediated neuropathology at different postnatal time points (new Fig.2A-D). These new pictures clearly indicate that from postnatal day 10 onwards Mac-3^{+} activated microglia are present only in the white matter of *Usp18*$^{-/-}$ mice but lacking in *Usp18*$^{+/+}$controls.
We agree that a temporal analysis of the LacZ expression as suggested by the referee would be of interest. However, *Usp18*$^{-/-}$ mice are very poor breeders and do not give rise to progeny at Mendalian ratio. Moreover, in our facility the ratio in heterozygous breeding pairs is 10 (*Usp18*$^{+/+}$) : 20 (U*sp18*$^{+/-}$) : 1 (*Usp18*$^{-/-}$) on a mixed background and even worse on C57Bl/6 background (see also Rempel et al., 2007, *Reprod Biol Endocrinol*. 26;5:13 and Ketscher et al., 2015, *Proc Natl Acad Sci U S A*. 112:1577-82). Due to the very limited number of expected ko mice further LacZ stainings would not be possible in a reasonable timeframe.
As suggested we also examined the number of oligodendrocytes in the absence of *Usp18*. We now include in the revised version of our manuscript the absolute numbers of Nogo^{+} oligodendrocytes in adult *Usp18*$^{+/+}$ and *Usp18*$^{-/-}$ (new Suppl. Fig. S4) and found no differences in any area examined. Therefore, we found no evidences that lack of *Usp18* may affect oligodendrocytes.

- Another issue in this study is the specificity of deletion in the Cx3cr1Cre:Usp18fl/fl line. One reviewer mentioned "The experimental design raises the question of a function in peripheral macrophages, which have access to perivascular and leptomeningeal CNS compartments in health and stream into inflamed CNS parenchyma". The authors responded "We acknowledge the reviewer´s comment. Indeed, we used the myeloid-restricted Cx3cr1Cre:Usp18fl/fl line that will delete Usp18 not only in microglia but also in other myeloid cells." But the text of the manuscript is not really accurate and did not take into account this most important issue. The authors stated "We next crossed these newly generated Usp18fl/fl mice with a transgenic line expressing the Cre recombinase under the control of the CX3CR1 promoter that drives recombination in myeloid cells in general (Yona et al., 2013) but only in microglia in the brain (Goldmann et al., 2013;Wieghofer et al., 2014)." The deletion will not only affect microglia in the brain if perivascular and leptomeningeal CNS macrophages are also targeted by the excision. The authors need to carefully address this issue at all the time points they investigated the presence of the pathology.

We agree with the reviewer's statement that our wording was incorrect. In fact, the constitutive CX3CR1 Cre line will in addition to microglia target all CX3CR1 expressing myeloid cells inside and outside the brain including peripheral monocytes, perivascular and leptomeningeal macrophages. We have now diligently and carefully modified the respective parts of the manuscript.

Finally, the authors stated "Cx3cr1Cre:Usp18fl/fl mice showed an Usp18 deletion only in microglia within the CNS (Fig 2C)." I presume that this was done in adult? The authors should verify the specificity of excision in all the different brain subpopulations at P4 and P10 to avoid any confounding factors, as the pathology starts at this early stage and not in adult.

We analyzed the deletion specificity in *Cx3cr1*^{Cre}:*Usp18*$^{fl/fl}$ mice using primary cultures of microglia, astrocytes, oligodendrocytes and neurons, respectively, as described in the m&m part, e.g. for microglia, astrocytes and oligodendrocytes from newborn mice and for neurons from E16 embryos. There are no established protocols to harvest these cells at high purity from adult mice.

- The different time points of analysis at the beginning of the paper are also confusing (it is really difficult for the readers to follow), as also initially highlighted by one of the previous reviewers. Authors should be more systematic and present side-by-side data of the early versus late time points of analysis: Is figure 1C adult stage? Why present in Fig1E histology of brain sections of

ten-day old and not adult? Why gene expression levels of chemokines and proinflammatory cytokines in the brains of four-day old pups and not ten-day old pups or adult?

We appreciate the suggestions and included a novel side-by-side comparison of the different postnatal developmental stages. The new figure 2A-D comprises histological data from postnatal days P0, P4, P10 and from adult mice, respectively. Furthermore, we included the expression of proinflammatory cytokines from P4, P7 and adult mice in the new Figure 2E-F as well as in the supplements. We hope that the new arrangement of the figures improved clarity.

- The authors reported a robust induction of chemokines at P4 that may attract monocytes. They also should look in adult. The authors stated that they did not see lymphocytes recruitment. What about monocytes? This should be done by flow cytometry that is more sensitive than immunochemistry and should reveal the presence of any type of infiltrating cells. This should also be done in adult.

We thank the referee for this advice. We now provide new immunhistochemical data depicting the absence of $CD45^+$ monocytes and $CD3^+$- and $B220^+$- lymphocytes in the brains of adult Usp18-deficient mice (new Suppl. Fig. S1). Monocyte and activated microglia share many myeloid marker and are therefore difficult to distinguish by FACS but they can potentially be differentiated by morphology because monocytes hardly express any processes whereas microglia do as recently demonstrated in $CX3CR1^{GFP}CCR2^{RFP}$ mice during EAE (Yamasaki et al., 2014, J Exp Med. 211:1533-49). The only $CD45^+$ cells in the CNS of USP18 ko mice were white matter microglia with processes but no monocytes (new Suppl. Fig. 1B).

- In Figure 3, authors are using the whole KO mouse for whole-genome gene expression and others experiments, while they just introduced a microglia specific USP18 KO model in figure 2. This is not logical. They should have used the conditional model rather than the full KO that can present confounding effects.

We acknowledge the reviewer's comment. However, the whole-genome gene expression array was performed in primary cultures of newborn pups from $Usp18^{-/-}$ mice. We wouldn`t expect any differences in primary cultures from $Usp18^{-/-}$ and $Cx3cr1^{Cre}$:$Usp18^{fl/fl}$ animals.

- The authors should discuss an important recent Nature paper related to their work (Human intracellular ISG15 prevents interferon-α/β over-amplification and auto-inflammation).

We now added and discussed this new paper in our revised manuscript.

Minor Points:

Figure 1E, expression of iNOS and S100a9: Authors should perform triple staining with Mac-3, iNOS and S100a9 to conclude that the cells expressing these factors are the same.

Unfortunately, we were technically not able to perform the requested triple stainings.

4) Figure 2D: Authors should show the low magnification view of Mac-3 staining in CX3CR1Cre:Ups18fl/fl mice to compare that in USP18-/- mice (Fig. 1E).

In order to allow a better assessment of the neuropathological changes found in $Cx3cr1^{Cre}$:$Usp18^{fl/fl}$ mice compared to $Usp18^{-/-}$ animals we improved this figure by adding several new Iba-1 immunohistochemical pictures at different time points of development (new Fig. 3D-I). This qualitative and quantitative analysis permits an easy comparison of the WMMA found in both genotypes (new Fig. 1E-L).

5) Figure Legends: There are many mistyping of Lac/ZlacZ. Correct to LacZ/LacZ.

We acknowledge this attentive point and corrected the mistyping's.

Accepted 17 March 2015

Thank you for submitting your revised manuscript. Your revision has now been re-reviewed by the referee and the comments are provided below. As you can see the referee appreciates the introduced changes and support publication here. I am therefore very pleased to accept the paper for publication.

REFEREE REPORT

Referee #1:

The authors addressed most of the issues that were raised and the manuscript has greatly improved. Good for publication!

