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OTUB1 inhibits CNS autoimmunity by preventing IFN-γ-induced hyperactivation of astrocytes

Xu Wang, Floriana Mulas, Wenjing Yi, Anna Brunn, Gopala Nishanth, Sissy Just, Ari Waisman, Wolfgang Brück, Martina Deckert, Dirk Schlüter

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1st Editorial Decision 29th Nov 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting. However, they also find that the analysis needs to be extended and further developed in order to consider publication here. Should you be ale to address the referees' concerns in full then I would be interested in considering a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the raised concerns at this stage.

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:
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Referee #1:

The authors describe analysis of OTUB1 regulation of astrocyte response to IFNgamma in MS and EAE. OTUB1 is a deubiquitinating enzyme normally expressed in the CNS by some neurons and oligodendrocytes. Findings include that expression in astrocytes was strongly and selectively upregulated in MS and in EAE. Mice lacking OTUB1 in astrocytes developed earlier and more severe EAE, without any alteration (apart from numbers) in infiltrating immune cell profile. Chemokine/cytokine production by OTUB1-negative astrocytes in response to interferon-gamma was enhanced, as were a smaller range of responses (STAT1, CXCL11, CCL2) to IFNbeta. This was attributed to loss of SOCS1 stabilization by OTUB1 (via K48 deubiquitination), leading to reduced inhibition of JAK2 and so enhanced JAK2 and STAT1 phosphorylation. Although there was release

from transcriptional control of SOCS1, there were higher levels of SOCS1 protein in OTUB1-sufficient astrocytes, presumably reflecting that OTUB1 normally stabilizes SOCS1 protein, confirmed by data for more rapid degradation in absence of OTUB1. Transfection with mutant OTUB1 constructs identified that the N-terminus of OTUB1 is normally required for its regulation of SOCS1/JAK2 downstream of IFN receptors.

This is a thorough and careful study. There is much interest in functional regulation via control of ubiquitination and recent papers identify a role for this pathway in neurological disease. Findings are reminiscent of this group's description of another astrocyte deubiquitinating enzyme A20, published in 2013. Novelty lies in regulation of IFNgamma as opposed to TNFalpha response, and a more complete analysis of mechanism.

Major concerns, aspects that require attention
No mechanism is proposed or discussed for the astrocyte-selective upregulation of OTUB1 in MS and EAE. This needs attention. The waning of OTUB1 expression at 22d pi in EAE points to inflammatory stimulus-dependence- has it been examined whether expression is sustained in a chronic EAE model?

Fig S6A shows downregulation of OTUB1 mRNA by IFNgamma, but Fig 5A shows no effect of IFNgamma on OTUB1 protein levels, both in primary astrocytes. How can these be reconciled?

The sentence lines 497-499 contains a missense comma and an unjustified presumption. There is no necessary conflict between the fact that a cytokine ameliorates MS and EAE and also induces STAT1 activation and chemokine production by astrocytes.

The sentence lines 514-518 is misleading, in suggesting a selective effect of OTUB1 deficiency on Th1 recruitment - the data show equivalently enhanced recruitment of Th1, Th17 and GM-CSF T cells, but no data are presented that Th1 directed (paved the way for) the other recruitments in the way the sentence suggests. The sentence should be rewritten to make clear that this is speculation - it is equally possible that recruitment of all 3 subsets was due to enhanced astrocyte-derived chemokines.

It would be helpful to show staining from normal/non-MS brain in Figure 1.

The references Millward et al 2010 and Chen et al 2007 (cited on page 13) do not describe blood-brain barrier effects of pertussis toxin, nor do they describe pertussis toxin-free EAE. The latter was described by McClain et al JI 2007, who showed disease onset at d9 pi in MOG35-55 induced EAE in C57BL/6 mice - the authors should discuss this discrepancy with their own pertussis-free EAE. On the other hand, the paper by Chen et al suggested a role for pertussis toxin distinct from BBB disruption (IL6 and Th17 induction, reduced Tregs) and this should be discussed in light of the increased IL-6 in GFAP-Cre OTUB1fl/fl mice as well as the lack of effect of OTUB1 deficiency on IL-17 responses.

The paper by Brambilla et al JI 2009 showing reduced disease and enhanced recovery from EAE in mice lacking astrocytic IkappaB alpha should be included in description and discussion of roles for NFkB-activated astrocytes.

The paper by Torre et al Nat Immunol 2016 that showed regulation of EAE by USP15 should also be cited.

Quantitations should be provided for western blots in supplementary figures S6 and S7

Neuroectodermal-directed or global OTUB1 ablation in this study was embryonic lethal, but an OTUB1 knockout mouse is advertised as commercially available. Can the authors comment?

Referee #2:

Wang and co-authors investigate the protein OTUB-1 during autoimmune neuroinflammation. They show that constitutive deletion of OTUB-1, which is embryonically lethal if constitutive in all cells

or in neural cells, produces no apparent phenotype if performed in astrocytes by using a GFAP-CRE mouse. In mice devoid of OTUB-1 they observe, however, a more severe EAE, both from a clinical and neuropathological point of view. Authors show in vitro that OTUB-1 de-ubiquitinates SOCS-1 which in turn is an inhibitor of IFNgamma signaling in astrocytes. Thus, the absence of OTUB-1 would determine increased degradation of SOCS-1 and therefore an augmented release of pro-inflammatory mediators by astrocytes in response to IFNgamma signaling, thus exacerbating EAE. The manuscript is interesting and addresses a relatively novel pathway involved in neuroinflammation. There several points and concerns, however, that need to be addressed.

- The first point that is made is that OTUB-1 is increased in astrocytes around human MS plaques. However no evidence is provided to show that OTUB-1 is not normally expressed in astrocytes in healthy tissue, nor comparison with normal appearing white matter. We are left with nice human pathology on brain biopsies of only positive specimens.
- EAE data raise some concerns. In fact, in figure 3E GFAP-CRE OTUB-1flox/flox EAE mice display a disease course that would be perfect for WT C57BL/6 mice, while OTUB-1flox/flox control mice appear to have a decreased EAE severity, also compared to the same control group in figure S5A. Declared sample size is 29 vs. 29, is this the merge of different independent experiments? How do you explain the difference?
- I perfectly know that it is convention to plot the mean clinical EAE score, which is obviously incorrect since EAE score is non parametric (median values would be more representative), however at least statistics should be correct: if you used t-test, as declared in the table legend, or one-way Anova, as declared in methods, please turn to a non-parametric test. Finally, comparing the day of onset using t-test is once again incorrect, differences in day of onset have to be evaluated using a Kaplan-Meyer survival curve.
- Following the interpretation proposed, one would expect EAE curves to be similar up to the peak of the disease and then GFAP-CRE OTUB-1flox/flox mice not recovering. Instead the disease is different from the beginning, being more severe at all time points but displaying the usual peak followed by a slight recovery that is typical for MOG-EAE in B6 mice.
- I'm puzzled by the interpretation of the role of IFNgamma. I'm not questioning the in vitro data, I think the demonstration of the signaling involved is a neat piece of in vitro science. But the discussion starts asserting that "IFN-γ plays a detrimental role in both MS and EAE". Then why IFNgamma knock-out mice display more severe EAE? Why neutralizing EAE with monoclonal Abs exacerbates EAE? Why delivering intrathecal IFNgamma ameliorates EAE? Further, the interferon system in humans and mice has not marginal differences also in the signaling pathways. Thus I found the discussion over-simplistic from this point of view, extrapolating to the in vivo disease the in vitro data.

Technical points

- The title of the first results paragraph. Data do not demonstrate that OTUB-1 induction ameliorates EAE but that the absence in astrocytes worsens it. It is not the same.
- For the inflammation in Fig. 3D there is no quantification reported thus discussing it as recovery vs. no recovery is not appropriate.
- Please do not use CRE+/- in mice nomenclature. CRE is a knock in of an exogenous gene, thus there is no negative allele.

In conclusion I think this is an interesting manuscript reporting novel data needing better definition and analysis, and a more thorough discussion.

Referee #3:

This manuscript investigates the function of OTUB1 in astrocytes during EAE. The main findings are that cell-type specific deletion of OTUB1 leads to a more severe clinical course of EAE including an increased T cell infiltration and augmentation of proinflammatory cytokines. Further in vitro experiments suggest that OTUB1 inhibits IFNg-induced JAK/STAT activation via K48 deubuquitination ans stabilisation of SOCS1. The clinical relevance of these findings are supported by immunohistochemical analysis of brain biopsies from MS patients.
Overall the manuscript is well written and the statements are supported by the experimental findings. Overall the manuscript is conclusive. The results are novel and add important information for neuroinflammatory processes.
There are a few minor comments:

- Line 299: It is stated that a detailed morphological analysis has been performed with reference to Fig. 2. However, the presented analysis is not very detailed. In particular, the morphology of the cells can hardly be seen in Fig. 2 F-H. There should be inserts with larger magnification so that single cells can be judged.
- In line 308 (heading) and on several othe occasions it is stated that OTUB1 expression in astrocytes ameliorates EAE. However, the authors have not investigated whether e.g. excess OTUB1 really ameliorates EAE. They have only indirectly concluded that this is the case because deletion led to more severe EAE. They should use rathe a term like „limits EAE severity" or „controls".
- While the EAE data and the in vitro data are quite detailed the data on MS tissue is rather scarce and particularly from a clinical point of view not sufficient. In particular it is not clear from what type of MS course (RRMS/SPMS/PPMS) the lesions were derived. What type of lesions were investigated? Early, acute? Chronic, inactive? This may be of great importance since the expression of OTUB1 may change during the course of lesion development. This has also implications on the function of astrocytes during different timepoints of the disease course. Thus, the characterisation of the lesions is required.

1st Revision - authors' response 23rd Jan 2019

Referee #1:

The authors describe analysis of OTUB1 regulation of astrocyte response to IFNgamma in MS and EAE. OTUB1 is a deubiquitinating enzyme normally expressed in the CNS by some neurons and oligodendrocytes. Findings include that expression in astrocytes was strongly and selectively upregulated in MS and in EAE. Mice lacking OTUB1 in astrocytes developed earlier and more severe EAE, without any alteration (apart from numbers) in infiltrating immune cell profile. Chemokine/cytokine production by OTUB1-negative astrocytes in response to interferon-gamma was enhanced, as were a smaller range of responses (STAT1, CXCL11, CCL2) to IFNbeta. This was attributed to loss of SOCS1 stabilization by OTUB1 (via K48 deubiquitination), leading to reduced inhibition of JAK2 and so enhanced JAK2 and STAT1 phosphorylation. Although there was release from transcriptional control of SOCS1, there were higher levels of SOCS1 protein in OTUB1-sufficient astrocytes, presumably reflecting that OTUB1 normally stabilizes SOCS1 protein, confirmed by data for more rapid degradation in absence of OTUB1. Transfection with mutant OTUB1 constructs identified that the N-terminus of OTUB1 is normally required for its regulation of SOCS1/JAK2 downstream of IFN receptors.

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Reply: We propose that the astrocyte-specific upregulation of OTUB1 in MS and EAE is induced by inflammatory stimuli. *In vivo*, astrocyte activation, severity of inflammation, and OTUB1 expression of activated astrocytes are positively correlated. Additionally, and as mentioned by the reviewer, OTUB1 expression in astrocytes was reduced at day 22 p.i., when inflammation was regressive in the spinal cord. We did not study OTUB1 expression in a chronic EAE model, because we further focused on the mechanism of OTUB1 function in astrocytes, which revealed that IFN-γ regulated OTUB1 function. As illustrated in Fig. 5, IFN-γ induced interaction of OTUB1 with SOCS1 (Fig. 5E) and K48 deubiquitination of SOCS1 (Fig. 5F) demonstrating that an inflammatory cytokine regulated OTUB1 function. We have included this proposed mechanism in the Discussion (lines532-535).

Fig S6A shows downregulation of OTUB1 mRNA by IFNgamma, but Fig 5A shows no effect of IFNgamma on OTUB1 protein levels, both in primary astrocytes. How can these be reconciled?
Reply: In Fig S6A (the new Fig EV5A), OTUB1 mRNA was downregulated upon IFN-γ, showing that IFN-γ signaling and OTUB1 act antagonistically. Of note, the mRNA levels of OTUB1 were downregulated by 20% after stimulation with IFN-γ for 2 hours (Fig EV5), which is a relatively slow process. Besides, 80% of mRNA can still generate an adequate amount of protein. In sharp contrast, the mRNA levels of SOCS1 were increased six times after treatment with IFN-γ for 1 hour (Fig. 5D). In addition, old OTUB1 protein needs to be degraded to show the difference in newly synthesized OTUB1 protein. As shown in Fig. 6C, OTUB1 protein is relatively stable and degradation of old GFP-OTUB1 was not observed within 9 hours. These synergistic factors might explain why we did not detected differences in OTUB1 protein levels in IFN-γ-stimulated cells up to 6 hours post stimulation (Fig 5A).

The sentence lines 497-499 contains a missense comma and an unjustified presumption. There is no necessary conflict between the fact that a cytokine ameliorates MS and EAE and also induces STAT1 activation and chemokine production by astrocytes.
Reply: Thank you for pointing out this mistake. We have rewritten this part in the text (lines 514-519).

The sentence lines 514-518 is misleading, in suggesting a selective effect of OTUB1 deficiency on Th1 recruitment - the data show equivalently enhanced recruitment of Th1, Th17 and GM-CSF T cells, but no data are presented that Th1 directed (paved the way for) the other recruitments in the way the sentence suggests. The sentence should be rewritten to make clear that this is speculation - it is equally possible that recruitment of all 3 subsets was due to enhanced astrocyte-derived chemokines.
Reply: We are sorry for this misleading statement. We have corrected it in the text stating that the increased production of chemokines leads to an augmented recruitment of encephalitogenic IFN-γ-, IL-17-, and GM-CSF-producing $CD4^+$ T cells to the CNS (lines 541-543).

It would be helpful to show staining from normal/non-MS brain in Figure 1.
Reply: The reviewer raises an important point. In our study, we focused on brain biopsies, because this tissue reflects by far better the *in vivo* situation as compared to MS tissue from autopsy cases. Furthermore, technically, postmortem tissue is not suitable for these studies as autolysis quickly alters CNS tissue. For ethical reasons, persons with normal brain do not undergo brain biopsy and, therefore, we cannot study astrocytic OTUB1 expression in brain biopsies of normal healthy brain. Instead, we have studied OTUB1 expression in biopsied tissues that show minimal pathological alterations and, thus, are close to normal brain. This included cases of brain biopsy due to astrocytoma (WHO grade II) in which we analyzed areas adjacent to the tumor without microscopical evidence for tumor infiltration. However, slight reactive changes including some astrocyte activation still may occur. Importantly as shown in the new Fig EV1B, astrocytes with the morphological features of resting and activated astrocytes were OTUB1-negative. Thus, astrocytic OTUB1 is strongly expressed in our MS cases, but not expressed in normal peritumoral tissue of astrocytoma. We would like to stress that the present study did not aim to provide a detailed analysis of OTUB1 expression in different subtypes of MS (see also Rev. 3, last point) and different CNS pathologies, but we strongly feel that our data clearly show strong astrocytic OTUB1 expression in MS but not in normal appearing brain tissue in the vicinity of a glioma.

The references Millward et al 2010 and Chen et al 2007 (cited on page 13) do not describe blood-brain barrier effects of pertussis toxin, nor do they describe pertussis toxin-free EAE. The latter was described by McClain et al JI 2007, who showed disease onset at d9 pi in MOG35-55 induced EAE in C57BL/6 mice - the authors should discuss this discrepancy with their own pertussis-free EAE. On the other hand, the paper by Chen et al suggested a role for pertussis toxin distinct from BBB disruption (IL6 and Th17 induction, reduced Tregs) and this should be discussed in light of the increased IL-6 in GFAP-Cre OTUB1fl/fl mice as well as the lack of effect of OTUB1 deficiency on IL-17 responses.

Reply:
The references Millward et al 2010 and Chen et al 2007 (cited on page 13) do not describe blood-brain barrier effects of pertussis toxin, nor do they describe pertussis toxin-free EAE.

Thank you for pointing out this mistake. Indeed, the BBB effect of pertussis toxin was initially described in Linthicum *et al* (Linthicum DS Cell Immunol, 1982). We have included this paper in the manuscript.

The latter was described by McClain et al JI 2007, who showed disease onset at d9 pi in MOG35-55 induced EAE in C57BL/6 mice - the authors should discuss this discrepancy with their own pertussis-free EAE.
Thank you for raising this question. EAE experiments are influenced by many factors such as the dose and quality of MOG peptide and adjuvant, the microbiota of the mice (which is strongly affected by the animal facility), and the way of induction. We and McClain *et al* used two different immunization methods: we immunized mice only once at four sites on the flank, whereas McClain *et al* immunized mice twice at two sites at the base of the tail. Furthermore, it is difficult to compare two EAE experiments from two different labs. The only thing changed in EAE experiments shown in Fig. 3E (with pertussis toxin) and 3F (without pertussis toxin) was the pertussis toxin. As shown in Fig. 3, control mice with normal EAE induction (Fig. 3E) started disease at day 12 p.i. In the absence of pertussis toxin, control mice showed strongly delayed and reduced EAE (Fig. 3F).

On the other hand, the paper by Chen et al suggested a role for pertussis toxin distinct from BBB disruption (IL6 and Th17 induction, reduced Tregs) and this should be discussed in light of the increased IL-6 in GFAP-Cre OTUB1fl/fl mice as well as the lack of effect of OTUB1 deficiency on IL-17 responses.
Reply: Chen *et al* have shown that pertussis toxin induces Th17 induction in an IL-6-dependent way in lymphatic organs and cultivated T cells. Of note, in our study, OTUB1 deletion was restricted to astrocytes in GFAP-Cre OTUB1$^{fl/fl}$ mice and the immune system was not deficient of OTUB1. Therefore, peripheral immune responses should be identical in OTUB1$^{fl/fl}$ and GFAP-Cre OTUB1$^{fl/fl}$ mice. Indeed, as shown in Fig EV4B-E, OTUB1$^{fl/fl}$ and GFAP-Cre OTUB1$^{fl/fl}$ mice exhibited equal T cell responses. Based on these data we suggest to keep the discussion on pertussis limited to astrocytes and the BBB.

The paper by Brambilla et al JI 2009 showing reduced disease and enhanced recovery from EAE in mice lacking astrocytic IkappaB alpha should be included in description and discussion of roles for NFkB-activated astrocytes.
Reply: Thank you for the suggestion. We have included this paper in the introduction (lines 72-74).

The paper by Torre et al Nat Immunol 2016 that showed regulation of EAE by USP15 should also be cited.
Reply: We have cited this paper and added it to the results (lines 505-506, 516-519).

Quantitations should be provided for western blots in supplementary figures S6 and S7
Reply: As suggested by the reviewer, we have added quantification to these figures (the new Fig EV5B and Appendix Fig S2).

Neuroectodermal-directed or global OTUB1 ablation in this study was embryonic lethal, but an OTUB1 knockout mouse is advertised as commercially available. Can the authors comment?
Reply: As shown in Fig S2 (the new Appendix Fig S1), either neuroectodermal-directed or global OTUB1 ablation was embryonic lethal. This finding is supported by a newly published paper by another research group (Pasupala N J Biol Chem 2018). In this paper, the authors stated that '*Otub1-deficient* (*Otub1*$^{-/-}$) *mice exhibit late embryonic lethality*'. In addition, we have shown in another study that only heterozygous OTUB1$^{+/-}$ could survive (Dong W J Am Soc Nephrol 2015). To the best of our knowledge, a conventional OTUB1 knockout mouse is not available.

References:

Dong W, Wang H, Shahzad K, Bock F, Al-Dabet MM, Ranjan S, Wolter J, Kohli S, Hoffmann J, Dhople VM, Zhu C, Lindquist JA, Esmon CT, Gröne E, Gröne HJ, Madhusudhan T, Mertens PR, Schlüter D, Isermann B. (2015) Activated Protein C Ameliorates Renal Ischemia-Reperfusion Injury by Restricting Y-Box Binding Protein-1 Ubiquitination. *J Am Soc Nephrol* 26:2789-2799

Linthicum DS, Munoz JJ, Blaskett A (1982) Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cell Immunol* 73: 299-310

Pasupala N, Morrow ME, Que LT, Malynn BA, Ma A, Wolberger C (2018) OTUB1 non-catalytically stabilizes the E2 ubiquitin-conjugating enzyme UBE2E1 by preventing its autoubiquitination. *J Biol Chem* 293:18285-18295

Referee #2:

Wang and co-authors investigate the protein OTUB-1 during autoimmune neuroinflammation. They show that constitutive deletion of OTUB-1, which is embryonically lethal if constitutive in all cells or in neural cells, produces no apparent phenotype if performed in astrocytes by using a GFAP-CRE mouse. In mice devoid of OTUB-1 they observe, however, a more severe EAE, both from a clinical and neuropathological point of view. Authors show in vitro that OTUB-1 de-ubiquitinates SOCS-1 which in turn is an inhibitor of IFNgamma signaling in astrocytes. Thus, the absence of OTUB-1 would determine increased degradation of SOCS-1 and therefore an augmented release of pro-inflammatory mediators by astrocytes in response to IFNgamma signaling, thus exacerbating EAE. The manuscript is interesting and addresses a relatively novel pathway involved in neuroinflammation. There several points and concerns, however, that need to be addressed.

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Reply: Thank you for the question, which was also asked by reviewer 1.

It would be helpful to show staining from normal/non-MS brain in Figure 1.
Reply: The reviewer raises an important point. In our study, we focused on brain biopsies, because this tissue reflects by far better the in vivo situation as compared to MS tissue from autopsy cases. Furthermore, technically, postmortem tissue is not suitable for these studies as autolysis quickly alters CNS tissue. For ethical reasons, persons with normal brain do not undergo brain biopsy and, therefore, we cannot study astrocytic OTUB1 expression in brain biopsies of normal healthy brain. Instead, we have studied OTUB1 expression in biopsied tissues that show minimal pathological alterations and, thus, are close to normal brain. This included cases of brain biopsy due to astrocytoma (WHO grade II) in which we analyzed areas adjacent to the tumor without microscopical evidence for tumor infiltration. However, slight reactive changes including some astrocyte activation still may occur. Importantly as shown in the new Fig EV1B, astrocytes with the morphological features of resting and activated astrocytes were OTUB1-negative. Thus, astrocytic OTUB1 is strongly expressed in our MS cases, but not expressed in normal peritumoral tissue of astrocytoma. We would like to stress that the present study did not aim to provide a detailed analysis of OTUB1 expression in different subtypes of MS (see also Rev. 3, last point) and different CNS pathologies, but we strongly feel that our data clearly show strong astrocytic OTUB1 expression in MS but not in normal appearing brain tissue in the vicinity of a glioma.

A detailed analysis of a large number of cases with different CNS pathologies is required to clearly define in which diseases and in which anatomic locations astrocytes express OTUB1. This is beyond the scope of this manuscript. Here, we mainly intend to illustrate that astrocytes express OTUB1 in MS and that astrocytes do not express OTUB1 or exhibit low OTUB1 expression in brain tissue with only minor morphologically detectable pathology.

- EAE data raise some concerns. In fact, in figure 3E GFAP-CRE OTUB-1flox/flox EAE mice display a disease course that would be perfect for WT C57BL/6 mice, while OTUB-1flox/flox control mice appear to have a decreased EAE severity, also compared to the same control group in figure S5A. Declared sample size is 29 vs. 29, is this the merge of different independent experiments? How do you explain the difference?
Reply: The Fig. 3E shows pooled data from 4 experiments with 7-8 mice per group. We have specified this in the figure legends (lines 948-949).

We understand the reviewer's concern that EAE might not be successfully induced because the peak clinical score of WT mice is 1.5 (Fig 3E). However, different clinical grading systems can be applied to score EAE. As indicated in Material and Methods, clinical signs of EAE were scored according to a scale of severity from 0 to 5 as follows: 0, no sign; 0.5, partial tail weakness; 1, limp tail; 1.5, slowing of righting; 2, partial hind limb weakness; 2.5, dragging of hind limb(s) without complete paralysis; 3, complete paralysis of at least one hind limb; 3.5, hind limb paralysis and slight weakness of forelimbs; 4, forelimb weakness; 5, moribund or dead. Of note, our score of 1.5 is equal to 2 in Mufazalov *et al* (Mufazalov EMBO J 2017) and 2-3 in Zabala A *et al* (Zabala A EMBO Mol Med 2018), respectively. In these studies, control mice displayed the same EAE severity as our control mice. Thus, we successfully induced EAE in control mice allowing a valid comparison of the disease between OTUB1$^{fl/fl}$ and GFAP-Cre OTUB1$^{fl/fl}$ mice.

- I perfectly know that it is convention to plot the mean clinical EAE score, which is obviously incorrect since EAE score is non parametric (median values would be more representative), however at least statistics should be correct: if you used t-test, as declared in the table legend, or one-way Anova, as declared in methods, please turn to a non-parametric test. Finally, comparing the day of onset using t-test is once again incorrect, differences in day of onset have to be evaluated using a Kaplan-Meyer survival curve.
Reply: Thank you very much for pointing out this mistake. We have re-analyzed the data according to your suggestions. Specifically, we used non-parametric test for EAE curves (Mann-Whitney U test), disease onset (Kaplan-Meyer survival curve followed by Gehan-Breslow-Wilcoxon test), and maximal scores (Mann-Whitney U test). The text was changed accordingly for the Material and Methods (lines 283-285) and table legend (lines 866-868).

- Following the interpretation proposed, one would expect EAE curves to be similar up to the peak of the disease and then GFAP-CRE OTUB-1flox/flox mice not recovering. Instead the disease is different from the beginning, being more severe at all time points but displaying the usual peak followed by a slight recovery that is typical for MOG-EAE in B6 mice.
Reply: According to the 'two wave theory', astrocyte-mediated production of proinflammatory cytokines and chemokines contributes to the disease onset of EAE. In this study, we show that OTUB1 inhibits IFN-γ-induced proinflammatory gene production in astrocytes, thereby ameliorating EAE. Therefore, we observed differences even at the onset of the disease. The similar observation was also made in other groups. Mice with astrocyte-specific deletion of IFN-γR (Ding X J Immunol 2015) and Act1 (Kang Z Immunity 2010), which resulted in reduced chemokine production in astrocytes, developed less severe EAE since the disease onset. Consistently, knockdown of TRAF3 (Zhu S J Exp Med 2010) in astrocytes promotes proinflammatory gene production in astrocytes, resulting in more severe EAE already at the disease onset. In addition, GFAP-Cre OTUB1$^{fl/fl}$ mice recover slightly after the peak, which is also seen in Zhu *et al* (Zhu S J Exp Med 2010). The slight recovery may be attributed to the apoptotic elimination of infiltrating leukocytes and remyelination. Interestingly, we have shown before that mice with targeted deletion of FasL (Wang X Eur J Immunol 2013) developed more severe EAE than control mice after the disease peak and could not recover from EAE due to an inability to induce the apoptotic elimination of infiltrating T cells from the CNS.

- I'm puzzled by the interpretation of the role of IFNgamma. I'm not questioning the in vitro data, I think the demonstration of the signaling involved is a neat piece of in vitro science. But the discussion starts asserting that "IFN-γ plays a detrimental role in both MS and EAE". Then why IFNgamma knock-out mice display more severe EAE? Why neutralizing EAE with monoclonal Abs exacerbates EAE? Why delivering intrathecal IFNgamma ameliorates EAE? Further, the interferon system in humans and mice has not marginal differences also in the signaling pathways. Thus I found the discussion over-simplistic from this point of view, extrapolating to the in vivo disease the in vitro data.
Reply: We agree with the reviewer that simply stating "IFN-γ plays a detrimental role in both MS and EAE" is inappropriate. Indeed, IFN-γ plays both detrimental and protective roles in MS and EAE, which are dependent on cell type, cell maturation status, dosage, and disease stage (reviewed by Arellano et al. Front Immunol 2015; Ottum et al. Front Immunol 2015). However, accumulative studies indicate that high amounts of IFN-γ in the CNS are disease-promoting. In high doses, IFN-γ aggravates EAE by inducing disease-worsening effects in CNS-resident cells including microglia, oligodendrocytes, and particularly, astrocytes.
We have changed the Discussion (lines 522-525) to make it more objective and scientific.

Technical points

- The title of the first results paragraph. Data do not demonstrate that OTUB-1 induction ameliorates EAE but that the absence in astrocytes worsens it. It is not the same.
Reply: Thank you for point out this mistake. The same concern was raised by Reviewer 3. We have corrected it as 'Ablation of OTUB1 in astrocytes aggravates EAE' (line 324). In addition, we have changed the statement in results (line 393), Table 1 (line 853), and Fig. 3 legend (line 931).

- For the inflammation in Fig. 3D there is no quantification reported thus discussing it as recovery vs. no recovery is not appropriate.
Reply: Regression of inflammation in OTUB1$^{fl/fl}$ mice is clearly evidenced by a lack of leukocyte infiltration and demyelination in this group at day 22 p.i. (Fig. 3D), which is in marked contrast to the prominent infiltrates in the spinal cord at day 15 p.i. (Fig 3B). We discussed the data more careful as an indication for regression of inflammation (lines 334-335).

- Please do not use CRE+/- in mice nomenclature. CRE is a knock in of an exogenous gene, thus there is no negative allele.
Rely: Thank you for pointing this mistake. We have corrected Cre$^{+/-}$ as Cre in the text (lines 125 to 127) and Appendix Fig S1.

In conclusion I think this is an interesting manuscript reporting novel data needing better definition and analysis, and a more thorough discussion.
Reply: Thank you for your comments. We have made changes according to your suggestions, which we think has strongly improved the quality of the manuscript.

References:

Arellano G, Ottum PA, Reyes LI, Burgos PI, Naves R. (2015) Stage-Specific Role of Interferon-Gamma in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *Front Immunol* doi: 10.3389/fimmu.2015.00492.

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Wang, X., Haroon, F., Karray, S., Martina, D., and Schluter, D. (2013) Astrocytic Fas ligand expression is required to induce T-cell apoptosis and recovery from experimental autoimmune encephalomyelitis. *Eur J Immunol* 43: 115-124.

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Zhu S, Pan W, Shi P, Gao H, Zhao F, Song X, Liu Y, Zhao L, Li X, Shi Y, Qian Y. (2010) Modulation of experimental autoimmune encephalomyelitis through TRAF3-mediated suppression of interleukin 17 receptor signaling. *J Exp Med* 207:2647-2662.

Referee #3:

This manuscript investigates the function of OTUB1 in astrocytes during EAE. The main findings are that cell-type specific deletion of OTUB1 leads to a more severe clinical course of EAE including an increased T cell infiltration and augmentation of proinflammatory cytokines. Further in vitro experiments suggest that OTUB1 inhibits IFNg-induced JAK/STAT activation via K48 deubuquitination ans stabilisation of SOCS1. The clinical relevance of these findings are supported by immunohistochemical analysis of brain biopsies from MS patients.
Overall the manuscript is well written and the statements are supported by the experimental findings. Overall the manuscript is conclusive. The results are novel and add important information for neuroinflammatory processes.
There are a few minor comments:
- Line 299: It is stated that a detailed morphological analysis has been performed with reference to Fig. 2. However, the presented analysis is not very detailed. In particular, the morphology of the cells can hardly be seen in Fig. 2 F-H. There should be inserts with larger magnification so that single cells can be judged.
Reply: The major aim of these double immunofluorescence studies was the identification of the cellular sources of OTUB1 expression. Therefore, we decided to provide the photomicrographs at a moderate magnification as shown here. Additionally, the first submitted version of text and figures was a merged PDF which unequivocally is associated with a reduction of figure quality. According to the Reviewer's comment, we have now included high-magnification inserts and submitted all figures showing histopathology as high resolution TIF files.

- In line 308 (heading) and on several othe occasions it is stated that OTUB1 expression in astrocytes ameliorates EAE. However, the authors have not investigated whether e.g. excess OTUB1 really ameliorates EAE. They have only indirectly concluded that this is the case because deletion led to more severe EAE. They should use rathe a term like „limits EAE severity" or „controls".
Reply: Thank you for pointing out this mistake. The same concern was raised by Reviewer 2. We have corrected the title as 'Ablation of OTUB1 in astrocytes aggravates EAE'(line 324). In addition, we have changed the statement in results (line 393), Table 1 (line 853), and Fig. 3 legend (line 931).

- While the EAE data and the in vitro data are quite detailed the data on MS tissue is rather scarce and particularly from a clinical point of view not sufficient. In particular it is not clear from what type of MS course (RRMS/SPMS/PPMS) the lesions were derived. What type of lesions were investigated? Early, acute? Chronic, inactive? This may be of great importance since the expression of OTUB1 may change during the course of lesion development. This has also implications on the function of astrocytes during different timepoints of the disease course. Thus, the characterisation of the lesions is required.
Reply: The aim of this analysis was to correlate data obtained in EAE with human MS as proof-of-principle. All samples are derived from patients with first episode of disease and clinically active neurological symptoms who underwent brain biopsy for establishment of diagnosis. These studies unequivocally identified activated astrocytes in the samples of these patients with actively ongoing inflammatory demyelination to express OTUB1. It will be very interesting to determine expression of OTUB1 in various stages and subtypes of MS; however, here we focused on treatment-naïve patients with first episode of MS, which also enables a comparison to our mouse studies focusing on acute EAE.

2nd Editorial Decision 15th Feb 2019

Thank your revised manuscript to The EMBO Journal. Your study has now been seen by referee #1 and 3 and their comments are provided below. The referees appreciate the introduced changes and support publication in the EMBO Journal.

Referee #1 has a remaining point that would be good to discuss. When you submit the revised version would you also please take care of the following editorial points

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REFEREE REPORTS:

Referee #1:

This is a resubmission of a paper describing OTUB1 regulation of astrocyte response to IFNgamma in MS and EAE. Significance of the study lies in general interest in functional regulation via control of ubiquitination and the role of this pathway in neurological disease.

The authors have responded to my (and two other reviewers) comments with new data and considerate and careful discussion. Some of my concerns were also raised by other reviewers and in my opinion they have been adequately addressed in this resubmission, with one exception.

I remain unsure why the upregulation of OTUB1 in MS and EAE was seen in astrocytes and not in other cell types. The authors propose that upregulation was induced by inflammatory stimuli, as already seemed likely in the initial study - why would these stimuli not also act on eg oligodendrocytes or neurons, or indeed any other cells? Discussion of this point would enhance the manuscript.

Referee #3:

The Points of this reviewer have been addressed adequately

2nd Revision - authors' response 20th Feb 2019

Referee #1:

This is a resubmission of a paper describing OTUB1 regulation of astrocyte response to IFNgamma in MS and EAE. Significance of the study lies in general interest in functional regulation via control of ubiquitination and the role of this pathway in neurological disease.

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Reply: Thank you for your question. Compared with astrocytes, neurons and oligodendrocytes possess less immune-regulating properties. Maybe that is the reason why proinflammatory stimuli have minor impact on neurons and oligodendrocytes. Of note, in addition to regulating inflammatory responses, OTUB1 has other important functions. For example, in neurons, OTUB1 has been shown to stabilize Tau (Wang P Acta Neuropathol 2017), indicating that neuron-specific OTUB1 might participate in neurodegenerative diseases. It is probable that, in neurons and oligodendrocytes,

OTUB1 plays other important roles, which are not affected by proinflammatory stimuli. We added these suggestions to the Discussion (page 21, lines 535-539).

Reference
Wang P, Joberty G, Buist A, Vanoosthuyse A, Stancu IC, Vasconcelos B, Pierrot N, Faelth-Savitski M, Kienlen-Campard P, Octave JN, Bantscheff M, Drewes G, Moechars D, Dewachter I (2017) Tau interactome mapping based identification of Otub1 as Tau deubiquitinase involved in accumulation of pathological Tau forms in vitro and in vivo. *Acta Neuropathol* 133: 731-749

Referee #3:

The Points of this reviewer have been addressed adequately

3rd Editorial Decision 28th Feb 2019

Thank you for submitting your revised manuscript to The EMBO Journal. I have now looked at everything and all looks good. I am therefore very pleased to accept the manuscript.

Congratulations on a nice study!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xu Wang, Dirk Schlüter
Journal Submitted to: the EMBO Journal
Manuscript Number: EMBOJ-2018-100947

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The group size was determined by using the G*Power 3.1.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The group size was determined by using the G*Power 3.1.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude animals from the experiments.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Sex- and age-matched animal were allocated randomly for the experiments.
For animal studies, include a statement about randomization even if no randomization was used.	Yes, we included this statement in the manuscript.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The EAE experiments were performed in a double-blind way.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes, we included this statement in the manuscript.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used a normality test in Graphpad Prism.
Is there an estimate of variation within each group of data?	Yes.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The information was included in the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Yes, this information was included in the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, this information was given in the manuscript.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirmed compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethics Committee of the University of Cologne, Gerrmany.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The statement is included in Material and Methods, section Histopathology.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No restrictions applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA