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NF-κB activation in astrocytes drives a stage-specific beneficial neuroimmunological response in ALS

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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 22nd December 2017

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been reviewed by referees their reports are provided below.

As you can see, the referees find the analysis interesting, insightful and support publication here. They raise a number of different concerns that I would like to ask you to address in 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 issues 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: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

It is well established that glial cells modulate the pathogenesis of motor neuron diseases such as amyotrophic lateral sclerosis (ALS). In this manuscript the authors studied the astrocytic-specific IKK2/NF-kB activation in the progress of the disease of the SOD1(G93A) mouse, a model for ALS. They propose that activation of the NF-kB pathway in astrocytes regulates microglia proliferation through Wnt5a signaling and contributes to a prolonged and milder presymptomatic phase, leading to a delayed disease onset, by decreasing motor-neuron degeneration. They use the appropriate set of experiments to address most of the main points that arise in such study.
Overall, this is a nice study supporting the hypothesis (although not being very new) that activated astrocytes modulate disease course and severity thought microglia activation that change their transcriptional machinery. However, the main weakness of the study is that several data are simply over interpreted and not all conclusions drawn are completely justified. Thus, the authors should be more cautious when interpreting the data.
For example, Fig. EV1O that shows several Iba-1+CD11b+ myeloid subsets during disease and these conclusions are not justified based on the data obtained. For these strong conclusions fate mapping tools would definitely be required.

Major points:
1. In figures that display astrogliosis or deramified microglia cells (ie. Fig2A, 2D, 2G, 4K, 6F), please include a nuclear marker (displayed merged or side by side). Otherwise it is difficult to assess whether there are indeed cells or just increased background of the staining.
2. The authors suggest that overexpression of IKK2 in GFAP expressing cells, leads in shift of astrocytes in an activated status, inducing astrogliosis. Astrocytes play an important role in the maintenance of the blood-CNS-barrier (here blood-spinal cord-barrier, BSCB). Activated astrocytes change their protein expression profile on their end-feet, leading to structural changes and leakage of the BSCB (Garbuzova-Davis, 2007). Did the authors investigate the integrity of the BSCB?
3. In combination with comment 2. The authors observed that 25% population of CD11b+ cells at P50 in both IKK and SOD1/IKK mice are also CD11c+ (FigEV1M,N). They claim that these cells are disease associated microglia (page 5). Although, these might as well be true CD11b and CD11c is also expressed by monocytes, blood-derived dendritic cells and macrophages, cells that contribute to ALS (Rusconi et al., 2017; Zhao et al., 2017). Since the BSCB might be compromised and they already see infiltration of CD3 cells at P50 a more thorough evaluation (eg. TMEM119+CD11c+) of the identity of these cells is suggested, to address peripheral contribution in the subsequent microglia activation and disease progression.
4. In Figure 4 the authors analyze the immune cells in the spinal cord by flow cytometry.
– Did the authors analyze also the P30 time point? By then, according to their previous data, there is already increased proliferation and deramification of microglia. It would be informative to include the early time point.
– In both IKK and SOD1/IKK groups already at P50 (and more at P90) they observe ~20% of non-microglia (CD11b+CD45low). Although a fraction of the CD11b+CD45high cells might be activated microglia, the rest are indicating again peripheral contribution (comments 4,5). How do the authors interpret this?
– In panel E-H they gate on CD11b+ cells (including microglia, perivascular & meningeal macrophages and infiltrating CD11b+ cells) what is the expression of the markers in CD11b+CD45low cells?
5. Regarding the use of the CB2 inhibitor. CB2 is also present in monocytes, macrophages, B-cells and T-cells (Galiègue et al., 1995; Centonze et al., 2008; Basu et al., 2011), which possibly contribute to the onset of the disease. The authors administered the drugs p.o., i.v. and i.p. suggesting that peripheral cells are also affected, which in turn can regulate the progression of the disease. How do the authors comment on that or how do they exclude the impact of the compounds on peripheral immune cells?
6. Did the authors identified the population of CD3 cells, are they CD8 or CD4, indicating a cytotoxic or protective response?
7. One of the major players in ALS pathology is the production of ROS which dysregulates glutamate control leading to excitotoxicity of the motor neurons (Rothstein et al., 1992). In the

SOD1-G93A the mitochondrial function is compromised and therefore affecting ROS production. Is this astrocytic NFkB activation regulates or suppresses ROS production?

Minor points:
1. Several errors have been detected regarding annotations in the Figures and reference of figure panels in the text, which confuse the reader. Indicatively, some examples are:
a. Page 4: 'Upon transgene activation (at P25), IKK-2CA mRNA and protein levels were persistently (at P50, P90 or P135; Fig 1B) upregulated in spinal cord in IKK and SOD1/IKK mice (Fig 1C-F) without interfering with the expression of mutant SOD1 (Fig 1C-F)' in figure 1 there is no panel B.
b. Figure 1 and 7: Several plots are shown with differently colored bars, but there is no indication of which groups these are!
c. Page 5: 'The increased cellularity in CD11b+/Iba1+/TMEM119+ cells was mainly due to the proliferation of two different microglia subpopulations: the Iba-1+CD45- and the Iba-1+CD45+ cells, taken together, accounted for 89.5{plus minus}4.2% PCNA+ (Fig EV2A-I) or Ki-67+ cells (Fig EV2J and K), with the latter representing roughly two-thirds of the PCNA+ cells (Fig EV2A-I)'. Again panel EV2J and K are missing from figure EV2.
d. Page 5: 'Therefore, NF-κB activation in astrocytes drives a neuroinflammatory response in IKK and SOD1/IKK mice, involving prominent microglial proliferation together with an important process of differentiation and polarization toward a reactive phenotype (Fig EVO), astrogliosis and leukocyte infiltration.' To which figure are they referring to? Is there a panel missing?
2. Regarding Materials and Methods.
a. Please provide the background of the SOD1-G93A mice and the gender of the mice used for the experiments (only in the legend of Fig1 it is indicated that male mice were used). To generate the double transgenic mouse the authors crossed C57BL/6 mice with NMRI mice and for the triple transgenic they crossed the aforementioned mixed background with the SOD1-G93A (background not mentioned).
b. It has been reported (Pfohl et al., 2015) that SOD1-G93A C57Bl/6 mice, when compared with SOD1-G93A B6SJL mice, display a delayed onset of the disease, increased lifespan and extended disease duration. Regarding gender, in both strains females appear to be more protected than males. Providing gender and background data, will assist in a more comprehensive interpretation of the results.
3. In Figure 6 the authors administer the Porcupine inhibitor C59 to block the WNT release.
a. In panel I the number of CD45+Iba1- is elevated in IKK and SOD1/IKK and comparing with Fig2H (CD45high cells) the numbers are almost doubled, suggesting again a noteworthy population or non-microglia immune cells. How do the authors comment on that?
b. In the corresponding text (page 9) they claim that they see a reduction of microglia density. In panel 6G they only display % of Iba1 area. Did the authors quantify the nuclei overlapping with Iba1?
4. Cite the following study on the role of astrocytic IKK2 for demyelination: PMID: 21310728

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Pfohl SR, Halicek MT, Mitchell CS (2015) Characterization of the contribution of genetic background and gender to disease progression in the SOD1 G93A mouse model of amyotrophic lateral sclerosis: A Meta-Analysis. J Neuromuscul Dis 2:137-150
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Rusconi M, Gerardi F, Santus W, Lizio A, Sansone VA, Lunetta C, Zanoni I, Granucci F (2017) Inflammatory role of dendritic cells in amyotrophic lateral sclerosis revealed by an analysis of patients' peripheral blood. Sci Rep 7:7853
Zhao W, Beers DR, Hooten KG, Sieglaff DH, Zhang A, Kalyana-Sundaram S, Traini CM, Hasley WS, Hughes AM, Sathe GM, Livi GP, Fan GH, Appel SH (2017) Characterization of gene expression phenotype in amyotrophic lateral sclerosis monocytes. JAMA Neurol 74(6):677-685

Referee #2:

In this study Alami et al. address the role of astrocytic NF-kB pathway activation in a mouse model of ALS. Their data show that activation of astrocyte NF-kB pathway induces Wnt 5A-dependent microglial proliferation and lymphocyte infiltration in both WT and pre-symptomatic ALS mice. This effect was associated with delayed disease onset and decreased histological alterations to motor neurons (mSOD1 aggregation and autophagy) but faster disease progression in SOD1G93A animals. Interestingly, this time course was correlated with an anti- to pro-inflammatory shift in the microglia/immune cells content in the spinal cord of ALS mice. Using a doxycyclin-dependent transgene expression, the authors confirmed dual astrocyte NF-kB control of (i) beneficial microglial activation at early stages of the disease, delayed disease onset whereas (ii) activation at later time points aggravated disease progression. Last, they show that modulation of early microglial activation through cannabinoid receptor 2 has protective effects on motor neuron histological alterations.

Overall, this is an interesting study that provides insights into temporally regulated neuroinflammatory signaling during disease progression in a model of ALS. Experiments performed in vivo are relevant to study glial-glial and glial-neuronal interactions, and the data provide mechanistic insights into the molecular pathways downstream of astrocyte NF-kB. While generally rigorously performed there are numerous errors, issues and questions that must be resolved in the data presented and other questions that should be addressed as detailed below.

Major Issues:
(1) Neuronal death should be assessed in the characterization of disease phenotype in Figure 3 and 5 because loss of motor neuron (MN)s correlates with disease progression in this model. The authors must clarify if MN death correlates with the microglia phenotype shift observed in SOD1(G93A)/IKK-CA mice with additional data.
(2) General comment- Almost of immunofluorescence staining images are saturated and prevent a correct identification and control of the staining itself. This is not very convincing although always quantified thoroughly but one might ask what those quantifications represent if performed with these images. Authors should modify these images and avoid saturation as much as possible. In some cases, DAPI is not necessary and just removing it will make the images clearer. This issue concerns the following figure panels: Figure 1G, I, K; Figure 2 D, J; Figure 4 I, K; Figure 6 F; Figure EV1 A, C, E, G, I, K, M; Figure EV2 A.
(3) General comment- For these histological figures to be self-explanatory, authors should include the antigen (for example, GFAP) on the image of the corresponding channel instead of the bottom of the panel. "Merge" should be used for composite images.
(4) Figure 1i Both p65 staining and DAPI are saturated. It makes it very difficult to identify nuclei and/ or cells and therefore is not convincing. From the quantification, the majority of these cells should be astrocytes, so authors should do a western blot from lumbar spinal cord of SOD1(G93A)-IKK2 CA and SOD1(G93A) controls to evaluate the ratio of phospho-p65 and total p65 levels, as a more quantitative measure of NF-kB p65 activation in these mice.
(5) Authors claim from the heatmap in Figure 6 that only Wnt5A is significantly upregulated in SOD1/IKK versus SOD1 mice but Wnt7a also seems even more upregulated, although its expression is more variable. A plot representing detectable Wnts (Wnt1, Wnt4, Wnt5a and Wnt7a) expression levels between groups is needed in Figure 6.
(6) There is no loss of function approach to confirm the specificity of Wnt5A involvement on astrocyte NF-kB-driven microglial proliferation. Porcn inhibitor Wnt-C59 is a generic Wnt inhibitor and could affect the expression of other Wnt family members. Authors mentioned "In all C59-treated mice Wnt5a immunoreactivity was markedly upregulated (>90%; not shown)", but what

about Wnt7a? Can the authors address this issue?

Other Specific Comments:
(7) Add more comprehensive introductory sentence on the NF-kB pathway in inflammation in the second introductory paragraph.
(8) The sentence reading, "in depth-analysis required a temporal control of NF-kB" is unclear.
(9) Regarding Figure 1 and related text:
a. Experimental groups should be defined more clearly in the first paragraph of the result section. Are the WT littermates also carrying the GFAP.tTA construct? Why the four groups aren't GFAP-tTA/tetO-IKK2-CA x SOD1G93A or WT and +/- DOX for each?
b. Between the text, the figure and the figure legends, homogenize IKK2CA writing (IKK2-CA, IKK2CA)
c. Figure 1C protein size in kDA should be added on the left hand side of the western blot
d. In general, the color code makes it very difficult to distinguish between IKK2CA and SOD1(G93A)-IKK2 CA groups (pastel green and blue)
e. When a value is undetectable in a group, put ND as non detectable instead of a blank (Figure 1D, E, N)
f. Figure 1F, problem with the scale (102 twice)
g. Figure 1G where is this field located, in the ventral horn grey matter? A low magnification image for both GFAP and IBA1 stainings is needed to visualize general IKK expression pattern. IBA1+ microglial cells are very difficult to identify as such on the bottom panel.
h. Figure 1K p65 staining does not look the same as in Figure 1i
i. Figure 1 lacks group labels

(8) Regarding Figure 2
a. Where are these quantifications done in the ventral spinal cord? Grey matter only? The GFAP staining in P50 and even P90 SOD1G93A mice alone is very surprisingly upregulated-if in grey matter-at that age.
b. General comments: the stars to show significance should be closer to the points that are significant, otherwise difficult to interpret.
c. Figure 2D, it is almost impossible to recognize microglia and their morphology (even if activated) in the IKK and SOD1/IKK groups.
d. Figure 2B and E authors should homogenize the y-axis label between % GFAP and IBA1+ area (% of total) or precise how this was quantified. I assumed from the methods that a ROI comprising the ventral horn was delineated and the % of "antigen+" area calculated as the ratio on the ventral horn area.

(9) Regarding Figure 3
a. Figure 3F and H low/high quartile should be represented as a ratio, it would simply the figure and be easier to visualize
b. Figure 3i cell volume or cell area/perimeter? How was this quantification done?

(10) In the result paragraph "Early-stage NF-kB activation in astrocytes enhances beneficial microglial activation", regarding the anti- and pro-inflammatory profile, explain the use of different markers to identify the heterogeneity of microglial/immune response in the result section rather than in the figure legend.

(11) Regarding Figure 5 and related text:
a. Figure 5E not clear on which groups were compared for statistical analysis from the bars with *** and ns
b. Is there an effect on astrocyte reactivity of the temporal regulation of astrocyte NF-kB between transgene activation time points?
c. The authors say that the extent of microglial polarization towards the pro-inflammatory phenotype (CD11b+CD68+) is comparable between SOD1/IKK-E and L and that there is a difference in the number of CD45+ cells. Could it also be due to a difference in terms of the number of anti-inflammatory microglia cells (CD11b/CD63 bright)? The authors should address this issue with more experimental data or explanation.

(12) Regarding Figure 5 and related text:

a. Figure 6E is this quantification per individual astrocyte?
b. Figure G to I ratio representation might be easier to visualize for these quantifications. There are a lot of groups and it is confusing.
c. Figure 6 K and M ratios low/high quartile. It is not clear between which groups the differences are significant for these plots.

(13) Regarding supplemental figures
a. Figure EV2 B and C change scale
b. Figure EV2 D-I almost exact same results for both time points, might not be necessary to show both but could be mentioned in the result section.

(14) The sentence mentioning a direct effect of astrocyte NF-kB on MN biology and the release of pro-inflammatory cytokines is out of place and should be revised.
(15) The first sentence of the second discussion paragraph on the absence of astrocyte proliferation versus astrocyte reactivity is unclear.
(16) Abbreviations are inconsistent, one example is: lcn2- lipocalin 2 abbreviated in the figure but not in results or figure legend. All abbreviations should be checked for consistent usage throughout the paper.

1st Revision - authors' response 26th March 2018

Point-by-point reply to the comments of the reviewers.

Referee #1:

Major points:
1. In figures that display astrogliosis or deramified microglia cells (ie. Fig2A, 2D, 2G, 4K, 6F), please include a nuclear marker (displayed merged or side by side). Otherwise it is difficult to assess whether there are indeed cells or just increased background of the staining.
>> We have amended the pictures accordingly and now we provide DAPI staining along with the GFAP, IBA1, CD45 and CD68 immunostaining in figure 2A, 2D, 2G, 4K (new 5K) and 6F (new 7G, new EV10). We have amended figure legends and captions accordingly.

2. The authors suggest that overexpression of IKK2 in GFAP expressing cells, leads in shift of astrocytes in an activated status, inducing astrogliosis. Astrocytes play an important role in the maintenance of the blood-CNS-barrier (here blood-spinal cord-barrier, BSCB). Activated astrocytes change their protein expression profile on their end-feet, leading to structural changes and leakage of the BSCB (Garbuzova-Davis, 2007). Did the authors investigate the integrity of the BSCB?
>> We agree with the reviewer on the importance of BSCB in contributing to neuroinflammatory and neurodegenerative processes in ALS. Therefore, we have included now an analysis of structural markers of Tight Junction (Tjs) complexes, relevant in maintaining the BSCB integrity (Claudin-V) and functional markers of BSCB permeability (parenchymal IgG immunostaining) for timepoints P30, P50 and P90. In WT mice, Claudin-V is visualized as an almost continuous ribbon inside the Collagen IV^{+} vascular basal membrane. However, already at P30, in SOD1 mice the Claudin-V ribbon becomes discontinuous, with focal breaks representing areas in which the BSCB has increased permeability. The length of the vascular segments showing disrupted Claudin-V was significantly higher in SOD1, SOD1/IKK and in IKK than in WT at P30 (Fig 3A,B) and was higher in SOD1/IKK than in SOD1 and IKK . We observed a progressive increase in the length of disrupted Claudin-V ribbon in IKK, SOD1 and SOD1/IKK at P50 and P90 with SOD1/IKK showing the greatest degree of disruption. Confirming the disruption of BSCB by astrocytic IKK activation and/or mutant SOD1 expression, we detected a significant leakage of IgG (Garbuzova-Davis et al., 2012) in the spinal cord parenchyma at P50 in IKK and SOD1/IKK mice (Fig 3C,D), confirming the neuroinflammatory component driven by astrocytic NF-κB, that became worse at P90. Thus, we show that activation of astrocytic IKK/NF-κB module causes BSCB disruption per se and enhances BSCB disruption in mice expressing mutant SOD1. However, the disruption of BSCB does not translate to a net toxic effect since BSCB is already significantly impaired during the early, neuroprotective phase. We report this data set in the new figure 3.

3. In combination with comment 2. The authors observed that 25% population of CD11b+ cells at

P50 in both IKK and SOD1/IKK mice are also CD11c+ (Fig EV1 M, N). They claim that these cells are disease associated microglia (page 5). Although, these might as well be true CD11b and CD11c is also expressed by monocytes, blood-derived dendritic cells and macrophages, cells that contribute to ALS (Rusconi et al., 2017; Zhao et al., 2017). Since the BSCB might be compromised and they already see infiltration of CD3 cells at P50 a more thorough evaluation (eg. $TMEM119^{+}CD11c^{+}$) of the identity of these cells is suggested, to address peripheral contribution in the subsequent microglia activation and disease progression.

>> We now provide further information to clarify the identity of $CD11c^{+}$ cells. We have included the results of the immunostaining for TMEM119 and CD11c, as suggested by the reviewer, in figure EV3N , O. We show that almost all of $CD11c^{+}$ cells are $TMEM119^{+}$, suggesting that the large majority of this population is of microglial origin. Only a tiny fraction of $CD11c^{+}$ appears to be $Tmem119^{-}$; this subpopulation is concentrated in clusters of smaller, round cells. Thus, the largest share of $CD11c^{+}$ cells represent microglia. We have amended the text to include this additional information. We have further excluded a peripheral origin for CD11c+ cells by considering the expression of the monocyte marker CD169 (new Fig EV3P-R). Almost all $CD11c^{+}$ were $TMEM119^{+}$ but consistently $CD169^{-}$, confirming their microglial identity (although we could identify a subset of $TMEM119^{+}$ microglia that was $CD169^{+}$, currently suggested to be early-activated microglia, Bogie et al., 2018). We also report on the time course of the $CD11b/CD11c^{+}$ microglia, whose fraction of the total $CD11b^{+}$ population remains stable between P50 and P90 (reported in Fig EV3S).

4. In Figure 4 the authors analyze the immune cells in the spinal cord by flow cytometry.
− Did the authors analyze also the P30 time point? By then, according to their previous data, there is already increased proliferation and deramification of microglia. It would be informative to include the early time point.

>> We have extended now the flow-cytometry analysis of immune cell populations and phenotypes to include the time point P30. The additional data show that the microglial population is already expanded after short term NF-κB activation in astrocytes (IKK and SOD1/IKK) at P30 (confirming histological data) whereas the subset of $CD11b^{+}/CD45^{high}$ cells are not yet elevated at this time point. There is a sharp increase in this population at p50 and also the fraction of $CD11b^{+}CD45^{low}$ further increases.
The immune cell population at P30 displays a relative enrichment in $CD163^{+}$ cells in SOD1/IKK mice compared to WT and SOD1 mice, together with low representation of $CD68^{+}$ cells. Thus, the polarization of the $CD11b^{+}$ immune cells at P30 appears very similar to that at P50. We have now added this new information in the results section and in the figure 5 and EV6.

− In both IKK and SOD1/IKK groups already at P50 (and more at P90) they observe ~20% of non-microglia (CD11b+CD45low). Although a fraction of the CD11b+CD45 high cells might be activated microglia, the rest are indicating again peripheral contribution (comments 4,5). How do the authors interpret this?

>> We do not want to state that there is no infiltration of peripheral immune cells upon NF-κB activation in astrocytes. However, an immediate consequence of NF-κB activation is expansion of microglia cells (see also TMEM119/IBA1 stainings). We think that the cells characterized as $CD11b^{-}/CD45^{high}$ can be classified as infiltrating peripheral immune cells and this group of cells is indeed increased in both IKK and SOD1/IKK mice. However, they reflect around 7-8% at p50 and around 10% at p90. At P30 this group of cells is only mildly enriched. In contrast, all cells represented by $CD11b^{+}/CD45^{high}$ might be either infiltrating cells or activated microglia cells as the reviewer indicated. These group of cells is almost absent at P30, whereas microglial $CD11b^{+}/CD45^{low}$ cells are already prominently expanded at this time point. Therefore we think that IKK/NF-κB activation in astrocytes induces at first microglia expansion and upon prolonged activation peripheral infiltration can be induced. In the discussion section, the role of infiltrating peripheral immune cells in phenotype development is now correspondingly noted.

− In panel E-H they gate on CD11b+ cells (including microglia, perivascular & meningeal macrophages and infiltrating CD11b+ cells) what is the expression of the markers in CD11b+CD45low cells?

>> We now performed the analysis of polarization markers expression CD163 and CD68 on two distinct populations of $CD11b^{+}/CD45^{low\text{-}intermediate}$ and $CD11b^{+}/CD45^{high}$ subsets (reported in figure EV6A-C). We show that a large fraction of $CD11b+/CD45l^{ow\text{-}intermediate}$ cells express high levels of CD163 at P30 and P50, but it declines at P90, whereas $CD11b^{+}/CD45^{high}$ cells do not express CD163 at any time point. Conversely, $CD11b^{+}/CD45^{high}$ show a strong increase in CD68 expression

from P30 and P50 (when it is comparatively low) to P90. Taken together with the demonstration that the large majority of CD11b$^+$ cells are TMEM119$^+$ (figure EV3), these data imply that polarization shifts occurs within distinct subset characterized by divergent activation state (identified by CD45 expression level).

5. Regarding the use of the CB2 inhibitor. CB2 is also present in monocytes, macrophages, B-cells and T-cells (Galiègue et al., 1995; Centonze et al., 2008; Basu et al., 2011), which possibly contribute to the onset of the disease. The authors administered the drugs p.o., i.v. and i.p. suggesting that peripheral cells are also affected, which in turn can regulate the progression of the disease. How do the authors comment on that or how do they exclude the impact of the compounds on peripheral immune cells?
>> We agree with Reviewer 1 about the possible contribution of peripheral immune system in modulating the onset and the progression of ALS. In fact, CB2 receptor is expressed at the highest level in B cells, macrophages, microglia and T cells. Although an indirect effect of CB2 agonists on microglial reactivity cannot be ruled out, it is noteworthy that the density of lymphocytes in spinal cord at P30 in WT and SOD1 mice was low (see also response to comment 4, FACS analysis at p30); moreover, we have verified that CB2 agonists do not exert any consistent effect on lymphocytes infiltration: CB2-A1, CB2-A2 and CB2-InvA do not change the number of CD4$^+$ cells in spinal cord, whereas CB2-A3 increases it (Figure 8G and H). Therefore, although we cannot rule out a possible additional effect on peripheral immune cells, it is likely that microglia is the main effector of the protective or detrimental effects of CB2-InvA or CB2-A, respectively. We have included additional data on the effect of CD4+ cells (Fig. 8G, H) and a note in the discussion to highlight this possible limitation.

6. Did the authors identified the population of CD3 cells, are they CD8 or CD4, indicating a cytotoxic or protective response?
>> We have now characterized the CD3$^+$ population along the lines suggested by Reviewer #1. We show that the largest majority of CD3$^+$ are CD4$^+$ and only a small minority is CD8$^+$. We have added the flow-cytometry data to figure EV8D and mentioned the results in the Results chapter.

7. One of the major players in ALS pathology is the production of ROS which dysregulates glutamate control leading to excitotoxicity of the motor neurons (Rothstein et al., 1992). In the SOD1-G93A the mitochondrial function is compromised and therefore affecting ROS production. Is this astrocytic NFkB activation regulates or suppresses ROS production?
>> Following the reviewer's suggestion, we have evaluated the levels of 8-hydroxydeoxyguanosine (8-OH-dG) and 4-hydroxy-2-noneal (4-HNE), commonly used for the evaluation of oxidative stress in tissue (Liou and Storz, 2015). Whereas the staining pattern of 4-HNE was not neuronal (and resembling astrocytic or microglial morphology), 8-OH-dG was detected in motoneurons (and in other neurons), both in nucleus and in cytoplasm (in agreement with reported patterns: Bender et al., 2013; Maity-Kumar et al., 2015). Immunostaining (new Fig 4K and L) for 8-OH-dG revealed that astrocytic NF-κB activation suppresses ROS production in SOD1/IKK samples at P50 but not at P90. This finding suggests that NF-κB in astrocytes interferes with ROS production in MN in a non-cell-autonomous manner in the early phase of ALS.

Minor points:
1. Several errors have been detected regarding annotations in the Figures and reference of figure panels in the text, which confuse the reader. Indicatively, some examples are:
a. Page 4: 'Upon transgene activation (at P25), IKK-2CA mRNA and protein levels were persistently (at P50, P90 or P135; Fig 1B) upregulated in spinal cord in IKK and SOD1/IKK mice (Fig 1C-F) without interfering with the expression of mutant SOD1 (Fig 1C-F)' in figure 1 there is no panel B.
>> We apologize for these errors and missing panels. We have now carefully addressed this issue and made sure that all annotations are depicted in a correct manner.

b. Figure 1 and 7: Several plots are shown with differently colored bars, but there is no indication of which groups these are!
>> We have now made sure that all figures contain legends clarifying the color code of the graphs and also unified the color code of the 4 experimental groups throughout the manuscript.

c. Page 5: 'The increased cellularity in CD11b$^+$/IBA1$^+$/TMEM119$^+$ cells was mainly due to the

proliferation of two different microglia subpopulations: the Iba-1^{+}CD45^{-} and the IBA1^{+}CD45^{+} cells, taken together, accounted for 89.5{plus minus}4.2% PCNA^{+} (Fig EV2A-I) or KI-67^{+} cells (Fig EV2J and K), with the latter representing roughly two-thirds of the PCNA^{+} cells (Fig EV2A-I)'. Again panel EV2J and K are missing from figure EV2.
>> We apologize for the errors and have now modified the figure (new EV4) in the correct manner. See also answer to specific comments 13 of referee #2.

d. Page 5: 'Therefore, NF-κB activation in astrocytes drives a neuroinflammatory response in IKK and SOD1/IKK mice, involving prominent microglial proliferation together with an important process of differentiation and polarization toward a reactive phenotype (Fig EVO), astrogliosis and leukocyte infiltration.' To which figure are they referring to? Is there a panel missing?
>> We apologize for this mistake. We have now modified the main text to include the correct call to the cartoon summarizing the microglial phenotype (Fig EV3K).
2. Regarding Materials and Methods.
a. Please provide the background of the SOD1-G93A mice and the gender of the mice used for the experiments (only in the legend of Fig1 it is indicated that male mice were used). To generate the double transgenic mouse the authors crossed C57BL/6 mice with NMRI mice and for the triple transgenic they crossed the aforementioned mixed background with the SOD1-G93A (background not mentioned).
b. It has been reported (Pfohl et al., 2015) that SOD1-G93A C57Bl/6 mice, when compared with SOD1-G93A B6SJL mice, display a delayed onset of the disease, increased lifespan and extended disease duration. Regarding gender, in both strains females appear to be more protected than males. Providing gender and background data, will assist in a more comprehensive interpretation of the results.
>> We now provide the full details of the genetic background of all mice (GFAP.tTA mice: C57BL/6 background; tetO7.IKK2-CA mice: NMRI background; high-copy SOD1-G93A line: C57BL/6 background) in the method section. Furthermore, we have added a graphical depiction of the exact breeding strategy and a clarification of the genotypes belonging to each experimental group in Fig 1A. The breeding strategy results in a mixed but clearly defined background of C57BL/6:NMRI (1:1) and importantly, all analysed animals (wt, single, double- and triple-tg) are littermates having the same mixed background. We also provide an additional experiment in which the triple-tg mice (SOD1/IKK in the main text) have been administered DOX through their lifespan, permanently silencing the IKK transgene expression. The survival and clinical progression of these mice was comparable to the GFAP.tTA/SOD1(G93A) mice (also continously administered DOX); thus, in absence of transgene activation, triple-tg mice have the same clinical progression of double-tg mice, ruling out any significant effects from C57BL/6:NMRI background or transgene interference (data reported in Fig EV5A, B).
We have also clearly stated that all experiments were performed with male mice, unless otherwise indicated, since male and female mice are known to have distinct progression rates. We now provide the clinical progression and survival data for female mice, too. Female SOD1 mice displayed, as previously reported, a slower progression rate; although overall survival was not modified in female SOD1/IKK mice compared to female SOD1, the presymptomatic phase (detected according to body-weight curves) was significantly longer in the former than in the latter (as observed in male mice); likewise, a strong trend toward shorter progression phase was observed in female SOD1/IKK mice. When the timing of clinical milestones was studied, female SOD1/IKK were shown to reach stage 1 and stage 2 later than female SOD1, but this effect disappeared for scores 3 to 5. Taken together, these data show that female mice were similarly affected by the activation of the IKK/NF-κB module as the male mice (see Fig EV5C-F).
We have also added the information regarding the effect of early and late activation of the IKK2/NF-κB on survival of female SOD1/IKK and IKK mice. In agreement with the outcome observed in males, early-only activation resulted in a significantly prolonged survival resulting, however, from a significantly prolonged presymptomatic phase followed by an actually shorter symptomatic phase, as observed in males. However, late-activation of the transgene results does not result in a significantly shorter survival; this is due to a larger variability in the female cohorts; in addition, we chose the P80 timepoint to activate the transgene, and this time point may not map on female progression in the same way it does for male progression (Fig EV9A-D).

3. In Figure 6 the authors administer the Porcupine inhibitor C59 to block the WNT release.
a. In panel I the number of CD45+Iba1- is elevated in IKK and SOD1/IKK and comparing with

Fig2H (CD45high cells) the numbers are almost doubled, suggesting again a noteworthy population or non-microglia immune cells. How do the authors comment on that?
>> We agree with the reviewer regarding the infiltration of peripheral immune cells upon NF-κB activation in astrocytes. Nevertheless, the reported discrepancy is due to the representation of two subsets of CD45$^+$ cells in the two graphs: whereas in Fig 2H we reported only the number of the brightest CD45 cells, in fig 6I we reported the total number of CD45 cells, therefore including microglia at different degree of activation. We have now re-quantified the number of CD45+ for figure 2H to include all CD45+ cells. The inclusion of a larger population now results in reconciled values for the two experiments. We have clarified the identity of the subpopulation depicted in each graph in the y axis, in the figure legends and in the text.

b. In the corresponding text (page 9) they claim that they see a reduction of microglia density. In panel 6G they only display % of Iba1 area. Did the authors quantify the nuclei overlapping with Iba1?
>> We now provide quantification of both the IBA1$^+$ cell number (number of microglial cells) and IBA1$^+$ area (which is dependent on microglial cell number and cell shape/size) in figure 7H and EV10D. The two measurements are in agreement, indicating that they reflect the increase in number and in reactivity of microglia.

4. Cite the following study on the role of astrocytic IKK2 for demyelination: PMID: 21310728
>> We now cite the recommended study in the introduction.

Referee #2:

Major Issues:
(1) Neuronal death should be assessed in the characterization of disease phenotype in Figure 3 and 5 because loss of motor neuron (MN)s correlates with disease progression in this model. The authors must clarify if MN death correlates with the microglia phenotype shift observed in SOD1(G93A)/IKK-CA mice with additional data.
>> We now provide data detailing the motoneuron density (number of VAChT$^+$ cells in ventral spinal cord area) at P38, P55, P90 and P120 for all four genotypes; MN density was obtained by sampling 10 histological sections of lumbar spinal cord between L3 and L5. Because of the transient nature of apoptosis, only a very small number of motoneurons was cleaved-caspase-3 positive at each given time point and therefore this readout could not be used. Our data show that WT and IKK mice showed a comparable and constant number of MN over time. SOD1 and SOD1/IKK mice showed a comparable number of MN at P38, while SOD1 mice displayed a significantly decreased number of MN at P55 (corresponding to the time of the first denervation), which declined further over time. Notably, the number of surviving MN was significantly larger in SOD1/IKK than in SOD1 mice at P55, P70, and P90, but no longer significant at P120. Together with the data on biomarkers (misfolded SOD1, p62, LC3A, 8-OH-dG) and on NMJ denervation, these new data contribute to confirm a direct neuroprotective effect of astrocytic IKK2/NF-κB early-phase activation. We have added the data to the results and in figure 4OP.

(2) General comment- Almost of immunofluorescence staining images are saturated and prevent a correct identification and control of the staining itself. This is not very convincing although always quantified thoroughly but one might ask what those quantifications represent if performed with these images. Authors should modify these images and avoid saturation as much as possible. In some cases, DAPI is not necessary and just removing it will make the images clearer. This issue concerns the following figure panels: Figure 1G, I, K; Figure 2 D, J; Figure 4 I, K; Figure 6 F; Figure EV1 A, C, E, G, I, K, M; Figure EV2 A.
>> All confocal images acquired within this project were checked to be not saturated and to have an histogram distribution spanning the 10-90 percentile of the dynamic range of the detectors (for 12-bits images, approximately between 400 and 3600 arbitrary units). Therefore, all the numerical data derived from image quantification were obtained from datasets within the linear range of the detectors. Contrast of the images was enhanced (only for display purposes and homogeneously across conditions) in order to enhance the presentation quality. We have modified the display parameters to allow for a wider dynamic range in the displayed images .

DAPI staining has been retained to guarantee the cellular identity of the structures displayed and to allow the assessment of cell density; please note that the maintenance of the DAPI has been requested by reviewer #1.

(3) General comment- For these histological figures to be self-explanatory, authors should include the antigen (for example, GFAP) on the image of the corresponding channel instead of the bottom of the panel. "Merge" should be used for composite images.
>> We have amended the panels depicting immune-fluorescence images to include the name of the antigen matching the color used for display, as recommended.

(4) Figure 1i Both p65 staining and DAPI are saturated. It makes it very difficult to identify nuclei and/ or cells and therefore is not convincing. From the quantification, the majority of these cells should be astrocytes, so authors should do a western blot from lumbar spinal cord of SOD1(G93A)-IKK2 CA and SOD1(G93A) controls to evaluate the ratio of phospho-p65 and total p65 levels, as a more quantitative measure of NF-kB p65 activation in these mice.
>> We have now replaced the panels depicting p65 and DAPI in order to provide a better identification of nuclei and cells; we have made sure that none of the pictures is saturated, while still providing sufficient contrast for proper visualization.
In addition, we now provide in figure 1E the western blot of total p65 and phosphorylated p65, together with the IKK and SOD1 blots. Our data clearly show a significant increase in the ratio of phosphorylated/total p65, confirming the activation of the NF-κB module, in agreement with the immunostaining findings. Quantification of the ratio of phospho-p65 and total p65 levels is shown in EV1C. *We have amended the text and the figure legends to include the new data.*

(5) Authors claim from the heatmap in Figure 6 that only Wnt5A is significantly upregulated in SOD1/IKK versus SOD1 mice but Wnt7a also seems even more upregulated, although its expression is more variable. A plot representing detectable Wnts (Wnt1, Wnt4, Wnt5a and Wnt7a) expression levels between groups is needed in Figure 6.
>> We now provide the bar plot representing Wnt4, Wnt5a and Wnt7a in Figure 7 and Wnt1 in Figure EV10A. We have amended the text to clarify that, although Wnt5a is not the only Wnt expressed, it is the only one whose expression pattern follows the IKK genotype and it is therefore the most likely candidate for IKK-related effects. In fact, Wnt7a is upregulated only in IKK but not in SOD1/IKK mice, making it unlikely that Wnt7a may be a strong mediator of astrocytic IKK2/NF-κB-induced microglial proliferation. Wnt1 is upregulated (compared to WT) in IKK but downregulated in SOD1 and SOD1/IKK mice and Wnt4 appears to be unaffected by IKK activation or mutant SOD1 expression. We have changed the text in the results chapter and the figure legends to detail the results.

(6) There is no loss of function approach to confirm the specificity of Wnt5A involvement on astrocyte NF-kB-driven microglial proliferation. Porcn inhibitor Wnt-C59 is a generic Wnt inhibitor and could affect the expression of other Wnt family members. Authors mentioned "In all C59-treated mice Wnt5a immunoreactivity was markedly upregulated (>90%; not shown)", but what about Wnt7a? Can the authors address this issue?
>> We agree with the reviewer that although Wnt5a is the only Wnt members whose expression is upregulated in IKK and SOD1/IKK mice (whereas Wnt7a and Wnt1 are downregulated in SOD1 and SOD1/IKK mice), and therefore it is the most likely mediator of IKK activation. Moreover, whereas WNT5a is expressed in astrocytes, as shown by the immunolocalization in $GFAP^+$ cells (Fig 7D), WNT7a was not expressed in astrocytes (see new Fig EV10B) neither in WT or SOD1 mice nor upon IKK2-CA expression (rather, WNT7a was expressed in a small number of clustered, round cells resembling infiltrating leukocytes).
We agree that the Porcupine inhibitor affects all WNT proteins. Indeed, WNT5a were downregulated in C59-treated mice, as verified by immunostaining (EV10C). We apologize for the typo: instead of "upregulated (>90%; not shown)", we meant "downregulated (levels <10% of the vehicle-treated"). We have amended the results and the discussion to address this possible limitation.
In addition, we have further expanded the exploration of the role of WNT proteins in microglia activation in ALS, showing that treatment with C-59 in the late phase of the disease (from P90 to P102) still results in the decrease in the number of $IBA1^+$ and $CD45^+$ cells (shown in Figure EV10F-H), indicating a persistent role of WNT proteins in mediating astrocytic NF-κB-dependent microglial expansion in ALS.

Other Specific Comments:
(7) Add more comprehensive introductory sentence on the NF-kB pathway in inflammation in the second introductory paragraph.
>> We have now added a comprehensive sentence describing the role of NF-κB in immune responses and inflammation, making reference to the review by Hayden and Ghosh (2012).
(8) The sentence reading, "in depth-analysis required a temporal control of NF-kB" is unclear.
>> We have replaced the quoted sentence "in depth-analysis required a temporal control of NF-κB" with " the elucidation of the role of NF-κB requires experimental control over the timing and the cellular subpopulations in which its activation takes place".

(9) Regarding Figure 1 and related text:
a. Experimental groups should be defined more clearly in the first paragraph of the result section. Are the WT littermates also carrying the GFAP.tTA construct? Why the four groups aren't GFAP-tTA/tetO-IKK2-CA x SOD1G93A or WT and +/- DOX for each?
>> We have amended the paragraph to clarify the genotype of each of the experimental groups "Four experimental groups were contrasted: the GFAP.tTA/(tetO)7.IKK2-CA/SOD1(G93A) (activation of astrocytic IKK2/NF-κB in mutant SOD1 ALS model, henceforth SOD1/IKK), GFAP.tTA/SOD1(G93A) mice (mutant SOD1 but no activation of IKK2/NF-κB; henceforth SOD1), GFAP.tTA/tetO.IKK2-CA mice (activation of astrocytic IKK2/NF-κB only, no mutant SOD, henceforth IKK) and GFAP.tTA (no activation of IKK2/NF-κB and no mutant SOD1, henceforth WT; Fig 1A)". Following the advice of the reviewer, we have now added, in Fig. 1A, B, and C, a graphical representation of the experimental groups and of the experimental design.
b. Between the text, the figure and the figure legends, homogenize IKK2CA writing (IKK2-CA, IKK2CA)
>> We have amended the text to eliminate inconsistencies; only the IKK2-CA writing is now used.

c. Figure 1C protein size in kDA should be added on the left hand side of the western blot
>> Protein size in kDA is now reported for each protein (IKK, p65, phospho-p65, SOD1, LCN2, IBA1 and GAPDH) investigated in the western blots in Fig 1E, EV1D and EV2C.

d. In general, the color code makes it very difficult to distinguish between IKK2CA and SOD1(G93A)-IKK2 CA groups (pastel green and blue)
>> We have replaced the color code throughout the manuscript to ensure an improved readability: we now use dark blue for SOD1/IKK, orange for IKK, green for WT and light blue for SOD1. We further added a scheme in figure 1A.

e. When a value is undetectable in a group, put ND as non detectable instead of a blank (Figure 1D, E, N)
>> We have amended the figures as recommended.

f. Figure 1F, problem with the scale (102 twice)
>> We apologize for the typo, which has been replaced with the correct labelling (10^3).
g. Figure 1G where is this field located, in the ventral horn grey matter? A low magnification image for both GFAP and IBA1 stainings is needed to visualize general IKK expression pattern. IBA1+ microglial cells are very difficult to identify as such on the bottom panel.
>>All images were obtained in the ventral horn grey matter (we have now clarified this point in the Methods section) and all the qualifications excluded the white matter. We now provide a low-magnification overview of the ventral horn displaying the strong expression of IKK2-CA, colocalized with $GFAP^+$ (new Fig 1F). We originally provided a single-optical section to demonstrate the lack of colocalization between IKK2-CA and microglial markers, which made the microglial cells difficult to appreciate. We have now replaced the microglial panel with a maximum-intensity-projection of 5 optical sections; in the new images the microglial morphology (EV2A, B) is more easily appreciated.

h. Figure 1K p65 staining does not look the same as in Figure 1i
>> The original pictures were obtained with two different batches of the same antibody, which displayed different performance. We have now performed new immunostaining using the better-

performing antibody; we have therefore replaced the former figure 1I (new Fig 1H) to ensure homogeneity.

i. Figure 1 lacks group labels

>> We apologize for the mistake; now the group labels have been added to the figure.

(8) Regarding Figure 2

a. Where are these quantifications done in the ventral spinal cord? Grey matter only? The GFAP staining in P50 and even P90 SOD1G93A mice alone is very surprisingly upregulated-if in grey matter-at that age.

>> All images were acquired in the ventral spinal cord and in the quantification only the grey matter was considered; we provide now a representative picture of the region of interest considered in figure 1F. Confocal imaging parameters were optimized in order to have intensity fluorescence within the linear range of the detectors. Immunostaining for GFAP was preceded by citrate-heat antigen retrieval to increase the sensitivity of the immunostaining and the specificity of the immunostaining was confirmed using two unrelated commercially-available anti-GFAP antibodies (rabbit polyclonal anti-GFAP from Abcam and mouse monoclonal anti-GFAP from Sigma). Increase in $GFAP^+$ processes, a proxy of astrogliosis, was detected at P50 (and further increased from P70 to P120) in agreement with previous publications identifying changes in GFAP already at about P60 (e.g. Ringer et al., 2009; Yang et al., 2011; Watanabe-Matsumoto 2017; Sunyach et al., 2012). The representative images were contrasted (with equal parameters across different genotypes) in order to enhance the differences and to highlight the processes of the astrocytes. We have changed the representative pictures and included a DAPI staining to highlight nuclei. We have specified in the Methods section the details of the region of interest considered for the quantification and the details of the procedure for GFAP immunostaining.

b. General comments: the stars to show significance should be closer to the points that are significant, otherwise difficult to interpret.

>> We have repositioned the asterisks in close proximity to the datapoint they refer to.

c. Figure 2D, it is almost impossible to recognize microglia and their morphology (even if activated) in the IKK and SOD1/IKK groups.

>> In order to allow the detailed examination of the cell morphology, we now provide high-magnification single optical section representative images of microglial cells corresponding to the figure 2D. We have added the new figures (EV2A and B) and changed the corresponding description in the figure legends.

d. Figure 2B and E authors should homogenize the y-axis label between % GFAP and IBA1+ area (% of total) or precise how this was quantified. I assumed from the methods that a ROI comprising the ventral horn was delineated and the % of "antigen+" area calculated as the ratio on the ventral horn area.

>> We have amended the figure to include a consistent y-axis labelling. We have also added to the Methods section a brief explanation of the quantification procedure for $GFAP^+$ and $IBA1^+$ area: " The quantification of the fraction of area occupied by $GFAP^+$ or $IBA1^+$ structures was performed as previously reported (Saxena et al., 2013); briefly, artifact-free ROI were considered in the ventral horn and for each we computed the ratio between the area displaying GFAP or IBA1 immunostaining above a set intensity threshold and the total area of the ROI. For quantitative analysis a minimum of 8 artifact-free sections per mouse were analyzed."

(9) Regarding Figure 3

a. Figure 3F and H low/high quartile should be represented as a ratio, it would simply the figure and be easier to visualize

>> In order to enhance the readability of the graphs and maintain low the number of data groups, we are now representing the data relative to misfolded SOD1 burden, LC3A burden and oxidative damage markers as single histograms depicting the "fraction of high-burden MN". We have considered the 75th percentile threshold in SOD1 mice (or SOD1 mice treated with vehicle in pharmacological experiments) as the high-burden threshold: for each experimental group, the percentage of MN whose fluorescence intensity exceeding the threshold has been calculated (being 25%, of course, in the single-tg or vehicle-treated SOD1 mice). We have changed the graphs in the figures 4, 7 and 8 and we have added the corresponding description in the methods section.

b. Figure 3i cell volume or cell area/perimeter? How was this quantification done?

>> The burden of p62 was defined as the percentage of cytoplasmic area in each MN occupied by p62-positive aggregates. For the quantification, we considered confocal stacks (6-8 optical sections thick) collapsed in maximum-intensity projections. The contour of each MN was manually drawn using the VAChT staining as reference; the surface of the cytoplasmic compartment was computed, excluding the nucleus. Within the same contour, the area occupied by p62 aggregates was computed by thresholding the p62 channel (to distinguish the bright p62 aggregates from the dimmer diffuse soluble p62 immunostaining in the cytoplasm). Although this approach does not measure the burden of aggregates in terms of the volume occupied, the use of a limited number of optical sections prevents biases due variable and depth-dependent penetration of the antibodies. We now provide a brief explanation of the p62 burden quantification in the Methods section.

(10) In the result paragraph "Early-stage NF-kB activation in astrocytes enhances beneficial microglial activation", regarding the anti- and pro-inflammatory profile, explain the use of different markers to identify the heterogeneity of microglial/immune response in the result section rather than in the figure legend.
>> We have removed the marker explanation from the figure legend and included it in the main text.

(11) Regarding Figure 5 and related text:
a. Figure 5E not clear on which groups were compared for statistical analysis from the bars with *** and ns
>> To avoid overloading the graph, we depicted only the statistical analysis related to the comparison of SOD1/IKK groups. We have now clarified this point in the figure legend.

b. Is there an effect on astrocyte reactivity of the temporal regulation of astrocyte NF-kB between transgene activation time points?
>> We have now performed immunostaining for GFAP with samples taken from the different transgene activation time points. Our findings reveal that the $GFAP^+$ area (% of total) is comparable in prolonged-, early-, early-short and late-activated SOD1/IKK mice, in which the area is also comparable to SOD1 mice. The $GFAP^+$ area of IKK mice activated in the prolonged, early or late modus are comparable but do not reach the level of SOD1/IKK of each group. Since only in the early-short IKK activation modus the $GFAP^+$ area appears to return to baseline value by P130, the pattern of astrocyte reactivity upon differently timed NF-κB activation resembles a delayed course in comparison to the one shown by microglia. We have now added a new graph (Fig 6E) to display the astrocyte reactivity in the different groups and included the findings in the main text.

c. The authors say that the extent of microglial polarization towards the pro-inflammatory phenotype (CD11b+CD68+) is comparable between SOD1/IKK-E and L and that there is a difference in the number of CD45+ cells. Could it also be due to a difference in terms of the number of anti-inflammatory microglia cells (CD11b/CD63 bright)? The authors should address this issue with more experimental data or explanation.
>> We have measured the number of anti-inflammatory microglia using the CD206 marker in samples obtained at P120 from early, late and short-early-activated SOD1/IKK mice (and to continuously-activated SOD1/IKK as well as to SOD1 mice). Notably, the number of $Iba1^+CD206^+$ cells was very low-to undetectable in all mice at P120, irrespective of the genotype and of the activation schedule (positive controls from P50 IKK contained the predicted number of CD206 cells). Therefore, anti-inflammatory $CD206^+$ microglia provides only a small contribution to the pathogenic process at P120. Because of the consistently low number of cells detected, we perceived it was not opportune to include a graph relative to this piece of evidence but we have now included this information in the text of the Results chapter.

(12) Regarding Figure 5 and related text:
a. Figure 6E is this quantification per individual astrocyte?
>> Former Figure 6E (now figure 7F) depicts the mean fluorescence intensity of WNT5a immunolabelling in $GFAP^+$ cells (having demonstrated that WNT5a is not expressed in other cells types, former figure 6D- now figure 7E). We have clarified this point in the text.

b. Figure G to I ratio representation might be easier to visualize for these quantifications. There are a lot of groups and it is confusing.

>> We have now changed the representation and depict only the fraction of high-burden cells, as described above (see also response to point 9a).

c. Figure 6 K and M ratios low/high quartile. It is not clear between which groups the differences are significant for these plots.
>> We have modified the graphs to represent only the high-burden fraction (see above) and we have repositioned the asterisks in close proximity to the groups they refer to.

(13) Regarding supplemental figures
a. Figure EV2 B and C change scale
>> We amended the scale in the graphs (now ranging 0-5 cells/10^4 mm^2) representing $PCNA^+$/Iba-1^+/$CD45^+$ and PCNA/Iba-1^-/$CD45^+$ to better represent the comparatively lower number of cells.
b. Figure EV2 D-I almost exact same results for both time points, might not be necessary to show both but could be mentioned in the result section.
>> Following the recommendation of the reviewer, we have streamlined the figure removing the graphs for the P90 time point.

(14) The sentence mentioning a direct effect of astrocyte NF-kB on MN biology and the release of pro-inflammatory cytokines is out of place and should be revised.
>> We have now revised the sentence as follows: "In the present work we have shown that astrocytic NF-κB regulates microglial activation and proliferation and markedly upregulates the neuroimmunological response in WT as well as in the SOD1(G93A) ALS model (although astrocytic NF-κB may also control additional disease-relevant pathways)."

(15) The first sentence of the second discussion paragraph on the absence of astrocyte proliferation versus astrocyte reactivity is unclear.
>> We have now replaced the sentence "Surprisingly, the intense microglial proliferation is accompanied by astrogliosis but not by a comparable expansion of the astrocyte population, suggesting that NF-κB activation may modify the homeostatic set-point between these two populations (De Biase et al., 2017)" with a more clear statement "Surprisingly, NF-κB activation resulted in the proliferation of microglia but not in the proliferation of astrocytes themselves, but rather in their hypertrophy (astrogliosis); thus, astrocytic NF-κB appears to regulate the relative density of these two glial populations and in particular to regulate the expansion of the microglia (De Biase et al., 2017)"

(16) Abbreviations are inconsistent, one example is: lcn2- lipocalin 2 abbreviated in the figure but not in results or figure legend. All abbreviations should be checked for consistent usage throughout the paper.

>> Abbreviations have been thoroughly checked and their use has been made homogeneous throughout the paper.

2nd Editorial Decision 20th April 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been re-reviewed by the two referees and as you can see below both referees appreciate the introduced changes and support publication here.

I am therefore happy to let you know that we will accept your manuscript for publication in The EMBO Journal. Before I can send you the formal acceptance letter there are just a few things to sort out in a final revision.

- Please include a running title in the manuscript text file

- You have at the moment 10 EV figures, but you can only have 5 EV Figures. I don't know if it is possible to combine some of them? Or alternatively you can use the appendix. Please also see our author guidelines http://emboj.embopress.org/authorguide#expandedview

- The call out for Fig. 7C missing.

- There are 2 Appendix Table files - Please combine them into one appendix file with a ToC.

- Fig 3C Will you please check the zoomed insert of the WT sample and if that is correct? It almost looks like the image has been flipped. Please also check the white boxes in IKK & SOD1 (p50) to double check that they accurately mark where the zoomed images are taken from.

- For the figures in Fig EV3 (A,C,E,G,I,L,N): The bottom panels look to be higher magnified views of the upper panels but this is not marked with a box. Also the figure legend is not very clear about that. Please take a look.

- I have attached an annotated word document from our publisher where they have marked several issues in the text - mainly figure legends - that should be addressed. Would you please use this document as a base for any other change you do and mark your response to the issues raised by the publisher. This is just so that it is easier for me to see your response.

- Lastly, we also encourage the publication of source data, particularly for electrophoretic gels and blots. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

I have provided a revision link below so that you can upload the modified files.

Congratulations on a nice study!

REFEREE REPORTS

Referee #1:

The authors did a great job by addressing all my concerns. There are no questions left. This is now a carefully revised and strong manuscript.

Referee #2:

The authors have provided a comprehensive set of responses to reviewer points. The paper is much improved and concerns regarding data have been adequately addressed. The results provide new insight into ALS pathology.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Francesco Roselli
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-98697R

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 χ2 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 sample size for in vivo and ex vivo analyses was chosen based on previous experiments and on similar studies performed by other groups. The number of independent animals/samples used in both in vivo and ex vivo experiments is mentioned in the respective figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to estimate sample size. The sample size was chosen based on previous experiments and on similar studies performed by other groups using the SOD1(G93A) ALS animal model.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from analyses.
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.	No specific randomization was used. Mice (littermates) were selected based on age and sex to build comparable groups of animals with similar age and sex distribution. Results were obtained from at least to independent litters.
For animal studies, include a statement about randomization even if no randomization was used.	No specific randomization was used. Results were obtained from at least to independent litters.
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.	Clinical scoring, body weight measurements and survival analysis were performed by an investigator (C.S.) blind to the genotype of the animals. Likewise, histological analysis were performed and quantified by an different investigator (N.O.) unaware of the genotypes by the time of the analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All animals from any given litter were considered for experiments and allocated to their genotype groups only after the completion of the observation/quantification/scoring. The investigator did not know the genotypes of littermates during analysis (e.g. clinical scoring).
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests are mentioned in the figure legends and further described in the Materials and Methods section. In particular, we used one-way ANOVA for the comparison of multiple groups, and two-way ANOVA when two variables were considered (e.g., genotype and drug treatment). Kaplan-Meyer was used for the comparison of survival and progression curves.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, normality of the data distribution was routinely assessed by Brown-Forshyte test.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	We have computed variance, standard deviation and standard error of the mean for each experimental group; for brevity, we have reported only standard deviation values (unless otherwise indicated). The specific estimate of variation used is reported in each figure legend.
Is the variance similar between the groups that are being statistically compared?	Variance of the groups has been tested and verified equal before adoption of parametric tests for comparisons.

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).	All antibodies used in this study are exactly specificed in the material and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in this study.

* 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.	Both female and male mice of different age and genotype were used in this study; detailled genotypes for each experimental group have been added in figures and in the main text; breeding strategy has been reported in figure 1A. Detailed information on animals is reported in Material and Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were performed in compliance with institutional guidelines (Tierforschungszentrum, Ulm) and German animal protection law and approved by Regierungspräsidium Tübingen (Tübingen, Germany).
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 comply with the ARRIVE guidelines. In addition, we have made all efforts to comply to the 3R guidelines, in order to minimize the number of animals subject to procedures or to any discomfort.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No experiments with human subjects were conducted.
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	No