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The NAD-dependent deacetylase sirtuin 2 is a suppressor of microglia activation and brain inflammation

Teresa Faria Pais, Eva M Szego, Oldriska Marques, Leonor Miller-Fleming, Pedro Antas, Patricia Guerreiro, Rita de Oliveira, Burcu Kasapoglu and Tiago Fleming Outeiro

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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.)

Editor: Karin Dumstrei

Additional Correspondence   22 February 2013

Thank you for submitting your manuscript to the EMBO Journal. 4 referees were asked to review the paper and I have now received comments back from 3 of them. I have provided the reports below. As you can see, significant concerns are raised. I would therefore like to give you an opportunity to consider the referees' reports and to provide me with a detailed point-by-point response outlining what experiments you can add to address the concerns raised before taking a decision.

The referees appreciate the potential interest of the findings reported and find the study timely. However, referee #1 also indicates that further in vivo analysis using either Sirt2 deficient mice or, even better, a microglia-specific conditional KO of Sirt2 is needed to strengthen the findings. Referees #2 and 4 also raise a number of important concerns that would have to be addressed. If you are able to significantly extend the manuscript along these lines then we would be interested in considering a revised version. As I don't know what data you have on hand or able to generate within a 3-6 months time frame it makes it difficult to invite a revision. I would therefore like to ask you to provide me with a detailed point-by-point response upfront before taking the decision. This is the most efficient way forward. Please do note that we would need significantly more in vivo analysis to support a role of Sirt2 in suppressing microglia activation for consideration here.

Looking forward to seeing the point-by-point response.

REFEREE REPORTS

Referee #1

The manuscript by Pais et al. reports on the role of SIRT2 in the control of microglial activation.

Sirtuin 2 is the member of a family of NAD-dependent deacetylase sirtuin. Numerous acetylated proteins are targeted by sirt2 activity, including different transcription factors, histones or cytoskeletal proteins. As a consequence sirt2 is involved in numerous cellular and physiological functions particularly in the brain.

In this study, the authors analyzed the function of sirt2 in microglia, which has never been investigated before. The main results of this study are:

1- sirt2 negatively regulates microglial activation, assessed by measurement of the production or expression different pro-inflammatory markers;
2- sirt2 negatively regulates microglial-induced neurotoxicity;
3- sirt2 is likely to mediates these effects through the deacetylation of NF-kB, a well characterized transcription factor involved in the transcription of different pro-inflammatory proteins.
The main conclusion of this study is that sirt2 is the main gatekeeper of microglial activation in the brain and could represent a potential target for therapeutic intervention in neuro-inflammatory disorders.

The finding that sirt2 regulates some facets of microglial activation is new, however this study remains limited by the fact that most experiments have been performed in vitro, often with the N9 microglial cell line, although a limited number of experiments were performed in primary microglial culture or with an in vivo model of microglial activation. In addition, protocol used to trigger microglial activation (cell stimunaltion with LPS+TNF or intracortical LPS) are of limited interest regarding physiological conditions.
Overall this study would be much more convincing if microglial activation was analyzed into details, in sirt2-deficient mice, ideally in microglia-specific sirt2 deficient mice, using appropriate in vivo physiopathological models of microglial activation.

Beside this general comment, this study also suffers from numerous experimental approximations and from the lack of interpretation of different results.

Major comments:

- On of the key results of the study is that sirt2 regulates NF-kB activity preventing its acetylation and thus its function as an initiator of inflammatory gene expression. Transcription of Il1 is known to be dependent of NF-kB activity, yet induction of Il1 expression by LPS is not affected in sirt2-deficient mice (Fig 1E). This discrepancy needs to be addressed.

- A closer analysis of microglia in sirt2-deficient mice is lacking. Indeed, the constitutive deletion of sirt2 might have some repercussion on the microglial physiology even in the absence of inflammatory challenge. The authors should analyze whether resting microglia phenotypes (i.e. morphology, density...) are affected by sirt2 deletion compared to wild type mice.

- The differences between sirt2 western blot experiments presented in figure 1B and 2A are unclear. First, LPS reduces the expression of sirt2 in brain extract, but this effect is not present in N9 cells stimulated with LPS+TNF. Second, the sirt2 large form and the phosphorylated shorted form are not apparent on western blot experiments from brain extracts. Is there any explanation for these differences?

- There are also discrepancies in Mpa21 expression between brain extract and N9 cells. In brain extract LPS triggers the expression of Mpa21 (Fig1E) while this is not present in N9 cells (Fig5B).

Other comments:
- Immunohistochemistry in panel 1A is of poor quality compared to panel D. In addition it is unclear why there is no Iba1 staining in PBS samples of panel C.

- Often data are normalized, sometimes not. All data should be expressed in the same way, and normalization should be used only when necessary.

- Student't test can not be used for multicomparison (Fig2, panel C). Please used Anova instead.

- There is no legend for Fig 2 panel F. Please revise.

- Material and method section is un-informative. Please provide more detailed experimental procedure instead of references. Origin of sirt2-deficient mice is lacking.

Referee #2

The manuscript by Faria Pais and colleagues elegantly and convincingly demonstrates an anti-inflammatory role of SIRT2 in activated microglia. Using a combination of approaches, including in vivo and in vitro studies in which expression levels of SIRT2 were manipulated using KO, KD and overexpression strategies, the authors demonstrate the capacity of SIRT2 to negatively affect several parameters linked to microglia activation, such as pro-inflammatory mediators' production and expression of activation markers.

Major remarks:

Although the data in Figure 1 and 2 convincingly demonstrate an inverse relationship between SIRT2 expression / activity and the production of pro-inflammatory mediators, the authors often propose or imply possible mechanisms at work, without really providing a firm demonstration.

1. In Figure 3, the authors juxtapose data concerning regulation of iNOS expression and activity with data related to cell survival. Although cautious in their interpretation, the authors leave the reader with the impression that these two observations, described in the same paragraph / figure are functionally linked. Modulation of NF-kB activity by SIRT2 could affect ROS/RNS production and possibly expression of pro-and anti-apoptotic genes such as Bim and Bcl-XL, as suggested by Lanzillotta A et al, Neurobiol Dis 2012. Although the experiment using conditioned media is in favor of the author's assumption (production of ROS/RNS), no experiment using pharmacological agents interfering with ROS/RNS production or biological activity has been performed to support their role. Such an experiment should be performed, and, depending to the result, the conclusions adapted.

2. Figure 5 appear as an attempt to support the mode of action of SIRT2 based on its ability to deacetylate K310 of NF-kB, as illustrated in the summary Figure. Again, the data showing increased basal and induced acetylation of K310 in SIRT2 KO cells (Figure 5C) are convincing. However, it is difficult to rationalize most of the observations reported in this study with this proposed mode of action, as implicitly admitted by the authors themselves. Indeed: (i) K310 is also a well described SIRT1 target, but KD of SIRT1 only recapitulates one of the key observations of the study, i.e. IL-6 overexpression (Fig S3); (ii) there is no clear relationship between the effect of shSIRT2 and the NF-kB-dependent status of genes examined in Figure 4. In other

words, only poly I:C affect expression of a prototypic NF-kB-dependent gene such as TNF (as discussed by the authors), while other TLR ligands show no effect. IL-10 production however, is increased in shSIRT2 expressing cells in response to both TLR4 and TLR2 ligands. The authors do not discuss the possibility that acetylation status of K310 in NF-kB could only affect a subset of NF-kB dependent genes (as suggested in the literature) and the only example they provide (Mpa21, Fig 5B) is somehow unrelated to the study. This referee is well aware of the putatively complex role of sirtuins in modulating a biological response, and pinpointing all mechanisms at work in a single study would be unrealistic. The authors should however attempt to better illustrate this biological complexity by exploring additional, non NF-kB related, site of action for SIRT2, rather than simply enumerating them (see discussion). A more detailed analysis of signal transduction (such as MAPK phosphorylation status), gene transcription (at least mRNA accumulation for iNOS, cytokines,..), and protein production in N9 and N9-shSIRT2 cells would be valuable in this context. Does SIRT2 only affect mRNA accumulation (in keeping with a simple, NF-kB-based explanation) or does it affect signal transduction as suggested for SIRT1 (see as for example Hong et al, JBC 2011 and Zhao et al, Exp. Neurol 2012) and/or protein translation?

Minor remarks:
1. Fig 1A: did the authors validate the SIRT2 staining using tissues from SIRT2 KO animals?
2. Fig1B:is expression of the long form of SIRT2 detectable in brain extracts, as in Fig2A ?
3. Fig2: why didn't the authors included a catalytically inactive SIRT2 mutant in their study?
4. Page 14, line 14: TLR3 is misspelled.

Referee #4

Overall the manuscript is important and timely. Yet there are some issues that require further clarifications:

• The authors have shown the expression of SIRT2 co-localized with Iba-1 activated microglia. It is not clear if the pictures are of naïve animal or LPS-stimulated animal. Moreover, it is documented that SIRT2 is expressed by neurons and oligodentrocytes and its expression by astrocytes is controversial. These staining are missing following the LPS induction.
• Figures 1(C+D) show enhanced Iba1 immunoreactivity and increase levels of pro-inflammatory cytokines in SIRT2 KO mice. However, in order to attribute these observations to microglia pro-inflammatory activity, RNA purification from specific microglia cells is required; the authors characterized the total milieu, rather than isolated microglia. Alternatively attempt should be made to use chimeric mice in which microglia are easily differentiated from infiltrating macrophages.
• In the in-vitro model, it is not clear why the authors stimulated the cells with LPS+TNF rather than with LPS, like the in-vivo model. Moreover, the control is not clear.
• In order to mimic better the in-vivo model, it is better to show SIRT2 expression also in primary microglia in-vitro (Figure2).
• The authors pointed that SIRT2 deficiency causes an increase in microglia apoptosis and decrease in HT22 cell viability. To complete this statement the authors should address this issue also in-vivo in the SIRT2 KO mice compare to the WT mice following LPS induction.
• SIRT2 KD cells activation with TLR4 and TLR2 caused an elevation in CD40 expression, IL-6 secretion and iNOS activity. However, TLR2 and TLR4 activation caused also elevation of IL-10 in SIRT2 KD cells. IL-10 is a well documented anti-inflammatory cytokine plays an important role in repair processes in the periphery and inside the CNS. Consequently, this result does not fit with the main massage

that SIRT2 regulates inflammation in microglia and its deficiency causes pro-inflammatory cascade.

Additional Correspondence   25 February 2013

I have just received the 4th referee report on your study - please see below. Referee #3 finds the analysis of high interest, but also raises similar issues as the other referees namely that some of the findings are just too preliminary at this stage. Please take the concerns raised below into consideration when you prepare your point-by-point responses. Do also note that I can extend the revision time up to 6 months, but also that for consideration here that the analysis would have to be significantly extended.

Looking forward to seeing your proposal

REFEREE REPORT

Referee #3

Remarks to the Author:
Sirtuins are a conserved family of deacetylases that have been implicated in the response of organisms to stress and in disease processes. SIRT2 remains the least understood sirtuin. A few SIRT2 targets have been elucidated (e.g. tubulin, FOXO1, NF-kB and recently receptor-interacting protein, RIP1) and has been implicated in the control of the cell cycle, adipose cell differentiation, and neurotoxicity. The latter phenotype was identified by the corresponding author using chemical inhibition (Outero et al., 2007). This manuscript, by Pais et al., present a set of results that indicate that SIRT2 is expressed and suppresses the inflammatory response of microglia, activation of which play an important role in the pathogenic and possibly the initiation of neurodegenerative diseases such as Parkinson's disease. Data is presented that the downstream pathways include SIRT2 dephosphorylation (on S331) and increases in acetylated NF-kB. The authors also test immortal and primary cell lines in some key experiments, which is of value. Overall the impact of the finding is of significance to a wide audience and raise the possibility that SIRT2 activation could be used to treat ROS-microglial-mediated neurodegenerative diseases. The problem with the manuscript is that it looks at each of the steps in the pathway very superficially; the level is far below the depth of investigation that would be expected of a journal such as EMBO J. At each step of the study, the authors do not go into sufficient detail to be certain that the chain of events shown in the model are likely to be correct.

Specific points:

1. Attempts to link the in vitro work to the in vivo findings are speculative. The reviewer appreciates that measuring acetylation changes in vivo are challenging but there are multiple steps in the model that could have been tested in vivo that were not.

2. The study fails to connect the first part of the work with phosphoSIRT2 with NF-kB acetylation. For example, considerably more could be done with the SIRT2 phospho mutants to show this step is relevant to the downstream steps in the model. Do the S331A or S331D mutants affect cell survival, NF-kB acetylation, are the effects NF-kB dependent, does NF-kB relocalize to the nucleus, to promoters?

3. In the SIRT2 KO mouse, are there gene expression changes consistent with the model? ROS? Staining for inflammatory markers? p65/NF-kB acetylation?

4. The authors developed an inhibitor of SIRT2 that was not put to use in this study for the cell work and, most importantly, in vivo. It is not clear why not. Is it suitable

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for mouse in vivo work? Are previous results questionable? An inhibitor was recently used in the Finkel RIP1 paper.

5. The authors have published that SIRT2 INHIBITION provides neuroprotection (e.g. Outeiro et al, 2007) and in this study they propose that SIRT2 should be ACTIVATED to achieve therapeutic benefit. This issue needs to be addressed in the text more that just saying SIRT2 "manipulation" could be beneficial.

6. Knowing if p/35-CDK5, or the p25 fragment, is the kinase for SIRT2 would be highly valuable in this model given the role of hyperactive p25 in Alzheimer's disease. This connection is not addressed experimentally or in the text.

7. Minor point: The Kazanstev paper which demonstrates that inhibition of SIRT2 is neuroprotective in cellular and invertebrate models of Huntington's was not cited or discussed.

1st Editorial Decision   01 March 2013

Thank you for submitting your detailed point-by-point response. I have now had the opportunity to take a careful look at it. I appreciate the proposed experiments and the inclusions of such data would strengthen the findings considerably. I would therefore like to extend an invitation to you to submit a revised manuscript that address the referees concerns as proposed in your response. We usually allow 3 months for revisions, but I can extend the deadline to 6 months in this case as I really would like a significantly revised manuscript back and I recognize that the proposed experiments will take some extra time.

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://www.nature.com/emboj/about/process.html

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

1st Revision - authors' response   21 June 2013

Referee #1

The manuscript by Pais et al. reports on the role of SIRT2 in the control of microglial activation.

Sirtuin 2 is the member of a family of NAD-dependent deacetylase sirtuin. Numerous acetylated proteins are targeted by sirt2 activity, including different transcription factors, histones or cytoskeletal proteins. As a consequence sirt2 is involved in numerous cellular and physiological functions particularly in the brain. In this study, the authors analyzed the function of sirt2 in microglia, which has never been investigated before. The main results of this study are:
1- sirt2 negatively regulates microglial activation, assessed by measurement of the production or expression different pro-inflammatory markers;
2- sirt2 negatively regulates microglial-induced neurotoxicity;
3- sirt2 is likely to mediates these effects through the deacetylation of NF-kB, a well characterized transcription factor involved in the transcription of different pro-inflammatory proteins.

The main conclusion of this study is that sirt2 is the main gatekeeper of microglial activation in the brain and could represent a potential target for therapeutic intervention in neuro-inflammatory disorders.

*The finding that sirt2 regulates some facets of microglial activation is new, however
this study remains limited by the fact that most experiments have been performed in
vitro, often with the N9 microglial cell line, although a limited number of
experiments were performed in primary microglial culture or with an in vivo model
of microglial activation. In addition, protocol used to trigger microglial activation
(cell stimunaltion with LPS+TNF or intracortical LPS) are of limited interest regarding
physiological conditions.*

*Overall this study would be much more convincing if microglial activation was
analyzed into details, in sirt2-deficient mice, ideally in microglia-specific sirt2
deficient mice, using appropriate in vivo physiopathological models of microglial
activation.*

We appreciate the reviewer's comments and we agree that a large number of *in vitro* experiments
were performed with a microglial cell line. Those helped us dissect the molecular mechanisms
involved in our *in vivo* demonstration of increased microglia activation upon LPS treatment. As
suggested, we performed a number of *in vivo* experiments to further detail our previous data
showing increased inflammatory response in SIRT2$^{-/-}$ mice. We analysed isolated cortical cells by
flow cytometry and concluded that there is a significant increase in the percentage of
microglia/macrophages that produce IL-6 in SIRT2$^{-/-}$ mice. The data is now shown in **Figure 1F
and Supplementary Figure 2**. We also observed that up-regulation of CD45, a marker of
microglia/macrophage activation, was similar between wt and SIRT2$^{-/-}$mice suggesting that SIRT2,
as shown in cell cultures, selectively inhibits specific pathways of microglia activation.

While we agree with the reviewer that it will be very interesting to address the role of SIRT2 in
other physiopathological models, we consider that, as a proof of concept, intracerebral LPS-injection
is a valid model. Moreover, the physiological relevance of LPS is also widely established (Rivest,
2003). LPS, a TLR4 ligand, has been used as a stimulus to study microglial-mediated innate
immune responses both *in vitro* and *in vivo*. The LPS receptor, TLR4, has been only described in
microglia in the CNS (Lehnardt et al, 2002). The probability of affecting directly these cells would
be much higher compared to other disease models where microglia would get activated as
consequence of events occurring in other brain cells, which are also depleted of SIRT2. In this
revised manuscript we added a paragraph to the introduction to better explain the use of LPS in the
context of the CNS inflammation.

Although we cannot exclude that lack of SIRT2 in other cells besides microglia, namely in
astrocytes, contribute to the increased inflammation reported in this study, microglia express and are
activated by TLR4 while astrocytic response was shown to happen as a consequence of TLR4
activation in microglia (Hanamsagar et al, 2012). We agree with the reviewer that microglia-specific
deletion of SIRT2 in mice is an interesting model but generating these animals is a lengthy process
that goes beyond the scope of this manuscript, making these experiments unrealistic in a reasonable
time-frame.

Major comments:
*- One of the key results of the study is that sirt2 regulates NF-kB activity preventing its acetylation
and thus its function as an initiator of inflammatory gene expression.*
*Transcription of Il1 is known to be dependent of NF-kB activity, yet induction of Il1 expression by
LPS is not affected in sirt2-deficient mice (Fig 1E). This discrepancy needs to be addressed.*

As mentioned by this reviewer, we detected increased levels of mRNA of several pro-inflammatory
factors in the brain of SIRT2$^{-/-}$mice but not of IL-1b. We also did not find induction of IL-1b in N9
cells that express reduced levels of SIRT2 (**Supplementary Figure 4D**). Since acetylation of NF-kB
is not the only mechanism that leads to activation of this transcription factor, one hypothesis is that
not all NF-kB-dependent genes are equally sensitive to NF-kB deacetlyation. We address this issue
in the Discussion section.

*- A closer analysis of microglia in sirt2-deficient mice is lacking. Indeed, the
constitutive deletion of sirt2 might have some repercussion on the microglial
physiology even in the absence of inflammatory challenge. The authors should
analyze whether resting microglia phenotypes (i.e. morphology, density...) are
affected by sirt2 deletion compared to wild type mice.*

As suggested by the reviewer, we counted the number of microglial cells on brain sections stained for Iba1 and we did not detect any significant difference in the number of microglial cells between wt and SIRT2$^{-/-}$ mouse brains under physiological conditions. This data is now included in **Supplementary Figure 2A**. Morphologically, it is very difficult to detect slight differences in microglial activation. However, we did not observe any significant differences in the mRNA levels of inflammatory cytokines between wt and SIRT2$^{-/-}$ mice injected with PBS indicating that SIRT2 is important to control microglia activation only above a certain threshold.

- The differences between sirt2 western blot experiments presented in figure 1B and 2A are unclear. First, LPS reduces the expression of sirt2 in brain extract, but this effect is not present in N9 cells stimulated with LPS+TNF. Second, the sirt2 large form and the phosphorylated shorted form are not apparent on western blot experiments from brain extracts. Is there any explanation for these differences?

We appreciate the reviewer's comment. We do not know whether the reduction of SIRT2 observed in brain extracts upon intracerebral LPS injection is solely due to the reduction of SIRT2 in microglial cells. All other cells in the brain were shown to express SIRT2 (Harting & Knoll, 2010) and microglial cells are 5-10% of the brain cell population. LPS reduced specifically SIRT2 expression, but not the expression of SIRT1. This suggested that SIRT2 could be involved in the inflammatory response in the brain.

The reviewer is correct in that the short splicing variant is the major isoform of SIRT2 in adult mouse brain as we show in comparison with other organs (**Supplementary Figure 1A**). On the other hand, the long SIRT2 isoform, but not the short, is expressed in the mouse brain at embryonic stages and post-natally until day 7 (Maxwell et al, 2011).

We agree that it would be interesting to assess dephosphorylation of SIRT2 in microglia *in vivo*. However, if it is an event that specifically happens in microglia it will be very difficult to detect by western blot since microglia cells represent only a small percentage of brain cells expressing this sirtuin.

- There are also discrepancies in Mpa21 expression between brain extract and N9 cells. In brain extract LPS triggers the expression of Mpa21 (Fig1E) while this is not present in N9 cells (Fig5B).

Although we agree with this is observation, we did not expect a completely identical gene transcription pattern between brain and cell cultures, since these are two different models. In N9 cells, based on a kinetic study (1h, 1h30 and 2h30) we selected the 2h30 time point because there was already higher mRNA induction expression of Mpa2l in SIRT2 Knock down (KD) cells.

Other comments:
- Immunohistochemistry in panel 1A is of poor quality compared to panel D. In addition it is unclear why there is no Iba1 staining in PBS samples of panel C.

We made attempts to improve the image on panel 1A but this was not trivial due to technical limitations. To detect colocalization of microglia with SIRT2, we had to use another anti-Iba 1 antibody instead of the antibody we used for the single staining. This latter antibody works better for immunohistochemistry but it is produced in rabbit as the available anti-SIRT2 antibody. The goat Iba1-antibody that we had to use for the colocalization study is less sensitive. Furthermore, SIRT2 stains fiber tracts and it makes even more difficult to detect individual cell staining. Also, we had to face the fact that when Iba1 is upregulated (under inflammation), the SIRT2 signal is decreased as we show by western blot (**Figure 1**). Therefore, unfortunately, we could not obtain better images than this one. In any case, we supported this *in vivo* observation with western blots showing the expression of SIRT2 in primary microglial cells as well as in other brain cells (**Supplementary Figure 1A**).

In order to have better images for Iba1 from PBS-treated mice, we used a higher concentration of anti-Iba1 antibody. We replaced the images on panel C by new ones where microglial staining is more visible in PBS samples.

- Often data are normalized, sometimes not. All data should be expressed in the same way, and normalization should be used only when necessary.

We agree with the reviewer and we avoided the normalization of the data so readers can have an idea of the absolute levels measured in the different assays. However, we had to do use normalization in some experiments for the following reasons: 1) In Figure 2, we normalized the expression of activation markers because these are average of different infection experiments. The variability between experiments involving infection and overexpression of the mutants is very high. Therefore we had to normalize the values to the control situation in each experiment; 2) Due to the normal and expected variability between experiments we had to normalize the MTT assays (Figure 5) as well. In the case of nitrites levels (Figure 5), we normalized it because it would be easier to compare the effect between N9 cells and primary cultures. When we used primary microglial cell, we obtained very few cells for the experiments and we seeded just 10^4 cells in 96 wells, as a result the levels of nitrites are much lower to those measured for N9 cells where 100 more cells were uses in 6 well plates.

- Student't test cannot be used for multicomparison (Fig2, panel C). Please use Anova instead.

As suggested, we applied ANOVA for Figure 2C and it is mentioned now in the figure legend.

- There is no legend for Fig 2 panel F. Please revise.

We thank the review for noticing this mistake in the original version. This has been corrected.

- Material and method section is un-informative. Please provide more detailed
experimental procedure instead of references. Origin of sirt2-deficient mice is
lacking.

As suggested by the reviewer, we have introduced further details in the Material and Methods section and we also mention there the origin of the SIRT2$^{-/-}$ mice, which are also acknowledged.

Referee #2

The manuscript by Faria Pais and colleagues elegantly and convincingly
demonstrates an anti-inflammatory role of SIRT2 in activated microglia. Using a
combination of approaches, including in vivo and in vitro studies in which
expression levels of SIRT2 were manipulated using KO, KD and overexpression
strategies, the authors demonstrate the capacity of SIRT2 to negatively affect several
parameters linked to microglia activation, such as pro-inflammatory mediators'
production and expression of activation markers.

Major remarks:
Although the data in Figure 1 and 2 convincingly demonstrate an inverse
relationship between SIRT2 expression / activity and the production of pro-
inflammatory mediators, the authors often propose or imply possible mechanisms at
work, without really providing a firm demonstration.

1. In Figure 3, the authors juxtapose data concerning regulation of iNOS expression
and activity with data related to cell survival. Although cautious in their
interpretation, the authors leave the reader with the impression that these two
observations, described in the same paragraph / figure are functionally linked.
Modulation of NF-kB activity by SIRT2 could affect ROS/RNS production and possibly
expression of pro-and anti-apoptotic genes such as Bim and Bcl-XL, as suggested
by Lanzillotta A et al, Neurobiol Dis 2012. Although the experiment using
conditioned media is in favor of the author's assumption (production of ROS/RNS),
no experiment using pharmacological agents interfering with ROS/RNS production
or biological activity has been performed to support their role. Such an experiment
should be performed, and, depending to the result, the conclusions adapted.

As suggested by the reviewer, we performed an experiment using pharmacological inhibitors. We stimulated SIRT2 KD microglia in the presence of N-acetyl-L-cysteine (NAC), a ROS scavenger, and L-N6-(1-iminoethyl)-L-lysine (L-NIL) an inhibitor of iNOS. These results are now shown in

Supplementary Figure 7. We found that both NAC and L-NIL reduced the levels of ROS, nitrites and neurotoxicity of microglial-condition medium. These results suggest that ROS and RNS production by microglia are required to mediate neurotoxicity. It is important to consider that ROS are also signaling molecules that regulate microglial activation (Roy et al, 2008) and, therefore, may also play an indirect role in the induction of microglial-mediated neurotoxicity

2. Figure 5 appear as an attempt to support the mode of action of SIRT2 based on its ability to deacetylate K310 of NF-kB, as illustrated in the summary Figure. Again, the data showing increased basal and induced acetylation of K310 in SIRT2 KO cells (Figure 5C) are convincing. However, it is difficult to rationalize most of the observations reported in this study with this proposed mode of action, as implicitly admitted by the authors themselves. Indeed: (i) K310 is also a well described SIRT1 target, but KD of SIRT1 only recapitulates one of the key observations of the study, i.e. IL-6 overexpression (Fig S3);

We were also intrigued by this observation. We hypothesize that this discrepancy can be due to different subcellular localization of the two sirtuins. It has been shown that SIRT2 deacetylates NF-kB in the cytoplasm and modulates its shuttling between the cytoplasm and the nucleus (Rothgiesser et al, 2010). On the other hand, SIRT1 is in the nucleus and although it has been shown to deacetylate NF-kB p65, SIRT2 was shown to do it more efficiently (Rothgiesser et al, 2010). For these reasons, the two sirtuins may have a different outcome on NF-kB-dependent gene expression. However, we cannot exclude that a stronger SIRT1 KD would be required to detect the same effects observed in SIRT2 KD cells.

(ii) there is no clear relationship between the effect of shSIRT2 and the NF-kB-dependent status of genes examined in Figure 4. In other words, only poly I:C affect expression of a prototypic NF-kB-dependent gene such as TNF (as discussed by the authors), while other TLR ligands show no effect. IL-10 production however, is increased in shSIRT2 expressing cells in response to both TLR4 and TLR2 ligands. The authors do not discuss the possibility that acetylation status of K310 in NF-kB could only affect a subset of NF-kB dependent genes (as suggested in the literature) and the only example they provide (Mpa21, Fig 5B) is somehow unrelated to the study. This referee is well aware of the putatively complex role of sirtuins in modulating a biological response, and pinpointing all mechanisms at work in a single study would be unrealistic. The authors should however attempt to better illustrate this biological complexity by exploring additional, non NF-kB related, site of action for SIRT2, rather than simply enumerating them (see discussion). A more detailed analysis of signal transduction (such as MAPK phosphorylation status), gene transcription (at least mRNA accumulation for iNOS, cytokines,..), and protein production in N9 and N9-shSIRT2 cells would be valuable in this context.
Does SIRT2 only affect mRNA accumulation (in keeping with a simple, NF-kB-based explanation) or does it affect signal transduction as suggested for SIRT1 (see as for example Hong et al, JBC 2011 and Zhao et al, Exp. Neurol 2012) and/or protein translation?

We appreciate these thoughtful comments. We agree that deacetylation of NF-kB may not be the sole mechanism regulated by SIRT2 in microglia though we show that transcription of many pro-inflammatory genes, transcriptionally activated by NF-kB, are affected. However, this is not the case of IL-1b, which is an agreement with the reviewer's suggestion that not all NF-kB-dependent genes will be equally affected by NF-kB deacetylation on K310. We also investigated signal transduction pathways by analysing the phosphorylation levels of ERK1/2 and p38. However, we did not detect significant differences between control and SIRT2 KD cells. These data was included in **Supplementary Figure 4** together with other pathways that were not affected by reduction of SIRT2 levels such as phagocytosis and glutamate production. qPCR for iNOS also shows that mRNA levels of this enzyme are also induced in the absence of SIRT2 (**Supplementary Figure 5**). Altogether, these data point to a role of SIRT2 at transcriptional level, which we suggest to require NF-kB deacetylation. We also analysed other parameters related to NF-kB activation such as kinetics of migration to the nucleus, IkB-alpha degradation and NF-kB phosphorylation on S536. These results are shown in the new **Supplementary Figure 8**. We think that a quantitative

proteomics between control and SIRT2 KD activated microglial cells could identify other endogenous substrates for SIRT2, though this would be a separated study and behind the scope of this work.

Minor remarks:
1. Fig 1A: did the authors validate the SIRT2 staining using tissues from SIRT2 KO
animals?

Yes, we did. We found no SIRT2 signal either on brain sections or on probes western blot from the SIRT2 KO animals.

2. Fig1B:is expression of the long form of SIRT2 detectable in brain extracts, as in
Fig2A ?

Yes, it is detected but at much lower levels compared to the short isoform as shown now in
Supplementary Figure 1.

3. Fig2: why didn't the authors included a catalytically inactive SIRT2 mutant in their
study?

Based on the observation that SIRT2 is dephosphorylated in N9 cells upon activation, we thought that the phosphorylation mutants described to have distinct levels of deacetylase activity would be more interesting to study, so we focused on those mutants.

4. Page 14, line 14: TLR3 is misspelled.

We thank the reviewer for pointing this out. This has been rectified.

Referee #3

Remarks to the Author:Sirtuins are a conserved family of deacetylases that have been implicated in the response of organisms to stress and in disease processes. SIRT2 remains the least understood sirtuin. A few SIRT2 targets have been elucidated (e.g. tubulin, FOXO1, NF-kB and recently receptor-interacting protein, RIP1) and has been implicated in the control of the cell cycle, adipose cell differentiation, and neurotoxicity. The latter phenotype was identified by the corresponding author using chemical inhibition (Outero et al., 2007). This manuscript, by Pais et al., present a set of results that indicate that SIRT2 is expressed and suppresses the inflammatory response of microglia, activation of which play an important role in the pathogenic and possibly the initiation of neurodegenerative diseases such as Parkinson's disease. Data is presented that the downstream pathways include SIRT2 dephosphorylation (on S331) and increases in acetylated NF-kB. The authors also test immortal and primary cell lines in some key experiments, which is of value. Overall the impact of the finding is of significance to a wide audience and raise the possibility that SIRT2 activation could be used to treat ROS-microglial-mediated neurodegenerative diseases. The problem with the manuscript is that it looks at each of the steps in the pathway very superficially; the level is far below the depth of investigation that would be expected of a journal such as EMBO J. At each step of the study, the authors do not go into sufficient detail to be certain that the chain of events shown in the model are likely to be correct.

Specific points:

1. Attempts to link the in vitro work to the in vivo findings are speculative. The reviewer appreciates that measuring acetylation changes in vivo are challenging but there are multiple steps in the model that could have been tested in vivo that were not.

We appreciate the reviewer's concern. Thus we performed several experiments that address i) microglial-specific production of pro-inflammatory cytokines such as IL-6, ii) oxidative stress and

iii) neurodegeneration *in vivo*. The results are now presented in **Figure 1**. We show that a higher percentage of microglia/macrophages produce IL-6 in SIRT2^{-/-} mice. Moreover, a stronger inflammatory response is associated with increased nitro-tyrosination in SIRT2 [-/-] mice. These results are in agreement with an inhibitor role of SIRT2 in ROS/RNS production that we demonstrate in microglial cell cultures. We also found a significant increase in neuronal cell death in SIRT2^{-/-} mice. The data is provided in Figure 1 and Supplementary Figure 2. The levels of neurodegeneration induced by LPS injection in the cortex were low has previously reported in comparison to other brain regions. (Kim et al, 2000).

2. The study fails to connect the first part of the work with phosphoSIRT2 with NF-
kB acetylation. For example, considerably more could be done with the SIRT2
phospho mutants to show this step is relevant to the downstream steps in the
model. Do the S331A or S331D mutants affect cell survival, NF-kB acetylation, are
the effects NF-kB dependent, does NF-kB relocalize to the nucleus, to promoters?

As suggested, we compared NF-kB p65 acetylation levels in microglial cells overexpressing the phosphorylation mutants. To avoid the interference of endogenous SIRT2, we performed those experiments is SIRT2 KD N9 cells. We found that overexpression of S331A SIRT2 mutant decreased the levels of NF-kB p65 acetylation. These data is in good agreement with our results showing that this mutant decreases the inflammatory response in microglia and is now included in **Figure 5C**. We did not address cell survival in this model because gene overexpression in microglial cells requires viral-transduction of cells for 48 hours, which are then further stimulated for 20 hours. We do not think this is an optimal system to address cell survival because cells are cultured for 3 days and there is already cell death due to the experimental conditions.

3. In the SIRT2 KO mouse, are there gene expression changes consistent with the
model? ROS? Staining for inflammatory markers? p65/NF-kB acetylation?

We confirmed that in SIRT2^{-/-} mice there is increased expression of pro-inflammatory markers such as IL-6, TNF, IP10 and Mpa2l, and in our revised version we also include new data of IL-6 increased production by microglia in SIRT2^{-/-} mice (**Figure 1**).
Taking into account that microglial cells are just 5-10% of the brain cells, it would be very difficult to detect differences in p65 acetylation levels between wt and SIRT2^{-/-} mice.
To address the production of ROS in vivo, we carried out the staining against nitro-tyrosine, which results from reaction with peroxynitrite. We observed an increase in protein nitro-tyrosination in SIRT2^{-/-} mice injected with LPS. These results support our *in vitro* data and are now provided in **Figure 1** of the revised manuscript.

4. The authors developed an inhibitor of SIRT2 that was not put to use in this study
for the cell work and, most importantly, in vivo. It is not clear why not. Is it suitable
for mouse in vivo work? Are previous results questionable? An inhibitor was recently
used in the Finkel RIP1 paper.

Our major aim was to understand the role of SIRT2 in brain inflammation. Therefore we avoided the use of inhibitors that have been shown to inhibit SIRT2, since we can never exclude off target effects. In addition, the inhibitors we previously described are not optimized for crossing the blood brain barrier, which would introduce an additional layer of complexity. The previous results are, by no means questionable, but they refer to effects in different cell types. In this study, we seeked to address our question at the molecular level using available molecular tools, which are cleaner.

5. The authors have published that SIRT2 INHIBITION provides neuroprotection (e.g.
Outeiro et al, 2007) and in this study they propose that SIRT2 should be ACTIVATED
to achieve therapeutic benefit. This issue needs to be addressed in the text more
that just saying SIRT2 "manipulation" could be beneficial.

We addressed this issue in the discussion (page 18) and we think the two possibilities are not mutually exclusive. It is also clear in the sirtuin literature that a tight control of the activity of these enzymes is critical, and it is likely that they play different roles in different cell types. This is well documented, and fits perfectly with our findings.

Regarding the "manipulation of SIRT2", we agree with the reviewer and changed the last sentence to: "However, previous data showing that SIRT2 inhibition is neuroptotective suggest that manipulation of SIRT2 activity should be regarded with caution in the context of neurodegenerative diseases, and further investigations will be required for the rational use of SIRT2 as a therapeutic target."

6. Knowing if p/35-CDK5, or the p25 fragment, is the kinase for SIRT2 would be highly valuable in this model given the role of hyperactive p25 in Alzheimer's disease. This connection is not addressed experimentally or in the text.

We also think this in an interesting subject. Further studies are required to prove that p35-CDK5 is indeed the kinase that phosphorylates SIRT2 in microglia. This will be very interesting to know due to the role of CDK5 in the CNS and in neurodegenerative diseases (Cheung & Ip, 2012). However, the upstream phopshorylation/desphosphorylation of SIRT2 is beyond the scope of this study.

7. Minor point: The Kazanstev paper which demonstrates that inhibition of SIRT2 is neuroprotective in cellular and invertebrate models of Huntington's was not cited or discussed.

As suggested by the referee this reference was included in the Discussion.

Referee #4

Overall the manuscript is important and timely. Yet there are some issues that require further clarifications:

• The authors have shown the expression of SIRT2 co-localized with Iba-1 activated microglia. It is not clear if the pictures are of naïve animal or LPS-stimulated animal. Moreover, it is documented that SIRT2 is expressed by neurons and oligodentrocytes and its expression by astrocytes is controversial. These staining are missing following the LPS induction.

We carried out the staining on slices of LPS-injected animals because LPS increases the staining for Iba1 and makes it easier to recognize microglial cells. Our aim with this image was just to show that microglial cells do express SIRT2 *in vivo*. Others have already demonstrated it for other cells including oligodendrocytes that express high levels of SIRT2 (Harting & Knoll, 2010). We also added to Supplementary Figure 1 a western showing expression of SIRT2 in primary microglia and astrocytes.

Figures 1(C+D) show enhanced Iba1 immunoreactivity and increase levels of pro-inflammatory cytokines in SIRT2 KO mice. However, in order to attribute these observations to microglia pro-inflammatory activity, RNA purification from specific microglia cells is required; the authors characterized the total milieu, rather than isolated microglia. Alternatively attempt should be made to use chimeric mice in which microglia are easily differentiated from infiltrating macrophages.

We understand the reviewer's concern. We agree that we cannot exclude the participation of astrocytes or of any infiltrating monocytes in the inflammatory response, as we already explained above. However, even if it happens, it would not change our interpretation and the message of our study, that SIRT2 does play a role in the control of that response. The use of chimeric mice is time-consuming and lately challenged by the scientific community because the irradiation and reconstitution with bone-marrow-derived cells is very artificial and non-physiological (Ransohoff, 2007). This is also far beyond the scope of our manuscript, and would not be doable within a realistic time frame.
However, as requested by the reviewer we looked to microglia in particular. We performed flow cytometry analysis of microglia within isolated cortical cells. The percentage of CD11b$^+$/CD45^{high} expressing cells did increase with LPS injection but was not different between wt and SIRT2$^{-/-}$ mice. This population can include both activated microglial cells and infiltrated monocytes. Nevertheless, in cortical cells isolated from LPS-injected SIRT2$^{-/-}$ there was a clear increase of CD11b positive

cells that produce IL-6. These results now more clearly support that SIRT2 deletion contributes to increase the inflammatory response by microglial cells.

In the in-vitro model, it is not clear why the authors stimulated the cells with LPS+TNF rather than with LPS, like the in-vivo model. Moreover, the control is not clear.

The use of LPS+TNF is as a general pro-inflammatory stimulus used in cell cultures to potentiate the activation of different signaling pathways. Nevertheless, we also tested other TLRs ligands (Figure 3) *in vitro* or just LPS without TNF. We used non-stimulated cells as control, cells just cultured in medium. We mentioned it in this new version when described the first results with N9 cells.

In order to mimic better the in-vivo model, it is better to show SIRT2 expression also in primary microglia in-vitro (Figure2).

We included in Supplementary Figure 1 a western comparing SIRT2 protein levels between primary cultures of microglia, astrocytes and hippocampal and cortical neurons

The authors pointed that SIRT2 deficiency causes an increase in microglia apoptosis and decrease in HT22 cell viability. To complete this statement the authors should address this issue also in-vivo in the SIRT2 KO mice compare to the WT mice following LPS induction.

As suggested, we addressed neurodegeneration in mice injected with LPS. To compare microglial responses between wt and SIRT2$^{-/-}$ mice, we used a very low amount of LPS (0.2 mg) to avoid overactivation of microglia and to better detect differences between genotypes. Studies reporting LPS-induced neurodegeneration inject 10 times more LPS (2-5 mg) (de Pablos et al, 2006). Therefore, we also injected wt and SIRT2 $^{-/-}$ mice with 5 mg of LPS to address neurodegeneration. We did observe a significant decrease in the number of neurons in the cortex of SIRT2$^{-/-}$ mice compared to wt (Figure 1).

SIRT2 KD cells activation with TLR4 and TLR2 caused an elevation in CD40 expression, IL-6 secretion and iNOS activity. However, TLR2 and TLR4 activation caused also elevation of IL-10 in SIRT2 KD cells. IL-10 is a well documented anti-inflammatory cytokine plays an important role in repair processes in the periphery and inside the CNS. Consequently, this result does not fit with the main massage that SIRT2 regulates inflammation in microglia and its deficiency causes pro-inflammatory cascade.

IL-10 induction upon activation of TLRs is part of a negative-feedback mechanism to restrain inflammatory response in macrophages. This mechanism involves a type I IFN-dependent secondary response (Chang et al, 2007). Therefore, increased IL-10 production in SIRT2 KD is probably a consequence of an enhanced inflammatory response and not a direct effect of SIRT2 in IL-10 gene transcription. However this is speculative because we did not specifically addressed IL-10 transcriptional regulation. We discuss this issue in the first paragraph of the Discussion section.

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2nd Editorial Decision   22 July 2013

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referees 1,2 and 4 and their comments are provided below.

Referees # 2 and 4 have some remaining minor issues that should be easily addressed, while referee #1 raises more significant ones. Referee #1 would like to see more in vivo data to support a role of Sirt2 in neurodegenerative disease (point #1). I recognize that this would clearly strengthen the data set, but also find that such analysis would be part of a separate story and is not needed for the present analysis. However, I would like to ask you to respond/resolve the issues raised concerning the quality of fig 1D and fig 1A, and provide a rational for why different LPS concentrations were used.

When you submit your revised version please make sure to include a point-by-point response. You can use the link below to submit your revised manuscript.

REFEREE REPORTS

Referee #1

In their revised manuscript, Pais et al. performed several additional experiments or provided specific explanation to answer the criticisms raised by the reviewers.
While some experiments or answers were satisfactory, other are not entirely acceptable.
Regarding my own comments, I still feel that in vivo experiments need to be improved and that more experiments should have been performed in Sirt2-/- microglia rather than using ShRNA-mediated knock down of Sirt2 in N9 cells.

First, regarding the model of intra cortical LPS injection, although it is a widely used model of neuro-inflammation, it poorly recapitulates real pathological conditions: it is rare that brain parenchyma is in direct contact with bacterial endotoxin. Importantly, activation of microglia is driven by sterile inflammation that occurs in numerous pathologies of the central nervous system (neuropathic pain, status epilepticus, Alzheimer disease...). I still believe that this study would be strengthen whether the role of Sirt2 was investigated in one of these models. For example it would be very informative for a broad category of readers to know whether SIT2 deletion has any repercussion on neuronal cell death in kainate-induced status epilepticus.

Second, from the pictures shown in figure 1D do not support the interpretation given by the authors. Microglial Iba1 immunostaining in SIRT2-deficient mice is clearly different from that of WT mice. In both type of staining (top and bottom row), a stronger signal is clearly observed in Sirt2-/- mice. In addition the confocal images (bottom row) shown that the morphology of Sirt2-/- microglia is very different from that of wild type: stronger intensity of Iba1 staining, thicker and shorter primary processes, all criteria that are strongly reminiscent of microglial activation. Either the pictures are not representative and misleading, or microglia from Sirt-/- mice is activated. There are numerous way of analyzing morphology and staining intensity of cells that could be used to further investigate potential change of the morphology of quiescent microglia. In addition, known function of resting microglia such as motility and or chemotaxis could have been investigated.
Microglial activation is complex and can adopt different states as a function of the triggering signals. These activation state do not obligatorily results in transcriptional pro-inflammatory cytokine up regulation and in proliferation. Since the authors only investigated LPS-evoked activation, the significance of their results remains limited.

In addition, goat-anti Iba1 antibodies that give good results by immunostaining are available (i.e. Abcam # Ab5076). Such antibody is likely to improved pictures in Fig. 1A.

The author provide additional data showing that cortical LPS injection leads to higher neurodegeneration in Sit2-/- mice. What is the rational for injecting 5 ug of LPS while 0.2ug was used in other experiment.

Finally, the authors argue that assessing Sirt2 dephosphorylation in vivo would be difficult because microglia only represent 5% of brain cell population. However microglia can be purified from discrete brain region in sufficient amount to perform biochemical experiments such as western blot. There is no doubt that performing key experiment in purified microglia would greatly improve the quality of this study.

Referee #2

The authors addressed all major comments that were raised during the primary review, and I therefore consider the manuscript acceptable for publication under its present form.

Minor remarks:

page 12: The authors inadequately refer to Supplementary Figure 5A,which does not illustrates levels of iNOS, as suggeste din the text

page 27: ...mice were used through an MTA and not "...and MTA...."

Figure 5: in panel C, the treatment (LPS + TNF) has been omitted.

Referee #4

Overall the authors have addressed most of the comments in a convincing way.

Minor comment:
The title and the abstract are not accurately describing the message of the manuscript. While the

effect on microglia are well presented and the results are convincing, the effect on brain inflammation should be more carefully presented, and should therefore be rephrased.

2nd Revision - authors' response   08 August 2013

Referee #1

In their revised manuscript, Pais et al. performed several additional experiments or provided specific explanation to answer the criticisms raised by the reviewers.
While some experiments or answers were satisfactory, other are not entirely acceptable.
Regarding my own comments, I still feel that in vivo experiments need to be improved and that more experiments should have been performed in Sirt2-/- microglia rather than using ShRNA-mediated knock down of Sirt2 in N9 cells.

First, regarding the model of intra cortical LPS injection, although it is a widely used model of neuro-inflammation, it poorly recapitulates real pathological conditions: it is rare that brain parenchyma is in direct contact with bacterial endotoxin. Importantly, activation of microglia is driven by sterile inflammation that occurs in numerous pathologies of the central nervous system (neuropathic pain, status epilepticus, Alzheimer disease...). I still believe that this study would be strengthen whether the role of Sirt2 was investigated in one of these models. For example it would be very informative for a broad category of readers to know whether SIT2 deletion has any repercussion on neuronal cell death in kainate-induced status epilepticus.

We thank the reviewer for the feedback and critical assessment. As we wrote in the manuscript, bacterial meningitis is a pathological condition that leads to TLR4 signaling and microglia activation by LPS. Besides, the study by Chakravarty et al (Chakravarty & Herkenham, 2005), suggests that even in endotoxemia induced by peripheral LPS, resident brain cells such as microglia can mediate an inflammatory response through TLR4. Nevertheless, we agree with the reviewer that we did not directly address the role of SIRT2 in other models of neurodegenerative diseases such as Alzheimer's disease (AD). This would be impossible for us in a reasonable time frame since it involves the crossing of SIRT2$^{-/-}$ mice with transgenic mouse models of AD. Moreover, these experiments would be more conclusive if performed with cell-specific SIRT2 KO mice because SIRT2 deletion can affect other cells besides microglia. We have overcome this issue by using LPS, which specifically triggers microglia cells through TLR4. Regarding the kainate-induced seizures model suggested by the reviewer, we agree it is very interesting as there are increasing evidences supporting the role of inflammation in epileptogenesis though this is not a simple model to our knowledge. Furthermore, it has been published that C57BL/6J (B6) mice are resistant to kainate-induced neuronal cell death even with high doses of kainate (McCord et al, 2008). Therefore, we think it is rather risky to invest in this model in SIRT2$^{-/-}$ mice, which have a B6 background. Our study shows, for the first time, that SIRT2 is important to regulate microglial responses and will pave the way for follow up studies from groups working in other neuroinflammatory diseases.

Second, from the pictures shown in figure 1D do not support the interpretation given by the authors. Microglial Iba1 immunostaining in SIRT2-deficient mice is clearly different from that of WT mice. In both type of staining (top and bottom row), a stronger signal is clearly observed in Sirt2-/- mice. In addition the confocal images (bottom row) shown that the morphology of Sirt2-/- microglia is very different from that of wild type: stronger intensity of Iba1 staining, thicker and shorter primary processes, all criteria that are strongly reminiscent of microglial activation. Either the pictures are not representative and misleading, or microglia from Sirt-/- mice is activated. There are numerous way of analyzing morphology and staining intensity of cells that could be used to further investigate potential change of the morphology of quiescent microglia. In addition, known function of resting microglia such as motility and or chemotaxis could have been investigated.
Microglial activation is complex and can adopt different states as a function of the triggering signals. These activation state do not obligatorily results in transcriptional pro-inflammatory cytokine up regulation and in proliferation. Since the authors only investigated LPS-evoked activation, the significance of their results remains limited.
We believe this comment may result from a misunderstanding. We do claim that there is an increase in microglia activation in SIRT2$^{-/-}$ mice injected with LPS as the images mentioned by the reviewer clearly show. We confirmed that the figure legend mentions that these images were obtained from

LPS-injected mice. The point previously raised by this reviewer was whether there were already differences between SIRT2$^{-/-}$ and WT mice even without any challenge. In this case, we did not detect any differences in the number of microglial cells or in pro-inflammatory cytokine profile between strains.

We did not address microglial proliferation, but we do show that this state of microglial activation is associated with increased pro-inflammatory cytokines such as IL-6 and TNF.

In addition, goat-anti Iba1 antibodies that give good results by immunostaining are available (i.e. Abcam # Ab5076). Such antibody is likely to improved pictures in Fig. 1A.

As suggested by the reviewer, we tried this other antibody, in an effort to improve the image. Unfortunately, we could not get a better image than the one included. We think that the levels of SIRT2 in microglia are lower than in other cells in the brain and, therefore, it is not easily detected by IHC. The western blot of primary cultures, clearly show that microglia express SIRT2 though at lower levels than astrocytes or neurons. The image in Fig. 1A shows another cell stained for SIRT2 that is not Iba-1 positive, which gave us confidence of co-localization in the Iba-1 stained cell. However, since we could not replace it with a better image according to this reviewer, we propose, if required by the editor, to remove this figure and replace it by the western blot of primary cultures, which clearly show the expression of SIRT2 in microglia.

The author provide additional data showing that cortical LPS injection leads to higher neurodegeneration in Sit2-/- mice. What is the rational for injecting 5 ug of LPS while 0.2ug was used in other experiment.
The reviewer is right, we have used different concentrations of LPS and we have explained the rationale to referee #4 in our previous response.
"As suggested, we addressed neurodegeneration in mice injected with LPS. To compare microglial responses between wt and SIRT2$^{-/-}$ mice, we used a very low amount of LPS (0.2 mg) to avoid overactivation of microglia and to better detect differences between genotypes. Studies reporting LPS-induced neurodegeneration inject 10 times more LPS (2-5 mg) (de Pablos et al, 2006). Therefore, we also injected wt and SIRT2 $^{-/-}$ mice with 5 mg of LPS to address neurodegeneration. We did observe a significant decrease in the number of neurons in the cortex of SIRT2$^{-/-}$ mice compared to wt (Figure 1). "

However, we thank the reviewer for raising this issue and to make it clear for the readers, we mention now the different doses in the Results section, where we wrote: "We used a higher dose of LPS in these experiments based on previous studies reporting LPS-induced neurodegeneration"

Finally, the authors argue that assessing Sirt2 dephosphorylation in vivo would be difficult because microglia only represent 5% of brain cell population. However microglia can be purified from discrete brain region in sufficient amount to perform biochemical experiments such as western blot. There is no doubt that performing key experiment in purified microglia would greatly improve the quality of this study.

We agree with the reviewer that microglia can be isolated from different brain regions by immunomagnetic separation as previously described (Nikodemova & Watters, 2012). However, in the mentioned study, the authors only performed flow cytometry analysis and they do not show any western blot. Moreover, they do not refer to the starting number of animals to obtain the purified microglial cells.
Based on our experience on detection of SIRT2 by western blot and microglia isolation from adult brains, we would have to start from a high number of animals that would have to be pooled.
Although we would like to study phosphorylation in purified microglia, we do not think these experiments are technically doable using a reasonable number of mice.
Referee #2 (Remarks to the Author):

The authors addressed all major comments that were raised during the primary review, and I therefore consider the manuscript acceptable for publication under its present form.
We appreciate the feedback from referee.

Minor remarks:

page 12: The authors inadequately refer to Supplementary Figure 5A,which does not illustrates levels of iNOS, as suggeste din the text
We thank the reviewer. This has been corrected to Supplementary Figure 5D.

page 27: ...mice were used through an MTA and not "...and MTA...."
This has been corrected.

Figure 5: in panel C, the treatment (LPS + TNF) has been omitted.

We thank the reviewer for noticing. We have added that to the Figure.

Referee #4

Overall the authors have addressed most of the comments in a convincing way.
We appreciate the feedback from referee.

Minor comment:
The title and the abstract are not accurately describing the message of the manuscript. While the effect on microglia are well presented and the results are convincing, the effect on brain inflammation should be more carefully presented, and should therefore be rephrased.
As suggested by the reviewer, we have added further details about the *in vivo* results to the Abstract. We also changed the running title to "SIRT2 modulates microglial activation".

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