

# SFRP1 modulates astrocyte to microglia cross-talk in acute and chronic neuroinflammation

Javier Rueda-Carrasco, Maria Martin-Bermejo, Guadalupe Pereyra, Maria Ines Mateo, Aldo Borroto, Frederic Brosseron, Markus Kummer, Stephanie Schwartz, Jose Lopez-Atalaya, Balbino Alarcon, Pilar Esteve, Michael Heneka, and Paola Bovolenta

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Bovolenta,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, referees #1 and #3 think that the findings are of interest. However, they both have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all their points need to be addressed, I will not detail them here.

Referee #2 is rather negative and questions the relevance of the findings obtained in a mouse model. However, after cross-commenting with the other referees, we think your data nevertheless will help to discover basic cellular mechanisms that could be relevant in a human setting. If you have any data, though, that would confirm your results in human cells, I would ask you to add these.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on  $n=2$  (the authors are then asked to present scatter plots or provide more data points).

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: <http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms>

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in the respective section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: <http://embor.embopress.org/authorguide#datadeposition>

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843  
(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843>)  
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

\*\*\* Note - All links should resolve to a page where the data can be accessed. \*\*\*

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of \*data citations in the reference list\* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: <http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: <http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis>

9) Please also note our new reference format:  
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling  
Editor  
EMBO Reports

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Referee #1:

In their work, Rueda-Carrasco et al. assessed the role of SFRP1 in microglia and astrocytes in chronic and acute neuroinflammation. Acute neuroinflammation, induced by LPS injection, increased the SFRP1 expression around the injection site. While lentiviral overexpression of SFRP1 increased astrocytic GFAP and microglia Iba1 expression, the absence of SFRP1 in knock mice reduced the signal upon LPS injection indicating a proinflammatory role of SFRP1. This role was further confirmed by an amelioration of the chronic inflammation and disease pathology in EAE mice upon SFRP1 KO. All in all, SFRP1 alters the microglial and astrocytic inflammatory response and microglial phagocytic capacity, however, SFRP1 depletion did not completely prevent microglial response to inflammatory insults. Thus, SFRP1 seems to be an enhancer but not a master regulator of

inflammation. As the authors found secretion of SFRP1 by astrocytes and not by microglia upon LPS stimulation *in vitro*, they describe SFRP1 as an astrocyte to microglia crosstalk protein. The transcriptomic analysis of SFRP1 KO microglia confirmed the authors' claims and highlighted the signalling molecules HIF and NF $\kappa$ B as underlying pathways.

Taken together, these data may be of interest to the glial community since it identifies a role of SFRP1 in the astrocyte-microglia crosstalk.

However, there are a couple of critiques, which need to be addressed.

### Major critiques

1. The authors should confirm the specificity of the SFRP1 antibody used for immunofluorescent stainings in Figure 1B by using SFRP1 KO animals, since the pictures shown give a rather diffuse staining pattern. Additionally, a quantification of the immunofluorescent signal of the  $\beta$ gal and SFRP1 staining, shown in Figure 1A/B, is required to state whether SFRP1 is only expressed by astrocytes (i.e. specificity) and to show its expression changes upon LPS injections. Of course, other measures to confirm specificity and expression levels on the protein levels are also fine (Western blotting or ELISA of sorted cells or similar).

2. In Figure 2, the authors show that overexpression of SFRP1 induces the expression of GFAP and Iba1 in astrocytes/microglia respectively. To show that this is due to microglial/ astrocytic reactivity several other activation markers have to be assessed as GFAP expression can be altered by non-pathological and pathological stimuli thus reflecting rather physiological adaptation. Astrocytic reactivity markers such as C3 and S100 $\beta$  and the microglial markers Clec7a and TMEM119 would help supporting the statement that SFRP1 overexpression increases glial reactivity.

3. The paper is based on the idea that there is an astrocyte to microglia crosstalk mediated by SFRP1. *In vitro* the authors nicely showed that astrocytes are the SFRP1 producing cells which are required for the immune response changes upon LPS treatment.

a. This referee would like to see that there is a lack of SFRP1 expression in microglia *in vivo* in the LPS and EAE model e.g. by ELISA or Western blot of isolated microglia and astrocytes in addition to the ELISA results of whole brain tissue.

b. The authors assume that microglia are responsible for the release of the measured cytokines in the *in vitro* experiment in Figure 4D. In this experiment, however, the cytokine profiles of pure astrocyte cultures treated with LPS in the wild type and SFRP1 KO setting are missing. These are required to see whether astrocytes on its own might be responsible for the cytokine changes found *in vitro*.

4. In Figure 4C, changes in the phagocytic capacity of SFRP1 KO microglia were tested using pHrodo E.coli particles *in vivo*. To clarify the contradicting results, the authors should test the phagocytic capacity of SFRP1 microglia after LPS stimulation *in vitro*. Here it would be required to address whether the presence of astrocytes is required for the phagocytic changes found in microglia.

5. In the first results section, the authors concluded: "Together these data show that astrocytes produce and secrete increased levels of SFRP1 in response to a bacterial lipopolysaccharide". However, the authors did not show that the SFRP1 measured by ELISA was specifically produced

or secreted by astrocytes in this section. A general measurement of whole brain SFRP1, including potential intracellular SFRP1, was performed here. Therefore, the respective experimental evidence for the astrocyte-specificity in this very setting has to be provided.

#### Minor critiques

1. Statistical testing in Figure 2: The authors analysed 3 animals per group and made several acquisitions per animals. It is unclear, whether the significance in each graph was calculated using the number of acquisitions or the number of animals as biological replicates. In case each acquisition was used, the data has to be retested by only using the mean per animal to avoid increasing the statistical power artificially. This applies to all figures shown in this paper.
2. In Figure 3C-D, the authors show the quantification of CD4+ cells, Iba1 immunoreactivity, MBP+ area and MBP immunoreactivity. However, in Figure 3C also GFAP images are shown and in the text the authors refer to reduced GFAP+ reactive astrocytes in SFRP1 KO animals. Thus, in Figure 3D, a quantification of the GFAP immunoreactivity is required to validate this statement.

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Referee #2:

The paper proports to describe the role of SFRP1 in modulating ADAM10 mediated pathways in various mouse models of inflammation. However the authors whilst stating that SFRP1 can modify both Wnt signalling and inhibit ADMA10 activity, completely neglect Wnt signalling, and their justification for doing so is not sufficient. Overall, the use of mouse models to mimic human inflammatory diseases is becoming rather inappropriate given than these models have failed to transfer to the clinic. I would have liked to have seen more of an attempt to align these results with human cells. Adam10 does not cleave TREM2 in humans. Because of this, I am not convinced this advances the field to any great degree.

A couple of small comments:

- M. Heneka does not appear in the author contributions.
- The coculture of astrocytes:microglia of 1:1 does not mimic in vivo ratios.

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Referee #3:

This manuscript, "SFRP1 shapes astrocyte to microglia cross-talk in acute and chronic neuroinflammation" by Rueda-Carrasco et al. seeks to examine the role of astrocyte-produced SFRP1's influence on microglia's role in neuroinflammation using both an LPS model and EAE. They found that SFRP1-/- mice have a reduced clinical score and severity of symptoms in the EAE model. They also demonstrate that LPS induces an increase in SFRP1 expression and reduced cytokine expression in response to LPS. This is an interesting paper that has a few issues that need to be clarified and expanded on.

- Much of the narrative of the manuscript is focused on the neurodegenerative disease, Alzheimer's disease, yet the manuscript experiments are using an LPS model and EAE, no AD models. There was also quite a bit of discussion around ADAM10. As pointed out, this could modulate TREM2 expression, however there were no experiments designed around this concept.

- The image used for CD45 in the WT/saline treatment in figure 2c, appears to have a lot of background.
- Can they show what cell type took up the LV to express SFRP1?
- In 3D, the number of CD4<sup>+</sup> cells should be confirmed with CD4<sup>+</sup>IBA1<sup>-</sup> cells, as monocytes can express CD4, since there is no CD3 co-staining.
- The data presented in figure 4C is confusing. pHrodo will only fluoresce when inside a low pH environment, but the number of positive cells is higher in the knockout but total fluorescence is lower. Is there less molecules per cell? And if so, why is that? Cell recruitment doesn't really make sense in the FACS experiment because it is only GFP<sup>+</sup> cells. Does this vary between the GFP<sup>+</sup>CD45<sup>low</sup> and GFP<sup>+</sup>CD45<sup>high</sup> cells? How exactly was the pHrodo fluorescence measured?
- In Figure 4D, addition of an astrocytes-only cell culture for comparison with the microglia and microglia + astrocyte co-cultures would be important to include. Astrocytes can be a source of cytokines such as IL-6.
- How does the LPS increase expression of astrocyte SFRP1? Is it via TLR4 on the astrocytes or does LPS stimulate the microglia which then signal to the astrocytes to upregulate SFRP1?
- Some in vitro mechanistic studies would add a lot to this paper. Experiments with purified SFRP1 directly on microglia +/- LPS would be helpful in understanding how it modulates their function, especially in dissecting out the relationship of LPS signaling and SFRP1 signaling. In the earlier experiments, LPS seemed to be required to see the effect of SFRP1 KO, but in the transcriptomics experiments there seems to be a bigger difference in the genotypes in the saline condition.
- What is the receptor on microglia for SFRP1? Can the pathway be manipulated in vitro to show that this is the mechanism of the findings?
- In terms of peripheral cell recruitment, is the reduction in the SFRP1 via microglia or another mechanism?

## Response to the Reviewers' Comments

We wish to thank you the reviewers of our manuscript for their insightful comments that have helped us improving the present study. We have carefully considered their criticisms/advices and we provide below a detailed response to their comments. We have highlighted in red all major changes in the text to facilitate their consideration but left in black minor corrections such as changes in the Figure nomenclature numbering etc.

### Referee #1:

*In their work, Rueda-Carrasco et al. assessed the role of SFRP1 in microglia and astrocytes in chronic and acute neuroinflammation. Acute neuroinflammation, induced by LPS injection, increased the SFRP1 expression around the injection site. While lentiviral overexpression of SFRP1 increased astrocytic GFAP and microglia Iba1 expression, the absence of SFRP1 in knock mice reduced the signal upon LPS injection indicating a proinflammatory role of SFRP1. This role was further confirmed by an amelioration of the chronic inflammation and disease pathology in EAE mice upon SFRP1 KO. All in all, SFRP1 alters the microglial and astrocytic inflammatory response and microglial phagocytic capacity, however, SFRP1 depletion did not completely prevent microglial response to inflammatory insults. Thus, SFRP1 seems to be an enhancer but not a master regulator of inflammation. As the authors found secretion of SFRP1 by astrocytes and not by microglia upon LPS stimulation in vitro, they describe SFRP1 as an astrocyte to microglia crosstalk protein. The transcriptomic analysis of SFRP1 KO microglia confirmed the authors' claims and highlighted the signalling molecules HIF and NFkB as underlying pathways. Taken together, these data may be of interest to the glial community since it identifies a role of SFRP1 in the astrocyte-microglia crosstalk. However, there are a couple of critiques, which need to be addressed.*

We thank the reviewer for the his/her general comment which indeed summarizes our message proposing the SFRP1 plays a role in enhancing inflammation (and indeed not as a master regulator). We however would like to stress that we observe increased *Sfrp1* expression not only around the injection site but also at quite a distance, consistent with the dispersible nature of the protein.

**Point 1.** *The authors should confirm the specificity of the SFRP1 antibody used for immunofluorescent stainings in Figure 1B by using SFRP1 KO animals, since the pictures shown give a rather diffuse staining pattern. Additionally, a quantification of the immunofluorescent signal of the  $\beta$ gal and SFRP1 staining, shown in Figure 1A/B, is required to state whether SFRP1 is only expressed by astrocytes (i.e. specificity) and to show its expression changes upon LPS injections. Of course, other measures to confirm specificity and expression levels on the protein levels are also fine (Western blotting or ELISA of sorted cells or similar).*

**Response.** We appreciate the comment. In this manuscript, we did not include a confirmation of the specificity of the antibody because we had already published its characterization (Esteve et al., 2019, Supplementary Fig. 1) and referred to it in the text. Supplementary Fig 1 in Esteve et al. 2019 shows that the antibody specifically recognizes radial precursors in the embryonic forebrain and that this staining is totally absent in *Sfrp1*<sup>-/-</sup> mice. We also showed with ELISA that the antibody specifically recognizes SFRP1 but not the highly related SFRP2. As stated in the text SFRP1 is a secreted and rather diffusible protein and therefore a “diffuse staining pattern” is not surprising. Furthermore, the absence of staining in saline-treated animals argues against the possible existence of unspecific binding or cross-reactivity in the adult brain. Nevertheless, and to provide additional support, we have included an image of antibody staining after saline and LPS injection in *Sfrp1*<sup>-/-</sup> mice (Fig 1D). This is now mentioned in the text (page 5). In regard to the statement that “*Sfrp1* is expressed only in astrocytes”, we apologize if this is the message that we transmitted. In the original version, the text read as follows: “ $\beta$ gal immunoreactivity ..... largely localized in GFAP<sup>+</sup> astrocytes but not in Iba1<sup>+</sup> microglial cells (Fig. 1A; S1A)”. In page 5 we have now modified the text to indicate that *Sfrp1* is also expressed by choroid plexus cells, as we have already reported (Esteve et al., 2019). More importantly, using RNAscope, we have previously reported the presence of *Sfrp1* mRNA in few Iba1<sup>+</sup> cells surrounding amyloid plaques present in the cortex of APP;PS1 mice (Esteve et al., 2019, Fig. 4). In mice injected with LPS we have observed expression in astrocytes, choroid plexus cells but basically in no Iba1<sup>+</sup> cells (nor in neurons as we reported in Esteve et al., 2019, Supplementary Fig. 7). This difference may be related to the state of microglial cells, which are known to respond differently in different pathological conditions (for a review Bachiller et al, 2018, now in the ref list). We have now referred to this discrepancy in page 5. The fact that microglial cells express very low or



no levels of *Sfrp1* is further supported by the fact that *Sfrp1* mRNA was not found among the transcripts detected in our RNAseq studies performed in FACS-sorted microglial cells. We apologize for not having explicitly mentioned this important point in the text. This is now clearly indicated in page 5 and 9. We have also included in the new EV3 the *Sfrp1* IGV profiles as compared to those of two microglial expressed genes, *Sall1* and *Aif1*. We provide the profiles for the four conditions of our RNA-seq analysis. In conclusion, astrocytes seem to be the main source of SFRP1 in the brain, as further supported by recent studies, which show an upregulation of *Sfrp1* expression in reactive astrocytes both after a demyelinating lesion (Huang et al., Cell Reports 33, 108394, 2020) and reactive gliosis induced by kainic acid (Garcia-Velazquez et al., 2021). These references have been included in the revised version of our manuscript (page 5). Additional support for our claim can be found in the VastBD database. We have also expanded our ELISA data previously shown in Fig. S2B, which quantified the release of SFRP1 in the culture media of isolated microglia and mixed astrocytes/microglia cultures. We now included the media of pure astrocytic cultures. These data, described in page 9 and illustrated in Fig 4D, confirm that astrocytes but not microglia produce SFRP1. We thus hope that these data provide convincing support for an astrocytic origin for most of the brain SFRP1. Thus, the effects observed on microglial cells cannot be cell autonomous. As a consequence, most of the SFRP1 protein present in brain lysates in the presence or absence of LPS (Fig. 1E) seems to have a predominant astrocytic origin, although we cannot discard that the choroid plexus could contribute to a fraction of it (see Esteve et al., 2019). Data in Fig. 1E have been obtained with a specific ELISA described in Esteve et al., 2019 and therefore already provide quantification of the expression as requested by the reviewer. ELISA determination is much more accurate than measuring levels of  $\beta$ gal immunoreactivity. This is why the immunohistochemical data shown here are meant to provide a qualitative analysis.

**Point 2.** *In Figure 2, the authors show that overexpression of SFRP1 induces the expression of GFAP and Iba1 in astrocytes/microglia respectively. To show that this is due to microglial/astrocytic reactivity several other activation markers have to be assessed as GFAP expression can be altered by non-pathological and pathological stimuli thus reflecting rather physiological adaptation. Astrocytic reactivity markers such as C3 and S100 $\beta$  and the microglial markers Clec7a and TMEM119 would help supporting the statement that SFRP1 overexpression increases glial reactivity.*

**Response.** We appreciate the comment and agree that the use of additional markers for supporting an activated state of astrocytes and microglial cells will help. Nevertheless, we would like to note that a very recent consensus report on the nomenclature, definition, markers etc for reactive astrocytes (Escartin et al, 2021 Nat Neurosci, now in the ref list) still considers GFAP as a useful marker. We now verified the presence of reactive astrocytes using Sox9 and S100 $\beta$  as additional markers according to Escartin et al., 2021. Immunostaining for the suggested C3 was rather inconsistent at least in our hands. We have used antibodies against CD45 and Clec7a as additional markers for activated microglial cells. The use of these new markers supports that SFRP1 gene addition is sufficient to activate glial cells. These data are now mentioned in page 6 and illustrated in new Fig EV1.

**Point 3.** *The paper is based on the idea that there is an astrocyte to microglia crosstalk mediated by SFRP1. In vitro the authors nicely showed that astrocytes are the SFRP1 producing cells which are required for the immune response changes upon LPS treatment. a. This referee would like to see that there is a lack of SFRP1 expression in microglia in vivo in the LPS and EAE model e.g. by ELISA or Western blot of isolated microglia and astrocytes in addition to the ELISA results of whole brain tissue. b. The authors assume that microglia are responsible for the release of the measured cytokines in the in vitro experiment in Figure 4D. In this experiment, however, the cytokine profiles of pure astrocyte cultures treated with LPS in the wild type and SFRP1 KO setting are missing. These are required to see whether astrocytes on its own might be responsible for the cytokine changes found in vitro.*

**Response.** a) As stated in our answer to point 1, the best evidence that microglial cells do not express *Sfrp1* mRNA is provided by our RNAseq data based on FACS isolated microglial cells in the presence or absence of LPS. Several additional evidences are described in our response to point 1. b) Astrocyte response to LPS seems to depend on the presence of functional microglia (Holm et al., 2012, in the ref list). This is why we did not include the levels of cytokine released in pure astrocytic culture treated with LPS, in the first place. However, we are grateful to the reviewer for raising this point, which allowed us to revise our

data obtaining a better picture of the cross talk between these two cell types. We have now repeated these experiments using cell cultures of just astrocytes, just microglia and mixed cultures of astrocytes and microglia (in a proportion of 2:1, to address point 2 of reviewer 2). These data are now reported in the new Fig 4 and described in page 9. The new data obtained with several independent biological replicas from both WT and *Sfrp1*<sup>-/-</sup> mice show that indeed astrocytes contribute to the overall secretion of cytokines and that this is in part modified by the absence of SFRP1. We thus concluded that astrocyte derived-SFRP1 influences the response of both astrocytes and microglia to an inflammatory stimulus, modifying their ability to secrete at least two specific cytokines: IL1 $\beta$  and IL10 (page 9).

**Point 4.** *In Figure 4C, changes in the phagocytic capacity of SFRP1 KO microglia were tested using pHrodo E.coli particles in vivo. To clarify the contradicting results, the authors should test the phagocytic capacity of SFRP1 microglia after LPS stimulation in vitro. Here it would be required to address whether the presence of astrocytes is required for the phagocytic changes found in microglia.*

**Response.** Our RNAseq results indicate that there is only a 6% difference between WT and KO microglia in homeostatic conditions. This is now better represented in the newly added Fig EV3. This is not surprising as *Sfrp1* is not expressed by microglial cells, as we underscored in our answer to points 1 and 3. This is why we tested phagocytic capacity of the different genotype/conditions in a physiological context in which cell non-autonomous effects could take place. Nevertheless, we have performed the suggested experiment following the manufacturer's recommendation of limiting pHrodo incubation to 1hr when used in cultured cells. The results are reported in Fig. EV2E and described in page 8. These data indicate that microglial exposed to astrocytes deficient in *Sfrp1* tend to have a less efficient phagocytic activity, although differences were not significant. This observation points to a cell non-autonomous effect, although the data do not provide an unequivocal explanation for the in vivo results, likely because in vivo cell-cell interactions are more complex than what can be reproduced in a test tube.

**Point 5.** *In the first results section, the authors concluded: "Together these data show that astrocytes produce and secrete increased levels of SFRP1 in response to a bacterial lipopolysaccharide". However, the authors did not show that the SFRP1 measured by ELISA was specifically produced or secreted by astrocytes in this section. A general measurement of whole brain SFRP1, including potential intracellular SFRP1, was performed here. Therefore, the respective experimental evidence for the astrocyte-specificity in this very setting has to be provided.*

**Response.** The reviewer is right. We apologize for this inaccuracy as we agree that we cannot conclude that all SFRP1 detected in the ELISA is derived only from astrocytes. We have modified the text to acknowledge the presence of *Sfrp1* mRNA in the choroid plexus. We have also modified the indicated sentence to better reflect our findings (bottom page 5).

### Minor critiques

**Point 6.** *Statistical testing in Figure 2: The authors analysed 3 animals per group and made several acquisitions per animals. It is unclear, whether the significance in each graph was calculated using the number of acquisitions or the number of animals as biological replicates. In case each acquisition was used, the data has to be retested by only using the mean per animal to avoid increasing the statistical power artificially. This applies to all figures shown in this paper.*

**Response.** In the revised figures, we have now reported the significance calculated according to the number of animals tested as well as that for the number of acquisitions.

**Point 7.** *In Figure 3C-D, the authors show the quantification of CD4+ cells, Iba1 immunoreactivity, MBP+ area and MBP immunoreactivity. However, in Figure 3C also GFAP images are shown and in the text the authors refer to reduced GFAP+ reactive astrocytes in SFRP1 KO animals. Thus, in Figure 3D, a quantification of the GFAP immunoreactivity is required to validate this statement.*

**Response.** We apologize because we realised that the text was confusing and did not reflect well our observations. GFAP does not show statistically significant difference between the

two genotypes and this is why we did not include the corresponding graph. However, what is consistently different is the absence of GFAP<sup>+</sup> pial disruption, present in WT but basically absent in the mutants. We have re-written this part of the results to reflect both observations (page 8).

## Referee #2:

*The paper proports to describe the role of SFRP1 in modulating ADAM10 mediated pathways in various mouse models of inflammation. However the authors whilst stating that SFRP1 can modify both Wnt signalling and inhibit ADMA10 activity, completely neglect Wnt signalling, and their justification for doing so is not sufficient. Overall, the use of mouse models to mimic human inflammatory diseases is becoming rather inappropriate given than these models have failed to transfer to the clinic. I would have liked to have seen more of an attempt to align these results with human cells. Adam10 does not cleave TREM2 in humans. Because of this, I am not convinced this advances the field to any great degree.*

**Response.** We regret that the reviewer does not found our work of significance. However, we would like to clarify a number of issues with which we respectfully disagree with him/her. First, our study shows that SFRP1 is part of the molecular signals that astrocytes provide to microglial cells to enhance their inflammatory response. In the presence of *Sfrp1*, microglial cells can foster the inflammatory response by enhancing the expression of members of the HIF pathway. This is a novel and previously unreported result that may explain the described, but poorly explored upregulation of SFRP1 in many human inflammatory conditions (as we discuss in our manuscript supported by a number of references). In contrast to what the reviewer states, we did not conclude that the role that SFRP1 has in inflammation is by modulating ADAM10 mediated pathways. In fact, the reviewer will not find the word ADAM10 nor in the abstract or in the results. In the introduction we clearly stated that SFRP1 acts on both ADAM10 and Wnt signalling and in this revised version we have balanced information related to Wnt signalling and ADAM10. We have also clarified that Wnt signalling implication in neurodegeneration has been mostly linked to synaptic plasticity but whether it has a relevant role in neuroinflammation is poorly explored (page 3). We have also mentioned that there are reports proposing SFRP1 interaction with other molecules/pathways (page 3). We discussed our data in view of a possible SFRP1 function through both ADAM10 (page 14) and Wnt signalling (page 15) on the basis of the data we found in the literature and our RNAseq data indicating no changes in the expression of Wnt signalling components in the different genotype/condition analysed. This was, and still is, clearly stated in the discussion. Thus, we have not “*completely neglect Wnt signalling*”. An activity mediated by ADAM10 (or a yet unidentified additional mechanism) may be more consistent with our and those available in the literature, but we have now deleted the sentence suggesting that this is a likely mechanism, to eliminate any bias.

In regard to the concern brought up by the reviewer that TREM2 cleavage by ADAM10 is not observed in humans, we have found reports showing that either ADAM10 or the related ADAM17 do shed human TREM2 (Thornton et al., EMBO Mol Med. 2017 Oct;9(10):1366-1378; Schlepckow et al., EMBO Mol Med. 2017 Oct;9(10):1356-1365; Feuerbach et al., Neurosci Lett. 2017 Nov 1;660:109-114). Furthermore, TREM2 variants at the ADAM cleavage side have been associated with Alzheimer’s disease (a disease in which we and others have demonstrated that SFRP1 expression is significantly elevated, Esteve et a., 2019 and Bai et al., 2020 in the ref list; Johnson et al., 2021, <https://doi.org/10.1101/2021.04.05.438450>, now added to the ref list).

Finally, we strongly disagree with the reviewer’s statement that “*the use of mouse models to mimic human inflammatory diseases is becoming rather inappropriate given than these models have failed to transfer to the clinic*”. It is true that some human diseases are not accurately replicated by animal models. However, they are the best approach to demonstrate cause-effect relationships since genetic manipulation is not allowed in humans. Ex vivo

experimentation with primary human brain cells is not as immediate as for example with blood-borne cell types. Of course, there are a few immortalized microglial and astroglial (mostly glioma) cell lines that could be used and co-cultured. But immortalized cell lines are not better models than mice. This is why we have used primary cultures from mice. It is indeed our plan for the future to go back to humans and for example test whether SFRP1 is increased in the CSF of MS patients or in patients with sepsis. However, obtaining these samples is not straightforward and requires support from clinicians as well as ethical approval.

Nonetheless, we would like to remind the reviewer that the present study aims to demonstrate a role for SFRP1 in neuroinflammation using a genetic system (a knockout mouse) to confirm our initial finding in humans (Esteve et al., 2019; Bai et al., 2020; Johnson et al., 2021). The results generated with knockout mice and the LPS and EAE models in the current manuscript show that SFRP1 is required for the activation and progression of neuroinflammatory diseases and that it might be relevant not only for AD, as was our initial hypothesis derived from studies in humans, but for other human neuroinflammatory conditions such as bacterial sepsis-derived inflammation (LPS) and MS, indicating that the mouse can still be a useful model to study the function of this protein.

**Point 1.** *M. Heneka does not appear in the author contributions.*

**Response.** M. Heneka appears in the manuscript as Michael T Heneka. In the authors' contributions he has been abbreviated as MTH. This may have confused the reviewer. As stated in our first version: JRC, MIM, **MTH**, PE and PB analysed and discussed the data.

**Point 2.** *The coculture of astrocytes microglia of 1:1 does not mimic in vivo ratios.*

**Response.** We agree that a 1:1 ratio might not represent the in vivo ratios, although cell proportions are very variable in different brain regions in both human and mice. Nevertheless, we have now repeated our co-cultures with a 2:1 (astrocytes vs. microglial) proportion, given that in the mouse cortex (the source of our cultures) the reported proportion between astrocytes and microglia is about 2:1 (Keller et al., 2018, in the ref list).

### **Referee #3:**

*This manuscript, "SFRP1 shapes astrocyte to microglia cross-talk in acute and chronic neuroinflammation" by Rueda-Carrasco et al. seeks to examine the role of astrocyte-produced SFRP1's influence on microglia's role in neuroinflammation using both an LPS model and EAE. They found that SFRP1<sup>-/-</sup> mice have a reduced clinical score and severity of symptoms in the EAE model. They also demonstrate that LPS induces an increase in SFRP1 expression and reduced cytokine expression in response to LPS. This is an interesting paper that has a few issues that need to be clarified and expanded on.*

**Point 1.** *Much of the narrative of the manuscript is focused on the neurodegenerative disease, Alzheimer's disease, yet the manuscript experiments are using an LPS model and EAE, no AD models. There was also quite a bit of discussion around ADAM10. As pointed out, this could modulate TREM2 expression, however there were no experiments designed around this concept.*

**Response.** We appreciate the comments of the referee about the relevance of the LPS and EAE models to determine the role of SFRP1 on neuroinflammation. We agree that the two models used are not models of Alzheimer's disease (AD). Nevertheless, we would like to underscore that the present study stems from our work related to the implication of SFRP1 in AD, Esteve et al., 2019 in the reference list). As we stated in the introduction, neutralization of Sfrp1 activity in an AD-like mouse model was associated with a substantial decrease on neuroinflammation. This preliminary observation linking SFRP1 with AD and neuroinflammation has been the trigger for the present study, which addresses the question of whether Sfrp1 could be directly implicated in inflammation. Testing this hypothesis in AD-like mice is rather difficult because amyloid plaque formation, synaptic loss etc complicate the interpretation of the data, as they are themselves causes of neuroinflammation. This is

why we resorted to use two different models that allowed testing the hypothesis directly. The use of LPS appeared as the most straight forward approach for testing our hypothesis since LPS is a clear proinflammatory insult that has been widely used in the literature. In addition, we decided to back our data with the EAE model, since it causes a chronic disease originated by an inflammatory component and resulting in permanent damage to the CNS. Considering our published data on the association between SFRP1 and neuroinflammation in AD and the current data demonstrating the role of SFRP1 in the LPS model and the EAE model, we propose that SFRP1 could be likely involved in multiple pathologies that curse with neuroinflammation, including septicaemias by gram-negative bacteria, multiple sclerosis and AD. This possibility is hinted in the concluding paragraph of the discussion (page15).

As we indicated in our comments in response to reviewer 2, we have introduced and discussed our data in view of a possible implication of ADAM10 and Wnt signalling. We could not find evidence of Wnt signalling involvement in our RNAseq data. In addition, data in the literature are rather controversial. This makes us think that SFRP1 effect on neuroinflammation may not be related to Wnt signalling. On the other hand, SFRP1 can interfere with ADAM10 mediated shedding of different substrates involved in microglial activation. We discussed the case of TREM2, CX3CL1 and CD200. We also indicated that we are not aware of good tools to test this possibility *in vivo*. We have performed experiments in cell lines transfecting the substrates and these *in vitro* experiments point in that direction. However, we have not included them in the manuscript as the context is rather artificial. We have therefore eliminated a sentence suggesting that SFRP1 might act though ADAM10 in this case, because we cannot demonstrate, at least with the tools we have in hands, that this is the case.

**Point 2.** *The image used for CD45 in the WT/saline treatment in figure 2c, appears to have a lot of background.* **Response.** We apologize for the poor choice of the image, which we have now replaced for one of higher quality.

**Point 3.** *Can they show what cell type took up the LV to express SFRP1?*

**Response:** The description of the cells that pick up the virus was already included in the text. We have now improved this description and included in Fig EV1B images showing that LV are picked up also by astrocytes. These changes are reported in page 6.

**Point 4.** *In 3D, the number of CD4+ cells should be confirmed with CD4+IBA1- cells, as monocytes can express CD4, since there is no CD3 co-staining.*

**Response.** We thank the reviewer for pointing this out. However, we basically did not detect CD4+/Iba1+ cells (monocytes) and this is why we assume that all CD4 positive cells were lymphocytes. This is now clarified in page 7.

**Point 5.** *The data presented in figure 4C is confusing. pHrodo will only fluoresce when inside a low pH environment, but the number of positive cells is higher in the knockout but total fluorescence is lower. Is there less molecules per cell? And if so, why is that? Cell recruitment doesn't really make sense in the FACS experiment because it is only GFP+ cells. Does this vary between the GFP+ CD45<sup>low</sup> and GFP+CD45<sup>high</sup> cells? How exactly was the pHrodo fluorescence measured?*

**Response.** We do not have a precise answer to the question of whether mutant microglial cells phagocytes less molecules per cells, as it is difficult to single out the particle in the FACS, which is the method we used to carry out this analysis. As we had indicated in the text, engulfment of less particles is a possibility, although the observed difference may also relate to slightly different lysosomal pH or a different rate of degradation. These explanations are provided in page 8. Fig EV2 and its legend describes how pHrodo fluorescence was measured. We also added a section in the methods related to this (page 19). We apologize if the difference in phagocytic ability between GFP<sup>+</sup>/CD45<sup>low</sup> and GFP<sup>+</sup>/CD45<sup>high</sup> cells was not well explained in the text. We have now clarified that basically only CD45<sup>hi</sup> cells have a

prominent phagocytic activity and this is why we focused only on those. We have improved the text referring to these results (page 8) and added a graph (Fig. EV2B) to show that CD11b<sup>+</sup>/CD45<sup>lo</sup>/GFP<sup>+</sup> surveying microglial cells showed a very limited phagocytic activity in both genotypes.

**Point 6.** *In Figure 4D, addition of an astrocytes-only cell culture for comparison with the microglia and microglia + astrocyte co-cultures would be important to include. Astrocytes can be a source of cytokines such as IL-6.*

**Response.** Thank you for making this important point that was also raised by Reviewer 1, Point 3b. Please read our answer to this point for details. In brief, we have performed new experiments using cultures of isolated astrocytes, isolated microglia and mixed ones. These data are shown in new Fig 4 and described in page 9.

**Point 7.** *How does the LPS increase expression of astrocyte SFRP1? Is it via TLR4 on the astrocytes or does LPS stimulate the microglia which then signal to the astrocytes to upregulate SFRP1?*

**Response.** This is indeed a very interesting possibility although we do not have a straightforward answer. Microglia seems to foster SFRP1 production as the release of SFRP1 into the supernatant of mixed astrocytes and microglia cultures is greater than that of astrocytic culture alone. Indeed, in the discussion (page 13) we propose “that up-regulation and release of SFRP1 from astrocytes occurs as part of their microglia-mediated early activation.” This is in line with the reviewer’s suggestion.

**Point 8.** *Some in vitro mechanistic studies would add a lot to this paper. Experiments with purified SFRP1 directly on microglia +/- LPS would be helpful in understanding how it modulates their function, especially in dissecting out the relationship of LPS signaling and SFRP1 signaling. In the earlier experiments, LPS seemed to be required to see the effect of SFRP1 KO, but in the transcriptomics experiments there seems to be a bigger difference in the genotypes in the saline condition.*

**Response.** We kindly disagree with the reviewer in the last part of his/her statement. We do not find inconsistency between our experiments. RNAseq analysis confirms that there is little difference between control and KO saline treated animal (see new Fig EV3 and Fig 5A and F). Indeed, in the PCA plot saline treated samples clustered more closely together and genotype variance is roughly 6% of the total variance of the samples. This is also visible in the heatmap in Fig. 5F. To clarify this crucial point, demonstrating the relevant role of SFRP1 in microglial response to LPS, we have added Fig EV3, which contains volcano plots that may facilitate the interpretation of our results (page 10). Following the reviewer’s suggestion of exploiting more the cultures to dissect the relationship of LPS and SFRP1 signalling, we have performed additional studies *in vitro* using ADAM10 specific inhibitors and Wnt antagonists. However, these studies did not provide clear results that could help illuminating on a specific mechanism. We have therefore not included them in the present manuscript.

**Point 9.** *What is the receptor on microglia for SFRP1? Can the pathway be manipulated in vitro to show that this is the mechanism of the findings?*

**Response.** This is a question that indeed we would like to answer in the future. We believe that addressing this question unfortunately requires specific proteomic approaches and a considerable number of experiments to demonstrate a cause-effect. All those new experiments are beyond the scope of the present study.

**Point 10.** *In terms of peripheral cell recruitment, is the reduction in the SFRP1 via microglia or another mechanism?*

**Response.** This is an interesting possibility. We believe that the most likely explanation of the observed reduced infiltration in *Sfrp1* ko mice in the EAE model is a better preservation of the ependyma layer (Fig. 3C). However, whether this is through microglia, astrocytes or a direct function of SFRP1 on ependyma cells or blood brain barrier integrity is difficult to determine at this point. Microglial cells have been recently reported to participate in the maintenance of

BBB integrity (Haruaka et al., Nat Comm. 2019 10:5816; Lou et al., 2016; PNAS 113:1074-9) but whether this function includes the activity of SFRP1 is so far unknown. We have not experimentally addressed this point since it seems to deviate from the main message of the present study.

Dear Prof. Bovolenta,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, both referees now support the publication of your study in EMBO reports. Nevertheless both have remaining concerns, comments and/or suggestions to improve the manuscript that I ask you to address in a final revised version of the manuscript. Please also provide a detailed point-by-point-response addressing the remaining points of the referees.

Moreover, I have these editorial requests:

- Please reduce the number of keywords to 5.
- The Data Availability section refers to large datasets that have been submitted to a public database. Thus, please remove the statement that materials will be available upon request, but please add a link to directly access the deposited dataset.
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- For the microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Presently, some panels do not have scale bars. Please place these in the lower right or left corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.
- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures), and that statistical testing has been done where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. If statistical testing was done but there is no significant difference, please also mark this in the diagrams (n.s.).
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I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely

Achim Breiling  
Editor  
EMBO Reports

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Referee #1:

The authors addressed our critics by performing several additional experiments underlining in a convincing manner that astrocytes are indeed the major SFRP1 producing cells. Further, they expanded their assessment of microglial and astrocytic activation upon SFRP1 overexpression by using appropriate established markers. The authors also included the missing and very important control group LPS-treated astrocytes in their cytokine ELISA assessment of in vitro cultures of SFPR1 KO mice. Importantly, they found that astrocytes respond to LPS treatment by cytokine release and thus, had to adapt their conclusion regarding the cell type (astrocytes vs. microglia) responsible for the changes in inflammation. The authors concluded that "astrocyte derived-SFRP1 influences the response of both astrocytes and microglia to an inflammatory stimulus, modifying their ability to secrete at least two specific cytokines: IL1b and IL10". We would like to emphasize that simply the lack of astrocytic IL-1b may also influence the release of IL-10 by microglia. The addition of a pHrodo phagocytosis assay of microglia in the presence of SFRP1 KO/wt astrocytes was highly appreciated, even though it unfortunately did not clarify the contradicting phagocytosis results.

Taken together, the authors addressed the major points of criticism with appropriate experiments or extensive explanations to our satisfaction.

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Referee #3:

The manuscript has been greatly improved. And the EAE experiments are of interest to the community. However, there are still some outstanding questions that can easily lead to misinterpretation of the results and are easy to address. With the knockout experiments, it appears that there are cell-intrinsic defects in the microglia, even though they do not produce SFRP1. This is shown in figure 4 (LPS stimulation of purified microglia from WT and SFRP1<sup>-/-</sup> mice), and the variance in the saline transcriptomics. This is most likely due to a developmental difference in the homeostatic population of microglia in the SFRP1<sup>-/-</sup> mice. Of course, it does seem that SFRP1 does indeed play a later role as shown by the elaborate LV experiment. In vitro experiments where WT microglia are treated with recombinant SFRP1 and LPS in parallel with WT microglia treated with supernatants from WT and SFRP1<sup>-/-</sup> astrocytes and LPS will clearly demonstrate that astrocytes are influencing microglia activation via SFRP1; clearing up any possible misinterpretations.

## Response the reviewers

### Referee 1

*The authors addressed our critics by performing several additional experiments underlining in a convincing manner that astrocytes are indeed the major SFRP1 producing cells. Further, they expanded their assessment of microglial and astrocytic activation upon SFRP1 overexpression by using appropriate established markers. The authors also included the missing and very important control group LPS-treated astrocytes in their cytokine ELISA assessment of in vitro cultures of SFRP1 KO mice. Importantly, they found that astrocytes respond to LPS treatment by cytokine release and thus, had to adapt their conclusion regarding the cell type (astrocytes vs. microglia) responsible for the changes in inflammation. The authors concluded that "astrocyte derived-SFRP1 influences the response of both astrocytes and microglia to an inflammatory stimulus, modifying their ability to secrete at least two specific cytokines: IL1b and IL10". We would like to emphasize that simply the lack of astrocytic IL-1b may also influence the release of IL-10 by microglia. The addition of a pHrodo phagocytosis assay of microglia in the presence of SFRP1 KO/wt astrocytes was highly appreciated, even though it unfortunately did not clarify the contradicting phagocytosis results. Taken together, the authors addressed the major points of criticism with appropriate experiments or extensive explanations to our satisfaction.*

**Response.** We thank the reviewer(s) for the positive comments about our revised manuscript. We appreciate also their comment related to the possible influence of astrocyte derived IL-1b over microglial derived IL-10, although we have been unable to find a suitable reference that support the statement. We also agree this the reviewer(s) that the in vitro experiments using pHrodo did not clarify the in vivo results. More importantly, we could not use long time of incubation with pHrodo in vitro as indicated by the manufacture. Therefore, we believe that we could not reproduce the complexity of in vivo interactions that underlying what seem contradicting results.

### Referee 3

*The manuscript has been greatly improved. And the EAE experiments are of interest to the community. However, there are still some outstanding questions that can easily lead to misinterpretation of the results and are easy to address. With the knockout experiments, it appears that there are cell-intrinsic defects in the microglia, even though they do not produce SFRP1. This is shown in figure 4 (LPS stimulation of purified microglia from WT and SFRP1<sup>-/-</sup> mice), and the variance in the saline transcriptomics. This is most likely due to a developmental difference in the homeostatic population of microglia in the SFRP1<sup>-/-</sup> mice. Of course, it does seem that SFRP1 does indeed play a later role as shown by the elaborate LV experiment. In vitro experiments where WT microglia are treated with recombinant SFRP1 and LPS in parallel with WT microglia treated with supernatants from WT and SFRP1<sup>-/-</sup> astrocytes and LPS will clearly demonstrate that astrocytes are influencing microglia activation via SFRP1; clearing up any possible misinterpretations.*

**Response.** We thank the reviewer for his/her positive comment and for indicating that our data suggests that there might be a developmental difference in the homeostatic population of microglia in the *Sfrp1*<sup>-/-</sup>. We had considered this possibility in the past and analyzed the presence of microglial cells in the early postnatal brain of the mutant mice, without finding any significant variations in the number and shape of the cells. A more detailed analysis may find subtle differences and thus we cannot fully discard this possibility. To account for this possibility, we have included a sentence in the discussion (page 14). However, as we have already stressed and documented, the differences between saline-treated wt and *Sfrp1*<sup>-/-</sup> microglial cell is very small and the identified DEGs do not seem to define any specific process at least using Gene Ontology Enrichment Analysis (Dataset EV1C; Fig 5 and Fig EV3). Furthermore, Fig EV4 clearly illustrates that the large bulk of DEG between wt and *Sfrp1*<sup>-/-</sup> is linked to LPS treatment. Part of the changes may be indirect and the result of astrocytes-microglia cross-talk but our data overall clearly show that SFRP1 is part of this cross-talk.

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#### A- Figures

##### 1. Data

The data shown in figures should satisfy the following conditions:

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

##### 2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

#### B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For each type of experiment, we performed a pilot study. We then used the freely available statistical power analysis program G Power (version 3.1.9.2; Faul, F., Erdfelder, E., Buchner, A., & Lang, A.-G. 2009) to determine the minimal sample size needed in each set of experiments. We used the criteria of a p-value of a maximum of 0.05 and a power of at least 0.8.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For each experiment in which animals were involved a minimal sample size of 3 animals per experimental condition was used. Each experiment was repeated at least 4 times to ensure no technical replication.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analysis with the exception of pre-established criteria. For Experimental Autoimmune Encephalomyelitis (EAE) weight control was established and two animals that presented no symptoms nor weight loss after disease induction were excluded from the final analysis.
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.	For each experiment, animals were divided into groups on the basis of their age, sex, and genotype. For EAE, only females were used as stated in the Methods section.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly assigned to different groups just taking age, sex and genotype as relevant matching criteria.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experimenter was blinded to the genotype of animals used, identification numbers were assigned to animals prior to genotyping and were used during the experiment. Usually, littermates from different genotypes were caged together after weaning and subjected to the same treatment blinding genotype to the experimenter.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were carried out with genotype blind to the experimenter.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, D'Agostino & Pearson and Shapiro-Wilk normality tests were assessed and non-parametric tests were used when not present or comparing ranks of scale e.g. EAE.
Is there an estimate of variation within each group of data?	Yes.

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<http://www.consort-statement.org>  
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<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum>  
  
<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://jii.biochem.sun.ac.za>  
<https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/>  
<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	Usually yes, but Welch's correction was applied in a condition of unequal variances.
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### 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).	Antibodies citation is included in the Material and Methods section and Table EV2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

### D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All details are included in the Material and Methods section. Mice were maintained in pathogen-free conditions at the CBMSO animal facilities, following current national and European guidelines (Directive 2010/63/EU).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures involving live vertebrates were approved by the CBMSO, CSIC and Comunidad Autónoma de Madrid ethical committees.
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 confirm compliance.

### E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

### F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.  Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNAseq dataset generated in this study is deposited at the European Nucleotide Archive public repository under the following accession numbers PRJEB36471/ERP119668 as indicated in the Material and Methods and Data Availability sections
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).	Dataset deposition meets journal's data policy.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

### G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No
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