nature portfolio

Peer Review File

BTK regulates microglial function and neuroinflammation – implications for multiple sclerosis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors intended to test whether tolebrutinib, an inhibitor of the enzyme BTK may provide therapeutic benefit within the CNS by targeting selective aspects of adaptive and innate immunity-mediated disease progression. This question is of outmost importance as therapeutic approaches to control the progressive aspect of MS are currently missing.

The authors investigated the effect of tolebrutinib in development of MOG 35-55 induced EAE as well as in the Cuprizone model. Furthermore, they provide an extensive in vitro analysis on BTK-dependent transcriptional signatures using mouse microglia, induced pluripotent stem cell-derived human microglia, and a human iPSC tri-culture system composed of neurons, astrocytes, and microglia.

This is an interesting manuscript and the effect on microglia is promising. Yet, in its current state, the work falls short in showing that tolebrutinib exerts these effects within the CNS in vivo independent of its immunmodulating properties in the periphery.

Specifically, BTKi is applied in MOG 35-55 EAE when the first clinical symptoms develop and accordingly at the max of CNS immune cell influx. One can easily envision that in this setting, a peripheral effect of tolebrutinib on myeloid cells and B cells may translate to an alteration of the invading immune cells (e.g. T cells), translating to an indirectly altered activation status of microglia. Unfortunately, the authors don't even control for these parameters. To show a direct effect of tolebrutinib within the CNS, the authors need to investigate its effect in two further EAE settings, namely in a) late-stage fully established EAE, and b) in adoptive transfer (passive) EAE, in which readily primed T cells only require reactivation in the CNS bypassing the periphery. Only a clinical and/or immunological effect in these settings would justify concluding that tolebrutinib exerts direct effect within the CNS.

Similarly, the authors perform their cuprizone experiments in wild-type mice, in which a contribution of the peripheral immune system cannot be excluded (as they also state in their manuscript). To more conclusively demonstrate an effect independent of the peripheral immune system, the authors would need to perform their cuprizone approach in e.g. RAG-deficient mice.

The in vitro analyses are extensive and interesting as they show that, in case tolebrutinib reaches relevant concentrations within the CNS it may alter the activation status of these cells. along the same lines of the two further points above, all results vitally depend on this precondition. Accordingly, this point needs to be strengthened using e.g. an in vitro model of the blood-brain-barrier in combination with their in vitro microglia settings. Can they measure relevant concentrations of tolebrutinib within the CNS in a setting of an intract blood-brain-barrier?

Reviewer #2

(Remarks to the Author)
The authors reported that a CNS-penetrant BTK inhibitor (BTKi) ameliorated EAE correspondent with reduced microglia activity. BTKi also lowered MS-relevant, BTK-dependent transcriptional signatures in vitro of mouse microglia, iPSC-derived human microglia, and in a tri-culture of neurons, astrocytes and microglia. They demonstrated that in MS tissue BTK was expressed in microglial cells, with increased levels in MS lesions. These results are interesting and important, and relevant to targeting BTK in the CNS in MS. The following are points to be considered:

1. In Figure 1C, when the tissue was taken for bulk RNAseq should be indicated within the figure. One may get the wrong impression that this is data from preventative EAE, while this is impressively from therapeutic intervention (ie after clinical signs were detected). Appreciating that this is not single-cell RNA sequencing, is there any capacity to differentiate the myeloid signal into monocyte-derived macrophages versus microglia? The separation of the effects of BTKi on microglia versus peripherally-derived macrophages would make a stronger case that the effects of BTKi in vivo is due to microglia versus macrophages, or not.

2. In the cuprizone experiments of Figure 2, the authors stated that the effects of BTKi was on microglia when they are not in a position to differentiate microglia from monocyte-derived or CNS-intrinsic macrophages. This theme permeates through the in vivo experiments and should be corrected (ie leave open the possibility that the in vivo effects could be contributed also by macrophages as the markers do not differentiate the myeloid populations).

3. The MS tissue results are particularly impressive. However, as with the mouse data, the authors did not discriminate between microglia and macrophages (IBa1+/CD68+ would imply that many of these are macrophages), so it would be fair to note the cells as 'microglia/macrophages' as opposed to only 'microglia'. Indeed, a BTKi working only in the periphery may potentially alter the properties of monocyte-derived macrophages when these enter the CNS.

4. In Figure 2, when BTKi is initiated and for what period could be illustrated in a schematic. Any comments on whether remyelination was enhanced in the cuprizone model, especially since this is such a commonly used model for de- and remyelination? The brief statement in the Discussion is not adequate.

5. The basis of the dose of BTKi used in mice could be described. The correspondence of the mouse dose to that used in humans could be explained. Are the concentrations in serum at steady state similar between the mouse and available human studies at the murine (this paper) and human (in clinical trials) doses used? In other words, are the doses and concentrations of BTKi in this paper relevant to those obtained in MS patients in clinical trials?

6. Last line of page 3: not sure what 'tool compound' means

7. The 'complexed immunoglobulin G (IgG), which is known to engage FcR that couples with BTK' is a sophisticated and physiological method to activate BTK in microglia. Can the authors provide more details in the Methods of this protocol, as many labs would wish to simulate this mode of activation? Any extra notes besides what is described? Was the same reagent (IgG complex from mouse serum) and protocol used for human and mouse cells? As an aside, does BTKi inhibit TLR signaling (eg by LPS, commonly used in many labs to activate microglia)?

8. Figure 3 shows BTKi preventing the IgG activation of microglia. The BTKi was applied before the stimulus. In MS brains, there would already be substantial BTK activation in microglia in plaques. Does the BTKi reduce the activation transcripts when BTKi is applied after cells have been stimulated with IgG?

9. Perhaps the authors could explain why only BTK (and not pBTK) antibody was used in in vivo experiments to characterize BTK, as Figure 3 (of pBTK and BTK) clearly shows that pBTK but not BTK informs on activity.

10. The data of Fig S3B that BTKi reduces Rgs1 of naïve brain is confusing. While preceding results suggest that BTKi reduces activated myeloid cells back to near baseline level, the data of Fig S3B would suggest that normal microglia is also inhibited to below basal level?

11. The last paragraph of Introduction summarizes the results of this paper, which are largely contained within the preceding abstract, and detailed in the proceeding Results section. Perhaps that paragraph in the Introduction could be substantially truncated to introduce the intent of this paper rather than to summarize the results.

12. Consider removing the triculture data. What is the composition of each of the cell subsets – how representative of that in the brain or in lesions? The schematic of Figure 5A (of the cell types) is confusing – momentarily, one gets the impression that these cells are studied in isolation. Basal response to tolebrutinib (or PRN?) in absence of IgG seems to be exaggerated compared to vehicle in absence of IgG. That BTK is only on microglia should be quantitated (doing cell counts). The inference that BTKi affects microglia that then changes neurons or astrocytes is speculative. Thus, the triculture data is equivocal.

13. This reviewer is of the opinion that the 'BTKi' label in figures and much of the text should be changed to the drug name, PRN2675 or just PRN. 'BTKi' infers that all available BTK inhibitors would have the same results but this may not be the case as there is a distinct effort in this field to separate BTKi's into those that are covalent inhibitors or not, and those with better selectivity to BTK versus other kinases. Note that 'tolebrutinib' was used to describe some of the data (eg page 12) – was tolebrutinib used or its analog PRN2675? If some experiments involved PRN while others tolebrutinib, this needs to be clarified. While PRN2675 has a 'single fluorine substitution for a hydrogen near the reactive moiety', this substitution is not

trivial. As well, in Discussion, page 31, the authors state ‘These data strongly support the concept that tolebrutinib is a potent regulator of neuroinflammation in vivo.’ Yet, unless this reviewer is mistaken or the description of the text is unclear, tolebrutinib was not used in this paper.

14. Figure S8 should be deleted as much of the schematic is not addressed by the results here. Also, it appears to be an advertisement for tolebrutinib, the authors’ company.

Reviewer #3

(Remarks to the Author)

BTK regulates microglial function and neuroinflammation in multiple sclerosis
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Abstract: Neuroinflammation in the brain and spinal cord, driven largely by central nervous system (CNS)-resident phagocytes, has been proposed as a significant contributor to disability accumulation in multiple sclerosis (MS) but has not been directly addressed therapeutically to date. Bruton’s tyrosine kinase (BTK) is expressed in B lymphocytes but also in innate immune cells including microglia; however, its role in these cells is poorly understood. Inhibition of BTK may provide therapeutic benefit within the CNS by targeting selective aspects of adaptive and innate immunity-mediated disease progression in MS, and is currently being investigated in phase 3 clinical trials across the spectrum of MS. We used a CNS-penetrant BTK inhibitor (BTKi) to demonstrate a robust in vivo effect of BTKi in mouse models of MS. We further identified BTK-dependent transcriptional signatures in vitro using mouse microglia, induced pluripotent stem cell (iPSC)-derived human microglia, and a complex human iPSC tri-culture system composed of neurons, astrocytes, and microglia. Further analysis of these specific transcriptomic signatures for the CNS-penetrant BTKi tolebrutinib in human microglia and the tri-culture revealed that tolebrutinib modulated neuroinflammatory pathways highly relevant to MS. Finally, we demonstrated that in MS tissue BTK was expressed in B cells as well as in microglial cells, with increased levels in MS lesions. Our data provide a strong rationale for targeting BTK in the CNS to modulate neuroinflammation and diminish the attendant disability accumulation.

PEER-REVIEW MANUSCRIPT

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

The authors show that BTKi PRN2675 (a tolebrutinib derivative) can ameliorate EAE in a therapeutic regimen (after symptom onset). They further show that this BTKi can modulate the inflammatory response consistent with microglia signature in the Cuprizone model and also in cell culture. In the cell culture, a tri culture model was used the reveal modulation of specific pathways when the 3 cells are cultured together. The use of human iPSC is a plus.
BTKis are extensively studies in MS, including clinical trials, which is also the case for tolebrutinib. Here, the effects of BTKi PRN2675 in two common MS mouse models (EAE&Cuprizone) are investigated, complemented with experiments in primary microglia/hiPSC-microglia.
Does the work support the conclusions and claims, or is additional evidence needed?
Study design, data analyses and methodology need major improvements. Key groups are missing: in almost all experiments, a control+BTKi group is missing.
Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
Yes, for details, please see coments below. Major issues are differential expression (DE) analysis, including definition of DE genes (DEGs) and statistical tests used. It is unclear which statistical tests were used for each figure and whether they are appropriate for the data and experimental design in question.
Largely, 4 kinds of experiments are performed, EAE, cuprizone, microglia, CNS-cell coculture. These 4 data sets are not, or poorly integrated. How do the data obtained in these 4 experiments groups integrate, for instance gene sets are not compared or integrated, in general the manuscript lacks somewhat in narrative.

Bulk RAseq is typically used. In EAE and cuprizone, cellular compositions of the tissue will change: loss of oligo’s, influx of immune cells, microglia proliferation etc etc. This has major implications on RNAseq analysis, data interpretation, the DEGs to be detected/expected. No deconvolution efforts were made to assess effects of treatments on cellular composition. All in all, aside from the methodological and statistical issues, this hampers data interpretation.
No validations on the effectivity of EAE/cup are offered, no IHC for lesion formation, effective de- and remyelination etc.
Is the methodology sound? Does the work meet the expected standards in your field?

Negative points: potentially serious methodological and statistical problems. Missing experimental group for EAE experiment (BTKi only, without EAE). Moreover, they do not show whether BTKi treatment improves remyelination in the cuprizone model (this fact is briefly mentioned as a future perspective during the discussion)
Positive points: tissue dissection in the cuprizone experiment (for corpus callosum only). Try to correlate results with available single cell data.
Is there enough detail provided in the methods for the work to be reproduced?
Not enough detail on key parts of methodology: statistical analyses, RNA-seq analyses and cell culture of cell lines. Which cell lines were used exactly and how were they obtained, cultured etc? Below a list of more specific comments:
Comment 1
Regarding all the results: statitstic tests are not clear. For each figure it should be clear which statistical test was used. The statistsical tests used are only mentioned in the methodology section and have no specific reference to which figure/results they refer too. Unclear. For more specific concerns about statistic, see comments below.
Comment 2
Regarding results from Figure 1E 1F, 2C 2G 2H, S2, 3J, 4D 4F: Normalized counts shouldn't be analyzed standalone, but DEseq2 differential analysis should reveal which genes are differentially expressed based on their model (and p value and logFC requirements). It is possible to set different contrasts and analyze interactions within DEseq2 pipeline, see http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#differential-expression-analysis and https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html#differential-expression-analysis for more information. Analyzing normalized counts apart from the differential expression analyzes,with ANOVA and T-tests are not appropriate because the p-value needs to be adjusted by all the comparisons and genes being tested and the nature of RNA-seq data, and DEseq2 (or other differential expression pipelines such as EdgeR/Limma/Voom) have models that account for that. Using normalized counts and analyze genes as qPCR proxy is not appropriate. Just use the p-value given by results() function in the DESeq2.
EdgeR/Limma/Voom method:
Law CW, Alhamdoosh M, Su S et al. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR [version 3; peer review: 3 approved]. F1000Research 2018, 5:1408 (https://doi.org/10.12688/f1000research.9005.3)
https://master.bioconductor.org/packages/release/workflows/vignettes/RNAseq123/inst/doc/limmaWorkflow.html
Comment 3
Regarding results from differential expression analyses and downstream analyses and all volcano plots: The definition of DEGs does not seem to be appropriate, based on the volcano plot. Page 35 (methods section): “(…) DEGs were determined using a 1.5-fold-change (FC) threshold and false discovery rate (FDR)-corrected p-value cut-off of 0.05.”
For that you should make use of the function results() in DEseq2 and specify lfcThreshold and altHypothesis arguments (building results table section at DEseq2 vignette (Bioconductor)
https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html#building-the-results-table and “Tests of log2 fold change above or below a threshold”section at
http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#:~:text=7401 980 8381-,Tests of log2 fold change above or below a threshold,-It is also).
Another example of properly tested DEG and corresponding volcano plot (https://www.nature.com/articles/s42003-023-04926-8) based on the TREAT method (https://doi.org/10.1093/bioinformatics/btp053 "Testing significance relative to a fold-change threshold is a TREAT” Davis J. McCarthy, Gordon K. Smyth) used in EdgeR/Limma/Voom pipeline.
Comment 4
Microscopy images and pictures: need to be of better quality (increase DPI/PPI resolution).
Comment 5
Ingenuity Pathway Analysis (IPA) figures: Figures lack clear indication of which experimental groups are being compared for the analysis (clear contrast is missing for some figure legends). For readability, please add this contrast as a title (e.g. BTKi vs vehicle). Also, not all figures indicate whether it is an enriched or downregulated pathway.
Comment 6
Comparing differential expressed genes Fig 4E Fig 5E: Venn diagrams result in “binary” data (yes or no categories). 4-way plots to correlate the log fold change of differentially expressed genes and to really show how similar or different (logFC = continuous value) the DEGs are in these venn diagrams is much more informative.
Comment 7
In page 3: why are there double quotes around “signature”?
Comment 8
In page 4: “The increased BTK gene expression after EAE induction was independent of B-cell-associated transcripts in the tissue, as assessed by Cd19 expression levels…” Cd19 is only one transcript and not transcripts? Or are the authors referring to ‘transcripts’ as mRNA molecules and not ‘genes’?
Is there another marker that could corroborate Cd19, maybe Cd20 and other lymphoid-lineage markers?
Comment 9
In Figure 1:
1) What is the effect of BTKi alone, without EAE induction? A control group with only BTKi is missing to assess whether BTKi has an effect or whether its effects are only present when EAE is induced (interaction). Can’t the users make use of the data from the Figure 2 (cuprizone experiment), there you have all 4 groups (including BTKi alone).
2) IPA results are not clear, are these pathways enriched or depleted? From which differential expression comparison? Which groups? For clarity add a title to figure 1 D of which specific comparison and whether the pathways are enriched or downregulated.
3) What determines the order of genes (rows) from panel C? Are genes from C also clustered or only clustered based on samples (supervised or unsupervised clustering?).
Comment 10
In Figure S1:

1) Not clear for panel C which genes were used (only downregulated, only upregulated, all DEGs from which comparison?).
Comment 11
In Figure 2:
Fig 2B) What is Digital signal? A bit more details are appreciated on the methodology based on HALO software.
Reproducibility issues: Which bregma point of the brain is being analyzed? The brain region heterogeneity is particularly important for the corpus callosum during the cuprizone model. There seems to be a lot of microgliosis, is this comparable to other cuprizone studies? There are different extents of demyelination and remyelination and potentially also microgliosis depending on the bregma point analyzed. See references:
Zhan J, Mann T, Joost S, Behrangi N, Frank M, Kipp M. The Cuprizone Model: Dos and Do Nots. Cells. 2020;9(4). doi:10.3390/cells9040843
Steelman AJ, Thompson JP, Li J. Demyelination and remyelination in anatomically distinct regions of the corpus callosum following cuprizone intoxication. Neurosci Res. 2012;72(1):32-42. doi:10.1016/j.neures.2011.10.002
Xie M, Tobin JE, Budde MD, et al. Rostrocaudal analysis of corpus callosum demyelination and axon damage across disease stages refines diffusion tensor imaging correlations with pathological features. J Neuropathol Exp Neurol. 2010;69(7):704-716. doi:10.1097/NEN.0b013e3181e3de90
Fig 2D) Some BTK positive cells on the right panel (Cuprizone 5W) do not seem to be IBA1 positive. Any idea why is that?
Comment 12
In Fig 3: are there stastitically significant differences between the protein in the western blots? Missing quantification panels. Is panel F the quantification of panel E? That is not evident, please clarify in figure legend.
Fig 3I, there is no indication of statistical significance. What was the statistical test? what are the statistically significant differences?
Fig 3H, again, as for other IPA plots, not clear which groups are being compared here. Please add a title to the figure with the correct contrast (e.g. IgG + tolebrutinib vs. IgG)
Comment 13
In page 15 and methodology in general: add which mouse microglia cell line was used. There is no description on the cell line used and how these cells were obtained and cultured.
Comment 14
Similarly, in page 17 and methodolgy in general, please add information on the cell line used. How were THP1 obtained, cultured?
Comment 15
In page 17 a grammar issue: “These data indicate that BTK activity can directly regulate the microglial transcriptional response in both mice and humans and potentially regulate the function of these cells.” I believe it is missing a word after “mice and humans”. Probably “cells” or “microglia”? Because “mice and human” are not “these cells”.
Comment 16
Fig 4: in panel C, why is there a cartoon of microglia “reactive” morphology? That is not described anywhere in the text or figure legend. Why is it specifically related to panel C? Unclear.
Comment 17
Fig S7: Improve quality of pictures.
Are these CD20+, BTK+ structures the perivascular cuffs where immune cells get “trapped” during MS?
Comment 18
In page 20: More detail is needed on how the single cell data was used as an atlas from the “tri-culture system (reference 27)”.
Comment 19
In page 29 DISCUSSION: discussion lacks a small introductory paragraph for smooth transition to the discussion.
Comment 20
Fig S8: The proposed mechanisms figure lacks references. The data from this manuscript do not suport this figure in its entirety.
Comment 21
On the animal experiments: The sex of the animals is indicated, but no reasoning is given why only one sex is being tested. Could the authors elaborate on that?
There is no indication of the housing conditions of the mice, were they single or group housed? This could have implications on replicability of the study since single vs group housing can affect the results.
Comment 22
In page 31: “The differences observed in our study between the in vitro BTK-dependent transcriptional signature of human and mouse microglia highlight the need to study more complex human models.” This statement lacks more references and in-depth discussion. For example, differences in cell culture conditions could have stronger effects than species differences. Microglia change completely when cultured with and without fetal bovine serum, and iPSC cultures are usually defined medium (without FBS) https://doi.org/10.1016/j.neuron.2017.04.043.
Comment 23
On page 40 Transcriptomic signature: Not enough details were provided, and a preprint from 2021 is referenced here

Reviewer #4

(Remarks to the Author)
I this manuscript, Gruber et al aim to determine the impact of BTK inhibitors on the response of microglia to several models of neuroinflammation including EAE, cuprizone demyelination, and mouse and iPSC 2D and 3D cultures. Untangling the biology of BTK in microglia is relevant, as most current knowledge has explored it in B cells. Although the authors provide a comprehensive and logically structured series of experiments to prove that a brain penetrant BTK inhibitor can change microglia transcriptomes, there are a number of major points I think should be addressed:

- In the introduction, the authors mention that ‘microglia are identified histologically as CD68+ MHCII+ cells”. I am not convinced this is a complete definition of these cells, not even at histological level. I suggest rewording it and provide a more comprehensive definition.
- In the EAE model, it is difficult to separate what are the direct effects on microglia or indirect effects from modulating other immune aspects. I think these set of experiments would greatly benefit from adding a side-by-side non-brain penetrant compound to be able to dissect better the contribution of different compartments
- The authors try to partially address that by adding a cuprizone model of demyelination, however the comparison made between the models is very poor. What are the changes in common between these systems? What are the changes in the microglia that can be observed in both models and therefore confidently say that due to direct microglia modulation?
- In general, the study has been performed from bulk tissue. Although this could be interesting to assess overall changes in the tissue, it makes it hard to make a point of specific transcriptional regulation of microglia. If the authors want to specifically investigate those, at least some key experiments should be performed in sorted microglia.
- I did not find a histological assessment of the cuprizone model. Is the treatment modifying the kinetic of de- or remyelination?
- The observation of differences between mouse and human need to be further elaborated. The article tries to present a new therapeutic avenue, and the effects observed in human systems become relevant. What are the differences between species in the experiments performed, and what can we learn from them?
- The tricultures is certainly very interesting, and again there are clear differences between 2D and 3D systems when it comes to microglia. I think the aurhors should elaborate on this, and even put more emphasis on those systems they consider relevant. The authors should also perform treatments of cultures lacking microglia (only neurons and astrocytes) to differentiate between direct and indirect effects.
- In figure 2C the authors talk about differences between time points but there is no statistical significance provided. Also in figure 2G, the statistical tests are not clear to me? What test was performed? And what significance is displayed?

Minor comments
In the intro reads both Corpus callosum and corpora callosa

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I recogize that the authors have provided an additional analysis to measure the effects on specific cell types in the CNS using single nuclei RNA sequencing. As the time-point of intervention has not changed, this approach unfortunately fails to answer whether immune cell function is directly altered within the CNS. As stated in my previous review, the authors need to investigate the effect of tolebrutinib in two further EAE settings, namely in a) late-stage fully established EAE, and b) in adoptive transfer (passive) EAE, in which readily primed T cells only require reactivation in the CNS bypassing the periphery. Only a clinical and/or immunological effect in these settings would justify concluding that tolebrutinib exerts direct effect within the CNS.

Reviewer #2

(Remarks to the Author)
The authors have addressed my concerns, thank you.

Reviewer #3

(Remarks to the Author)
Gruber ad colleagues went at length to address the issues raised and added extensive new data and analyses. Key groups were added that solidify the conclusions drawn.
No further comments, a well performed and complete study.

Reviewer #4

(Remarks to the Author)
Thanks to the authors for the extensive revision. In general, all my concerns have been adequately addressed, with the exception of the two points below:

“In the EAE model, it is difficult to separate what are the direct effects on microglia or indirect effects from modulating other immune aspects. I think these set of experiments would greatly benefit from adding a side-by-side non-brain penetrant compound to be able to dissect better the contribution of different compartments. The authors try to partially address that by adding a cuprizone model of demyelination, however the comparison made between the models is very poor. What are the changes in common between these systems? What are the changes in the microglia that can be observed in both models and therefore confidently say that due to direct microglia modulation?”

What I meant here is that the effect of the inhibitors directly on the adaptive immune system at systemic level were not controlled. Although the authors provided RNA-seq evidence of transcriptomic effects in the microglia which are very interesting, my point stands that in the current setting, it could well be that the inhibitor is acting systemically, and only having an indirect effect on microglia. Could it be possible to perform at least one experiment with a non-brain penetrant molecule to be able to pull this confounding effects apart?

“In figure 2C the authors talk about differences between time points but there is no statistical significance provided”
I still cannot see the statistics in the figure provided

Comments on the new data provided
Figure 1G – The transcriptomic changes seem to be larger in the naïve than in the EAE mice. The authors should discuss the possibility that BTKi affect the CNS in the absence of pathology, and given that they propose it as a therapeutic tool, at least discuss what are the implications of the changes observed.
Figure 1I - The granulocyte degranulation term in pathways analysis is not very informative, given that the transcriptomes come from microglia, not granulocytes. I recommend the authors to look into what genes are in this pathway and perhaps assigned a different name that is more meaningful in this particular context.
Figure 1H – What is the relevance of the genes displayed and why were they selected? It could be relevant if the authors showed also the expression of genes from the homeostatic signature.
Figure 2G – It could be very relevant to adjust the genes displayed based on the sequencing in 1H, and find common hubs in both EAE and Cuprizone model

Minor
3I – not clear what the x-axis legend means

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors added very interesting mechanistic data, which improved the manuscript substantially. I still believe an interrvention strategy in established EAE or in passive EAE would have been the better strategy to prove a direct effect within the CNS, but must agree that the data provided get relatively close to the point requested.

Reviewer #4

(Remarks to the Author)
Thanks to the authors for such an extensive revision. I do not have any further comments and congratulate the authors for a nice piece of work.

Dear Reviewers

We would like to thank all the reviewers for going through our manuscript: NCOMMS-23-22421: "BTK regulates microglial function and neuroinflammation in multiple sclerosis". We were happy to see the positive comments about our study. Over the last few months, we have performed a range of new experiments to address the concerns of the reviewers. We kindly thank them for their insights. In particular, we have performed additional *in vivo* experiments, including an EAE study and a cuprizone study. Furthermore, we have examined the role of BTK on multiple cell types by doing single cell sequencing in the EAE model. Finally, based on the comments of the reviewers we have extensively updated methods section around the statistical methods used, as well as updated some of the analyses employed in this study to harmonize them across different datasets. We believe that these suggestions from the reviewers have helped us generate a more comprehensive data set that supports our key findings. Furthermore, these data help strengthen the key hypothesis of our study which highlights the importance of BTK a deleterious inflammatory mediator in innate immune cells, including macrophages and microglia. As detailed in the point-by-point responses, we have strengthened the impact and novelty of the manuscript by conducting more in-depth analyses.

Please find below a point-by-point response to each of the reviewer's comments. We sincerely appreciate your time and consideration in revisiting our manuscript and eagerly await your feedback.

Point by point response:

Reviewer comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors intended to test whether tolebrutinib, an inhibitor of the enzyme BTK may provide therapeutic benefit within the CNS by targeting selective aspects of adaptive and innate immunity-mediated disease progression. This question is of outmost importance as therapeutic approaches to control the progressive aspect of MS are currently missing.
The authors investigated the effect of tolebrutinib in development of MOG 35-55 induced EAE as well as in the Cuprizone model. Furthermore, they provide an extensive in vitro analysis on BTK-dependent

transcriptional signatures using their mouse microglia, induced pluripotent stem cell-derived human microglia, and a human iPSC tri-culture system composed of neurons, astrocytes, and microglia.

This is an interesting manuscript and the effect on microglia is promising. Specifically, BTKi is applied in MOG 35-55 EAE when the first clinical symptoms develop and accordingly at the max of CNS immune cell influx. One can easily envision that in this setting, a peripheral effect of tolebrutinib on myeloid cells and B cells may translate to an alteration of the invading immune cells (e.g. T cells), translating to an indirectly altered activation status of microglia. Unfortunately, the authors don't even control for these parameters. To show a direct effect of tolebrutinib within the CNS, the authors need to investigate its effect in two further EAE settings, namely in a) late-stage fully established EAE, and b) in adoptive transfer (passive) EAE, in which readily primed T cells only require reactivation in the CNS bypassing the periphery. Only a clinical and/or immunological effect in these settings would justify concluding that tolebrutinib exerts direct effect within the CNS.

The reviewer rightly points out that just from the results of the EAE study alone it is difficult to determine whether the effect of BTK inhibition on the severity of the model is through its effect on microglia directly or a secondary effect through infiltrating cells. The goal of this study was to show that BTK is expressed in immune cells and in particular macrophages and microglia in the CNS and that inhibition of this kinase modulates inflammatory responses. We have now clearly stated this throughout the text.

Furthermore, to better support this conclusion, we have performed an additional EAE experiment in which we measure the effects on specific cell types in the CNS using single nuclei RNA sequencing (**Fig. 1**). Using this approach, we were able to identify both microglia and macrophages in the CNS and show that BTK inhibition alters the transcriptional signature in both these cells within the CNS.

Similarly, the authors perform their cuprizone experiments in wild-type mice, in which a contribution of the peripheral immune system cannot be excluded (as they also state in their manuscript). To more conclusively demonstrate an effect independent of the peripheral immune system, the authors would need to perform their cuprizone approach in e.g. RAG-deficient mice.

In the results text we have reworded our introduction to the cuprizone mouse model, to avoid the insinuation that peripheral lymphocyte infiltration can be completely ruled out. While involvement of adaptive immunity is possible, it remains true that cuprizone model induction does not depend upon adaptive immunity. Therefore, there is relevance in employing the cuprizone model to accompany EAE experiments, for which we now present single-cell data.

The in vitro analyses are extensive and interesting as they show that, in case tolebrutinib reaches relevant concentrations within the CNS it may alter the activation status of these cells. along the same lines of the two further points above, all results vitally depend on this precondition. Accordingly, this point needs to be strengthened using e.g. an in vitro model of the blood-brain-barrier in combination with their in vitro microglia settings. Can they measure relevant concentrations of tolebrutinib within the CNS in a setting of an intract blood-brain-barrier?

In this revised manuscript we have included exposure data of the BTK inhibitor used in the *in vivo* studies (Fig. S11). We show that in mouse the molecule reaches pharmacologically relevant concentrations in the CNS.

In addition, we have recently submitted a manuscript comparing the activity and pharmacology of 3 different BTK inhibitors (Turner et al. Under Review; data summary below). A key finding in that study is that in both NHP and human studies tolebrutinib reaches pharmacologically relevant concentrations in the CSF.

[figure redacted]

PRN2675 dosing for in vivo studies was based on the observed brain penetrance and pharmacokinetics of this molecule (Fig. S11) and the efficacious dose observed in the tolebrutinib Ph2 clinical study (60 mg) (Reich et al 2021, Lancet Neurol). Based on simple allometric scaling, a dose of 60 mg in human translates to approximately 12.3 mg/kg in mouse (Nair and Jacob 2016, J Basic Clin Pharm). Tolebrutinib is about 40% more potent biochemically than the tool molecule, thus we chose the 15 mg/kg dose. Furthermore, based on the pharmacokinetics for the in vivo tool BTK inhibitor, the CSF concentration at 1hr (5 mg/kg) is in the ~2ng/mL (~4nM) range, which is similar to the concentration observed in the phase 1 study for tolebrutinib. The dose proportionality is semi-linear, so the exposure in CSF would be in the low double-digit nM range with the 15 mg/kg dose. Furthermore, based on CSF exposures for the tool molecule and clinical CSF data from a Ph1 study with tolebrutinib, we expect a similar level of free CNS exposure and BTK inhibition in mice dosed with PRN2675 at 15mg/kg once daily. Thus, for this irreversible inhibitor we predict achievement of near complete CNS target occupancy after several days of the 15 mg/kg dose. Additional data regarding modeling CNS BTKi with tolebrutinib can be found at https://www.biorxiv.org/content/10.1101/2024.03.25.586667v1. This rationale has now been described in the Materials and Methods.

References:

- A. B. Nair and S. Jacob, A simple practical guide for dose conversion between animals and human. J Basic Clin Pharm 7(2), 27-31 doi: 10.4103/0976-0105.177703
- D. S. Reich, D. L. Arnold, P. Vermersch, A. Bar-Or, R. J. Fox, A. Matta, T. Turner, E. Wallstrom, X. Zhang, M. Mares, F. A. Khabirov, A. Traboulsee, Tolebrutinib Phase 2b Study Group, Safety and efficacy of tolebrutinib, an oral brain-penetrant BTK inhibitor, in relapsing multiple sclerosis: a

phase 2b, randomised, double-blind, placebo-controlled trial. Lancet Neurol 20(9), 729-738 doi: 10.1016/S1474-4422(21)00237-4

Reviewer #2 (Remarks to the Author):

The authors reported that a CNS-penetrant BTK inhibitor (BTKi) ameliorated EAE correspondent with reduced microglia activity. BTKi also lowered MS-relevant, BTK-dependent transcriptional signatures in vitro of mouse microglia, iPSC-derived human microglia, and in a tri-culture of neurons, astrocytes and microglia. They demonstrated that in MS tissue BTK was expressed in microglial cells, with increased levels in MS lesions. These results are interesting and important, and relevant to targeting BTK in the CNS in MS. The following are points to be considered:

1. In Figure 1C, when the tissue was taken for bulk RNAseq should be indicated within the figure.

We have now included that information in the figure legend. "Spinal cord tissue was collected after 10 days of vehicle or BTK treatment and RNA-seq was performed," as well as in the main text.

One may get the wrong impression that this is data from preventative EAE, while this is impressively from therapeutic intervention (ie after clinical signs were detected).

We thank the reviewer for pointing this out. We have now clarified that the dosing was therapeutic in the main results section in addition to the figure legend.

Appreciating that this is not single-cell RNA sequencing, is there any capacity to differentiate the myeloid signal into monocyte-derived macrophages versus microglia? The separation of the effects of BTKi on microglia versus peripherally-derived macrophages would make a stronger case that the effects of BTKi in vivo is due to microglia versus macrophages, or not.

We agree that distinguishing the transcriptional effects in microglia versus other cell types is relevant to the conclusions drawn in this manuscript. We have therefore performed an additional EAE experiment in which we measure the effects on specific cell types in the CNS using single nuclei RNA sequencing (Fig. 1). In this manner, we have distinguished which transcriptional effects occur specifically within microglia, as opposed to monocyte-derived macrophages. In microglia, BTK inhibition induced a robust transcriptional response in both naive and EAE conditions. Moreover, we found that BTKi reversed the EAE-induced increase in multiple disease-associated microglial genes (Fig. 1H). These new data are now a key feature of this study.

2. In the cuprizone experiments of Figure 2, the authors stated that the effects of BTKi was on microglia when they are not in a position to differentiate microglia from monocyte-derived or CNS-intrinsic macrophages. This theme permeates through the in vivo experiments and should be corrected (ie leave open the possibility that the in vivo effects could be contributed also by macrophages as the markers do not differentiate the myeloid populations).

We thank the reviewer for this comment, and we fully agree a key component of the effect of BTK inhibition can also be on monocyte derived cells that have entered the CNS. We have altered the text in reference to the cuprizone experiments to highlight this point and to emphasize that the CNS penetrant

nature of a BTK inhibitor will allow for inhibition of microglia, monocyte-derived, and CNS-intrinsic macrophages. With respect to the EAE *in vivo* experiments, we have now employed single nuclei RNA sequencing, as described above, allowing us to distinguish effects on microglia.

3. The MS tissue results are particularly impressive. However, as with the mouse data, the authors did not discriminate between microglia and macrophages (IBa1+/CD68+ would imply that many of these are macrophages), so it would be fair to note the cells as 'microglia/macrophages' as opposed to only 'microglia'. Indeed, a BTKi working only in the periphery may potentially alter the properties of monocyte-derived macrophages when these enter the CNS.

We thank the reviewer for this comment and recognize that we cannot here discriminate between microglia and macrophages. We have therefore altered the relevant text in both results and discussion sections to reflect this point.

4. In Figure 2, when BTKi is initiated and for what period could be illustrated in a schematic. Any comments on whether remyelination was enhanced in the cuprizone model, especially since this is such a commonly used model for de- and remyelination? The brief statement in the Discussion is not adequate.

We thank the reviewer for this comment and have added a new figure in which we examine the role of BTK in remyelination. Our data show that acute BTK inhibition does not measurably affect remyelination in the cuprizone model. While 5 days of BTK inhibition is sufficient to alter cuprizone-induced transcriptional changes in the CNS (Fig. 2E), it is not sufficient to enhance remyelination (Fig. S3).

5. The basis of the dose of BTKi used in mice could be described. The correspondence of the mouse dose to that used in humans could be explained. Are the concentrations in serum at steady state similar between the mouse and available human studies at the murine (this paper) and human (in clinical trials) doses used? In other words, are the doses and concentrations of BTKi in this paper relevant to those obtained in MS patients in clinical trials?

PRN2675 dosing for in vivo studies was based on the observed brain penetrance and pharmacokinetics of this molecule (Fig. S11) and the efficacious dose observed in the tolebrutinib Ph2 clinical study (60 mg) (Reich et al 2021, Lancet Neurol). Based on simple allometric scaling, a dose of 60 mg in human translates to approximately 12.3 mg/kg in mouse (Nair and Jacob 2016, J Basic Clin Pharm). Tolebrutinib is about 40% more potent biochemically than the tool molecule, thus we chose the 15 mg/kg dose. Furthermore, based on the pharmacokinetics for the in vivo tool BTK inhibitor, the CSF concentration at 1hr (5 mg/kg) is in the ~2ng/mL (~4nM) range, which is similar to the concentration observed in the phase 1 study for tolebrutinib. The dose proportionality is semi-linear, so the exposure in CSF would be in the low double-digit nM range with the 15 mg/kg dose. Furthermore, based on CSF exposures for the tool molecule and clinical CSF data from a Ph1 study with tolebrutinib, we expect a similar level of free CNS exposure and BTK inhibition in mice dosed with PRN2675 at 15mg/kg once daily. Thus, for this irreversible inhibitor we predict achievement of near complete CNS target occupancy after several days of the 15 mg/kg dose. Additional data regarding modeling CNS BTKi with tolebrutinib can be found at https://www.biorxiv.org/content/10.1101/2024.03.25.586667v1. This rationale has now been described in the Materials and Methods.

References:

- A. B. Nair and S. Jacob, A simple practical guide for dose conversion between animals and human. J Basic Clin Pharm 7(2), 27-31 doi: 10.4103/0976-0105.177703
- D. S. Reich, D. L. Arnold, P. Vermersch, A. Bar-Or, R. J. Fox, A. Matta, T. Turner, E. Wallstrom, X. Zhang, M. Mares, F. A. Khabirov, A. Traboulsee, Tolebrutinib Phase 2b Study Group, Safety and efficacy of tolebrutinib, an oral brain-penetrant BTK inhibitor, in relapsing multiple sclerosis: a phase 2b, randomised, double-blind, placebo-controlled trial. Lancet Neurol 20(9), 729-738 doi: 10.1016/S1474-4422(21)00237-4

6. Last line of page 3: not sure what 'tool compound' means

We have removed the term "tool compound". Instead, we specify that in this manuscript both tolebrutinib and PRN2675 were employed to achieve BTK inhibition, where PRN2675 differs from tolebrutinib by a single fluorine substitution for a hydrogen near the reactive moiety. While *in vitro* experiments were performed using both tolebrutinib and PRN2675, PRN2675 was used for all *in vivo* experiments, due to ongoing tolebrutinib Phase 3 clinical trials. To rectify any further confusion, we now refer to PRN2675 and tolebrutinib directly in the figure legends and text, instead of simply BTKi.

7. The 'complexed immunoglobulin G (IgG), which is known to engage FcR that couples with BTK' is a sophisticated and physiological method to activate BTK in microglia. Can the authors provide more details in the Methods of this protocol, as many labs would wish to simulate this mode of activation? Any extra notes besides what is described? Was the same reagent (IgG complex from mouse serum) and protocol used for human and mouse cells?

We thank the reviewer for pointing out this opportunity for added clarification. IgG complexed from mouse serum was only applied to murine cells. The complexed IgG applied to human cells was Fc OxyBURST™ (10 µg/mL; Invitrogen, F2902), which is rabbit polyclonal anti-BSA antibody complexed with BSA. For clarity, we have now specified in every figure legend whether complexed *mouse* IgG was employed or complexed IgG in the form of Fc OxyBURST™. We have also stated in the Materials and Methods section that complexed IgG from mouse serum was only applied to murine cells and that experiments with human cells were performed with Fc OxyBURST™.

We have updated the Materials and Methods section with additional details related to the complexed mouse IgG employed here. Specifically, we now state that cells were treated with 400 µg/mL of the complexed mouse IgG stock we generated for 15 minutes at 37°C.

As an aside, does BTKi inhibit TLR signaling (eg by LPS, commonly used in many labs to activate microglia)?

We do not believe that BTKi inhibits TLR signaling. In the iPSC-derived human microglia employed in this manuscript, we observe that tolebrutinib blocks LPS + nigericin mediated IL-1β release only at micromolar concentrations (well above the expected effective concentration range). This data is summarized below, in panel A (note that NLRP3 inhibitor MCC950 is a positive control for inhibition). We therefore believe this to be an off-target effect. This conclusion is broadly supported by existing literature, where blockage of LPS-mediated IL-1β release is consistently demonstrated at elevated BTK inhibitor concentrations. In fact, as shown below in panel B, Mao et al demonstrated that the BTK inhibitor ibrutinib could effectively block mouse IL-1β secretion in BTK knock-out animals. Again, at concentrations above the expected effective range (Ibrutinib has a reported IC50 of 0.5 nM).

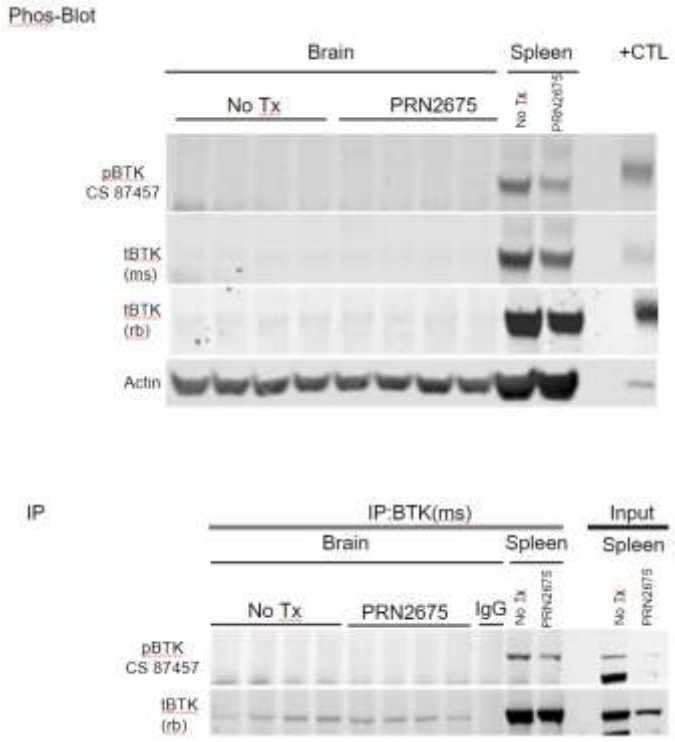
[figure redacted]

8. Figure 3 shows BTKi preventing the IgG activation of microglia. The BTKi was applied before the stimulus. In MS brains, there would already be substantial BTK activation in microglia in plaques. Does the BTKi reduce the activation transcripts when BTKi is applied after cells have been stimulated with IgG?

To address this question in as functionally relevant a manner as possible, we repeated the experiment outlined in Fig. 5C-D, with an altered design. In cultures of iPSC-derived microglia we introduced an IgG stimulus for 24 hours, removed the cell culture media and quickly washed the cells, and then re-applied IgG alongside BTKi for another 24 hours. Chemokine and cytokine secretion was then assessed using multiplex panels. We find that application of BTKi 24 hours after the introduction of IgG stimulus profoundly reverses the stimulated chemokine and cytokine secretion profile (Fig. S6).

9. Perhaps the authors could explain why only BTK (and not pBTK) antibody was used in in vivo experiments to characterize BTK, as Figure 3 (of pBTK and BTK) clearly shows that pBTK but not BTK informs on activity.

Unfortunately, we were unable to detect phosphorylated BTK in brain tissue by both immunostaining and Western blot. We believe this is both because the levels of BTK are low and the sensitivity of the commercially available phospho-antibodies. This is exampled in the figure below, in which we can detect low levels of BTK in the brain as compared to spleen. Furthermore, we were unable to detect pBTK in healthy mouse brain, via total brain homogenate or immunoprecipitated total BTK. Therefore, such data was not included in the manuscript, but the presence of total BTK in the human brain and the increase with disease suggest that there is activation, which is potentially transient that occurs *in vivo*.



10. The data of Fig S3B that BTKi reduces Rgs1 of naïve brain is confusing. While preceding results suggest that BTKi reduces activated myeloid cells back to near baseline level, the data of Fig S3B would suggest that normal microglia is also inhibited to below basal level?

We thank the reviewer for this question and point out that these results are not in opposition to one another. There remains the possibility that *Rgs1* expression in mouse microglia is IgG enhanced via multiple mechanisms beyond those which are BTK-dependent. Thus, BTKi treatment of IgG stimulated microglia may only reduce *Rgs1* expression back to baseline, while treatment of unstimulated cells may

reduce expression below baseline. This is consistent with our in vitro data where baseline BTK phosphorylation can be rapidly reduced following inhibitor treatment.

11. The last paragraph of Introduction summarizes the results of this paper, which are largely contained within the preceding abstract, and detailed in the proceeding Results section. Perhaps that paragraph in the Introduction could be substantially truncated to introduce the intent of this paper rather than to summarize the results.

We thank the reviewer for this comment and have revised and truncated the last paragraph of the introduction.

12. Consider removing the triculture data. What is the composition of each of the cell subsets – how representative of that in the brain or in lesions? The schematic of Figure 5A (of the cell types) is confusing – momentarily, one gets the impression that these cells are studied in isolation. Basal response to tolebrutinib (or PRN?) in absence of IgG seems to be exaggerated compared to vehicle in absence of IgG. That BTK is only on microglia should be quantitated (doing cell counts). The inference that BTKi affects microglia that then changes neurons or astrocytes is speculative. Thus, the triculture data is equivocal.

BTK average expression had been quantitated in the microglia, astrocytes, and neurons that comprise this tri-culture (Fig. 5H), however, as requested by the reviewer, we have now included in Fig. 5H information related to the percentage of cells in which BTK transcripts were identified. For added clarity we have also added this information to the figure legend (BTK transcripts identified in 30.5, 0.9, and 0.9 percent of microglia, astrocytes, and neurons, respectively, with Seurat normalized average expression levels of 0.376, 0.004, and 0.007).

These values highlight our finding that in this tri-culture system, there is little-to-no expression of BTK in neurons and astrocytes. This strongly supports our hypothesis that BTK inhibition influences neuronal and astroglial signaling indirectly via microglia, consistent with the highly integrative nature of these cell types in the CNS, including in the context of neuroinflammation. Indeed, this same tri-culture system has been employed in prior work to demonstrate a role for microglia in iron-induced neurotoxicity, exampling the utility of this system in untangling non-cell autonomous disease-relevant effects (Ryan et al 2023, Nat Neurosci).

In further support of our hypothesis that BTK inhibition influences neuronal and astroglial signaling in these tri-cultures indirectly via microglia, we have repeated the experiment described in Fig. 5C-D, this time quantifying chemokine and cytokine secretion in cultures with and without microglia (i.e. only neurons and astrocytes) (Fig. S8). Broadly, we see minimal effects from either IgG stimulation or BTK inhibition in cultures lacking microglia.

Irrespective of non-cell autonomous effects on neuronal and astroglial signaling, the presence of these additional cell-types may modulate the basal microglial gene expression profile, function, and responsiveness to immunological stimuli and BTK inhibition, as evidenced in the following reference provided by Reviewer #3 (Bohlen et al 2017, Neuron).

As described in the Materials and Methods (Human *in vitro* microglia culture and tri-culture cell culture procedures) astrocytes, neurons, and microglia were seeded at 1.5×10^4, 3.5×10^4, and 2.0×10^4 cells/well, respectively. This yields cultures that consist of approximately 60% astrocytes, 25% neurons,

and 15% microglia, as described in a textually referenced publication (Citation#28, Ryan et al 2023, Nat Neurosci). These cell densities were established during efforts to optimize the health of our tri-cultures.

Other groups have arrived at similar ratios, when generating triple co-culture systems. For example, Luchena et al (2022) generated a system consisting of a 2:5:1 astrocyte, neuron, microglia plating ratio, which is quite similar to ours (1.5×10^4, 3.5×10^4, and 2.0×10^4 cells/well). Meanwhile, the cellular composition of the human brain appears to be somewhat controversial and to vary by region.

Finally, as also requested, we have rearranged the schematic of Fig. 5A to clarify that these cells are studied together in co-culture.

References:

- S. K. Ryan, M. Zelic, Y. Han, E. Teeple, L. Chen, M. Sadeghi, S. Shankara, L. Guo, C. Li, F. Pontarelli, E. H. Jensen, A. L. Comer, D. Kumar, M. Zhang, J. Gans, B. Zhang, J. D. Proto, J. Saleh, J. C. Dodge, V. Savova, D. Rajpal, D. Ofengeim, T. R. Hammond, Microglia ferroptosis is regulated by SEC24B and contributes to neurodegeneration. Nat. Neurosci. 26, 12-26 (2023). doi: 10.1038/s41593-022-01221-3
- C. J. Bohlen, F. C. Bennett, A. F. Tucker, H. Y. Collins, S. B. Mulinyawe, B. A. Barres, Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures. Neuron. 94(4), 759-773 (2017). doi: 10.1016/j.neuron.2017.04.043
- C. Luchena, J. Zuazo-Ibarra, J. Valerio, C. Matute, E. Alberdi, E. Capetillo-Zarate, A Neuron, Microglia, and Astrocyte Triple Co-culture Model to Study Alzheimer's Disease. Front. Aging. Neurosci. 14:844534 (2022). doi: 10.3389/fnagi.2022.844534

13. This reviewer is of the opinion that the 'BTKi' label in figures and much of the text should be changed to the drug name, PRN2675 or just PRN. 'BTKi' infers that all available BTK inhibitors would have the same results but this may not be the case as there is a distinct effort in this field to separate BTKi's into those that are covalent inhibitors or not, and those with better selectivity to BTK versus other kinases. Note that 'tolebrutinib' was used to describe some of the data (eg page 12) – was tolebrutinib used or its analog PRN2675? If some experiments involved PRN while others tolebrutinib, this needs to be clarified. While PRN2675 has a 'single fluorine substitution for a hydrogen near the reactive moiety', this substitution is not trivial. As well, in Discussion, page 31, the authors state 'These data strongly support the concept that tolebrutinib is a potent regulator of neuroinflammation in vivo.' Yet, unless this reviewer is mistaken or the description of the text is unclear, tolebrutinib was not used in this paper.

We thank the reviewer for this comment. In this manuscript, both tolebrutinib and PRN2675 were employed to achieve BTK inhibition. While *in vitro* experiments were performed using tolebrutinib and PRN2675, only PRN2675 was used for *in vivo* experiments, due to ongoing tolebrutinib Phase 3 clinical trials.

To rectify any confusion, we now directly refer to PRN2675 and tolebrutinib in the Results text and figure legends, instead of simply BTKi.

14. Figure S8 should be deleted as much of the schematic is not addressed by the results here. Also, it appears to be an advertisement for tolebrutinib, the authors' company.

To alleviate concerns about Fig. S8 (which is now Fig. S10) we have added to the figure legend citations for what our manuscript does not show but is in the literature, as suggested by Reviewer 3. We have also replaced all mentions of tolebrutinib in the figure with "BTKi." If these changes do not satisfy the reviewers, we will remove the figure in its entirety.

Reviewer #3 (Remarks to the Author):

The authors show that BTKi PRN2675 (a tolebrutinib derivative) can ameliorate EAE in a therapeutic regimen (after symptom onset). They further show that this BTKi can modulate the inflammatory response consistent with microglia signature in the Cuprizone model and also in cell culture. In the cell culture, a tri culture model was used the reveal modulation of specific pathways when the 3 cells are cultured together. The use of human iPSC is a plus.
BTKis are extensively studies in MS, including clinical trials, which is also the case for tolebrutinib. Here, the effects of BTKi PRN2675 in two common MS mouse models (EAE&Cuprizone) are investigated, complemented with experiments in primary microglia/hiPSC-microglia.

Does the work support the conclusions and claims, or is additional evidence needed? *Study design, data analyses and methodology need major improvements. Key groups are missing: in almost all experiments, a control+BTKi group is missing.*

We thank the reviewer for this suggestion and have now included the control+BTKi group in all the *in vivo* experiments.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
Yes, for details, please see coments below. Major issues are differential expression (DE) analysis, including definition of DE genes (DEGs) and statistical tests used. It is unclear which statistical tests were used for each figure and whether they are appropriate for the data and experimental design in question.

In every relevant figure legend, we now include a definition of DEGs and also the statistical approaches employed.

Largely, 4 kinds of experiments are performed, EAE, cuprizone, microglia, CNS-cell coculture. These 4 data sets are not, or poorly integrated. How do the data obtained in these 4 experiments groups integrate, for instance gene sets are not compared or integrated, in general the manuscript lacks somewhat in narrative.

We thank the reviewer for their feedback. To better integrate the datasets presented in this manuscript, we have added four-way plots which directly compare differential gene expression results across datasets. In doing so, we highlight the genes which are commonly differentially expressed across models and especially likely to be modulated by BTKi directly in microglia and to be relevant to pathological neuroinflammation. Specifically, we compare the following:

- Fig. 3I – EAE pseudo-bulk microglia differential expression results for EAE + PRN2675 vs EAE + vehicle (Fig. 1G, right) and *in vitro* mouse microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 3G).
- Fig. 4E - human iPSC microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 4B) and *in vitro* mouse microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 3G).
- Fig. 5E – human iPSC triculture differential expression results for IgG + tolebrutinib vs. IgG (Fig. 5B) and human iPSC microglia monoculture differential expression results for IgG + tolebrutinib vs. IgG (Fig. 4B).

These raw data will also be provided, so that others may pursue additional inquiries.

Bulk RAseq is typically used. In EAE and cuprizone, cellular compositions of the tissue will change: loss of oligo's, influx of immune cells, microglia proliferation etc etc. This has major implications on RNAseq analysis, data interpretation, the DEGs to be detected/expected. No deconvolution efforts were made to assess effects of treatments on cellular composition. All in all, aside from the methodological and statistical issues, this hampers data interpretation.

We thank the reviewer for this comment and agree regarding the limitations and considerations necessary in interpreting bulk RNA sequencing data. To enhance our insight into differential gene expression, we have performed an additional EAE experiment in which we employ single nuclei RNA sequencing to measure effects within specific cell types (Fig. 1). In this manner, we have distinguished which transcriptional effects occur specifically within microglia, as opposed to monocyte-derived macrophages and intruding adaptive immune cells. In microglia, BTK inhibition induced a robust transcriptional response in both naive and EAE conditions. Moreover, we found that BTKi reversed the EAE-induced increase in multiple disease-associated microglial genes (Fig. 1H). As the reviewer suggested, EAE disease induction elicited a change in CNS cellular composition, which included an expansion in microglia, macrophage, and T-cell populations. Therapeutic BTK inhibition, however, did not notably affect the composition of these cell types.

No validations on the effectivity of EAE/cup are offered, no IHC for lesion formation, effective de- and remyelination etc.

With respect to the EAE model, we point to Fig. 1A, in which we demonstrate clinical symptoms consistent with successful disease induction. These symptoms are ameliorated by BTKi. In Fig. 1B, we further quantify plasma Neurofilament heavy chain (NfH), demonstrating elevated NfH levels which are ameliorated by BTKi. To confirm effectivity of cuprizone treatment, we have added a new figure in which we confirm demyelination in the disease model. Here, we also examine whether acute BTK inhibition measurably enhances remyelination (Fig. S3).

Is the methodology sound? Does the work meet the expected standards in your field? *Negative points: potentially serious methodological and statistical problems. Missing experimental group for EAE experiment (BTKi only, without EAE). Moreover, they do not show whether BTKi treatment improves remyelination in the cuprizone model (this fact is briefly mentioned as a future perspective during the discussion) Positive points: tissue dissection in the cuprizone experiment (for corpus callosum only). Try to correlate results with available single cell data.*

We have performed an additional EAE experiment, employing single nuclei RNA sequencing, in which we now include a non-EAE with BTKi group (Fig. 1D-I). Moreover, in the newly added remyelination experiment described below, we also include a non-cuprizone with BTKi group (Fig. S3).

Regarding the cuprizone model, we have added a new figure in which we examine the role of BTK in remyelination. Our data show that acute BTK inhibition does not measurably affect remyelination following cuprizone administration. While 5 days of BTK inhibition is sufficient to alter cuprizone-induced transcriptional changes in the CNS (Fig. 2E), a comparable 4 days does not measurably enhance remyelination (Fig. S3).

Is there enough detail provided in the methods for the work to be reproduced?
Not enough detail on key parts of methodology: statistical analyses, RNA-seq analyses and cell culture of cell lines. Which cell lines were used exactly and how were they obtained, cultured oetc?

We thank the reviewer for the following comments and have addressed each, as described below, including information regarding statistical analyses, RNA-seq analyses, and cell lines.

Below a list of more specific comments:
Comment 1
Regarding all the results: statitstic tests are not clear. For each figure it should be clear which statistical test was used. The statistsical tests used are only mentioned in the methodology section and have no specific reference to which figure/results they refer too. Unclear. For more specific concerns about statistic, see comments below.

As stated in our response to this reviewer's earlier comment, we now include in every relevant figure legend a definition of DEGs and also the statistical approaches employed. Responses to more specific concerns have been provided below.

Comment 2
Regarding results from Figure 1E 1F, 2C 2G 2H, S2, 3J, 4D 4F: Normalized counts shouldn't be analyzed standalone, but DEseq2 differential analysis should reveal which genes are differentially expressed based on their model (and p value and logFC requirements). It is possible to set different contrasts and analyze interactions within DEseq2 pipeline, see
http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#differential-expression-analysis and
https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html#differential-expression-analysis for more information. Analyzing normalized counts apart from the differential expression analyzes,with ANOVA and T-tests are not appropriate because the p-value needs to be adjusted by all the comparisons and genes being tested and the nature of RNA-seq data, and DEseq2 (or other differential expression pipelines such as EdgeR/Limma/Voom) have models that account for that. Using normalized counts and analyze genes as qPCR proxy is not appropriate. Just use the p-value given by results() function in the DESeq2.
EdgeR/Limma/Voom method:
Law CW, Alhamdoosh M, Su S et al. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR

[version 3; peer review: 3 approved]. F1000Research 2018, 5:1408 (https://doi.org/10.12688/f1000research.9005.3) https://master.bioconductor.org/packages/release/workflows/vignettes/RNAseq123/inst/doc/limmaWorkflow.html

We thank the reviewer for this comment and agree that the bulk RNA-sequencing analysis details needed further clarification. To address this comment, in each figure caption, we have provided information on how the RNA-seq data was quantified (i.e., DESeq2 normalized counts or log2(FPKM+1)), how p-values were calculated (i.e., with DESeq2 or with a general linear model in Array Studio), and the fold-change and p-value thresholds used to determine significantly differentially expressed genes.

Comment 3

Regarding results from differential expression analyses and downstream analyses and all volcano plots: The definition of DEGs does not seem to be appropriate, based on the volcano plot. Page 35 (methods section): "(…) DEGs were determined using a 1.5-fold-change (FC) threshold and false discovery rate (FDR)-corrected p-value cut-off of 0.05."
For that you should make use of the function results() in DEseq2 and specify lfcThreshold and altHypothesis arguments (building results table section at DEseq2 vignette (Bioconductor) https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html #building-the-results-table and "Tests of log2 fold change above or below a threshold"section at http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#:~:text=7401 980 8381-,Tests of log2 fold change above or below a threshold,-It is also). Another example of properly tested DEG and corresponding volcano plot (https://www.nature.com/articles/s42003-023-04926-8) based on the TREAT method (https://doi.org/10.1093/bioinformatics/btp053 "Testing significance relative to a fold-change threshold is a TREAT" Davis J. McCarthy, Gordon K. Smyth) used in EdgeR/Limma/Voom pipeline.

As mentioned above, in each figure caption, we have now provided information on how the RNA-seq data was quantified (i.e., DESeq2 normalized counts or log2(FPKM+1)), how p-values were calculated (i.e., with DESeq2 or with a general linear model in Array Studio), and the fold-change and p-value thresholds used to determine significantly differentially expressed genes.

Comment 4

Microscopy images and pictures: need to be of better quality (increase DPI/PPI resolution).

We thank the reviewer for drawing this to our attention. We have now provided higher resolution images for this manuscript.

Comment 5

Ingenuity Pathway Analysis (IPA) figures: Figures lack clear indication of which experimental groups are being compared for the analysis (clear contrast is missing for some figure legends). For readability, please add this contrast as a title (e.g. BTKi vs vehicle). Also, not all figures indicate whether it is an enriched or downregulated pathway.

All Ingenuity Pathway Analysis (IPA) figures are now appropriately titled, with the specific experimental groups being compared. They now also indicate whether each pathway is predicted to be activated or inhibited. All EnrichR pathway analysis figures (i.e. Fig. 2F) also specify compared groups; however, it

should be noted that pathway analyses performed with EnrichR do not generate predictions regarding pathway activation or inhibition.

Comment 6
Comparing differential expressed genes Fig 4E Fig 5E: Venn diagrams result in "binary" data (yes or no categories). 4-way plots to correlate the log fold change of differentially expressed genes and to really show how similar or different (logFC = continuous value) the DEGs are in these venn diagrams is much more informative.

We agree with the reviewer that 4-way plots are more informative to the reader. As requested, we have replaced both Fig. 4E and Fig. 5E with such 4-way plots.

Comment 7
In page 3: why are there double quotes around "signature"?

As suggested, we have removed the double quotation marks around "signature".

Comment 8
In page 4: "The increased BTK gene expression after EAE induction was independent of B-cell-associated transcripts in the tissue, as assessed by Cd19 expression levels…" Cd19 is only one transcript and not transcripts? Or are the authors referring to 'transcripts' as mRNA molecules and not 'genes'? Is there another marker that could corroborate Cd19, maybe Cd20 and other lymphoid-lineage markers?

We have rewritten this portion of the results to discuss a newly added single nucleus RNA sequencing study, performed with EAE mouse spinal cord. The grammatical inconsistency referenced by the reviewer therefore no longer appears in the text.

In this new single nucleus RNA sequencing dataset, we did not identify any B-cell populations. This is consistent with the earlier bulk RNA sequencing dataset (Fig. S1C), in which we showed that *Cd19* transcripts were not increased with EAE induction.

To further corroborate *Cd19*, as suggested by the reviewer, we have now included a additional B-cell markers *Cd20, Cd40, and Cd80* from the bulk RNA sequencing experiment (Fig. S1C).

Comment 9
In Figure 1:
1) What is the effect of BTKi alone, without EAE induction? A control group with only BTKi is missing to assess whether BTKi has an effect or whether its effects are only present when EAE is induced (interaction). Can't the users make use of the data from the Figure 2 (cuprizone experiment), there you have all 4 groups (including BTKi alone).
2) IPA results are not clear, are these pathways enriched or depleted? From which differential expression comparison? Which groups? For clarity add a title to figure 1 D of which specific comparison and whether the pathways are enriched or downregulated.
3) What determines the order of genes (rows) from panel C? Are genes from C also clustered or only clustered based on samples (supervised or unsupervised clustering?).

1) We have performed an additional EAE experiment, employing single nuclei RNA sequencing, in which we now include a non-EAE with BTKi group (Fig. 1D-I).

2) As described above in our answer to this reviewer's Comment 5, all Ingenuity Pathway Analysis (IPA) figures are now appropriately titled, with the specific experimental groups being compared, and also indicate whether each pathway is predicted to be activated or inhibited.
3) Regarding the order of genes (rows) from panel C, we now specify in the Materials and Methods section that this was determined by unsupervised hierarchical clustering, performed in RStudio (R 4.0.2) with the pheatmap package (version 1.0.12), using Euclidean distance and complete linkage.

Comment 10
In Figure S1:
1) Not clear for panel C which genes were used (only downregulated, only upregulated, all DEGs from which comparison?).

We have chosen to remove Fig. S1C, as it is now redundant to Fig. S1B in which we employ Ingenuity Pathway Analysis (IPA). In the legend for Fig. S1, we now specify which genes were used.

Comment 11
In Figure 2:
Fig 2B) What is Digital signal? A bit more details are appreciated on the methodology based on HALO software.

We thank the reviewer for pointing out this confusion. The digital signal is simply the immunohistochemical signal measured by the HALO software for each image, which is then overlayed back over the original image. This clarification has been added to the Immunohistochemistry section within the Materials and Methods.

Reproducibility issues: Which bregma point of the brain is being analyzed? The brain region heterogeneity is particularly important for the corpus callosum during the cuprizone model. There seems to be a lot of microgliosis, is this comparable to other cuprizone studies? There are different extents of demyelination and remyelination and potentially also microgliosis depending on the bregma point analyzed. See references:
Zhan J, Mann T, Joost S, Behrangi N, Frank M, Kipp M. The Cuprizone Model: Dos and Do Nots. Cells. 2020;9(4). doi:10.3390/cells9040843
Steelman AJ, Thompson JP, Li J. Demyelination and remyelination in anatomically distinct regions of the corpus callosum following cuprizone intoxication. Neurosci Res. 2012;72(1):32-42. doi:10.1016/j.neures.2011.10.002
Xie M, Tobin JE, Budde MD, et al. Rostrocaudal analysis of corpus callosum demyelination and axon damage across disease stages refines diffusion tensor imaging correlations with pathological features. J Neuropathol Exp Neurol. 2010;69(7):704-716. doi:10.1097/NEN.0b013e3181e3de90

We thank the reviewer for pointing out this detail. We now specify in the Materials and Methods section that brains from the cuprizone experiment (Fig. 2B) were dissected coronally between Bregma –1.0 and –1.7 mm. As the reviewer points out, the consistency and extent of demyelination and microgliosis within the corpus collosum are brain region-dependent. The study provided by the reviewer - Steelman et al. (2012) - concluded that the callosum caudal to –0.5 mm Bregma demonstrated nearly complete demyelination. Therefore we would expect prominent demyelination within the region examined in our study. Steelman et al. also concluded that microgliosis correlated with demyelination. In examining Figure

4 of their study, the extent of microgliosis they observe does seem consistent with what we report in our Fig. 2B.

Fig 2D) Some BTK positive cells on the right panel (Cuprizone 5W) do not seem to be IBA1 positive. Any idea why is that?

Regarding Fig. 2D, there are instances of imperfect overlap between the the IBA1 and BTK signals, despite clearly co-occurring within the same cell. This could be due to slight differences in subcellular localization of the proteins. This situation is exampled in Fig. S9, where the labeling of sparsely distributed CD68 and BTK positive cells makes clear that cells are co-labelled, despite subcellular regions of non-overlapping signal.

Comment 12
In Fig 3: are there statistically significant differences between the protein in the western blots? Missing quantification panels. Is panel F the quantification of panel E? That is not evident, please clarify in figure legend.

The Western blots displayed in subpanels A, C, and E are intended to be visual representations only. Statistical significance cannot be meaningfully calculated in subpanels A and E, as only one replicate is shown for several conditions. These Western blot images are provided specifically to support the dose-response ELISA quantifications (subpanels B and D) and Western blot-quantified effects of IgG +/- BTKi (subpanel F).

As the reviewer points out, the Western blot image shown in subpanel E displays one datapoint per condition, which are used in the subpanel F quantification. To alleviate confusion, we have now specified in the Fig. 3 legend that subpanel F is a Western blot quantification of the effects exampled in subpanel E.

Fig 3I, there is no indication of statistical significance. What was the statistical test? what are the statistically significant differences?
Fig 3H, again, as for other IPA plots, not clear which groups are being compared here. Please add a title to the figure with the correct contrast (e.g. IgG + tolebrutinib vs. IgG)

We have opted to remove Fig. 3I, to focus on the genes shown in Fig. 3J. The full dataset will be made available in the supplement. We have replaced Fig. 3I with a four-way plot, comparing EAE pseudo-bulk microglia differential gene expression results (Fig. 1G, right) with *in vitro* mouse microglia differential gene expression results (Fig. 3G).

Regarding Fig. 3H, as requested, all Ingenuity Pathway Analysis (IPA) figures are now appropriately titled with the specific experimental groups being compared.

Comment 13
In page 15 and methodology in general: add which mouse microglia cell line was used. There is no description on the cell line used and how these cells were obtained and cultured.

We thank the reviewer for pointing out this oversight. In both the Figure 3 legend and Materials and Methods sections, we now specify that the BV-2 mouse microglia cell line was employed. We also include sourcing and culturing information in the Materials and Methods section.

Comment 14
Similarly, in page 17 and methodolgy in general, please add information on the cell line used. How were THP1 obtained, cultured?

The thank the reviewer for pointing out this oversight. We have now specified in the Fig. S7 legend and Materials and Methods section that the THP1-Lucia NF-κB monocytic cell line (Invivogen) was used. We also include sourcing and culturing information in the Materials and Methods section.

Comment 15
In page 17 a grammar issue: "These data indicate that BTK activity can directly regulate the microglial transcriptional response in both mice and humans and potentially regulate the function of these cells." I believe it is missing a word after "mice and humans". Probably "cells" or "microglia"? Because "mice and human" are not "these cells".

The reviewer is correct that a grammatical mistake was made here. We have correctly specified "mouse and human microglia."

Comment 16
Fig 4: in panel C, why is there a cartoon of microglia "reactive" morphology? That is not described anywhere in the text or figure legend. Why is it specifically related to panel C? Unclear.

We have removed the word "reactive" from the figure, leaving the microglial descriptor simply as "inflammatory," which relates to panel C.

Comment 17
Fig S7: Improve quality of pictures.
Are these CD20+, BTK+ structures the perivascular cuffs where immune cells get "trapped" during MS?

The BTK positive CD20/B-cells were present almost exclusively in the perivascular space in progressive MS patient tissue. We have now stated this in the legend. We have also provided higher resolution images for the manuscript.

Comment 18
In page 20: More detail is needed on how the single cell data was used as an atlas from the "tri-culture system (reference 27)".

To clarify, we are referencing prior work in which a single cell atlas was derived from this tri-culture system (i.e. reference 27). We have modified the relevant sentence, to further specify that we are referencing prior work.

Comment 19
In page 29 DISCUSSION: discussion lacks a small introductory paragraph for smooth transition to the discussion.

We thank the reviewer for their comment and have added to the first paragraph of the Discussion, to facilitate a smoother transition.

Comment 20
Fig S8: The proposed mechanisms figure lacks references. The data from this manuscript do not suport this figure in its entirety.

We have now added to the figure legend citations for what our manuscript does not show but is in the literature. We have also replaced all mentions of tolebrutinib in the figure with "BTKi." If these changes do not satisfy the reviewers, we will remove the figure in its entirety.

Comment 21
On the animal experiments: The sex of the animals is indicated, but no reasoning is given why only one sex is being tested. Could the authors elaborate on that?
There is no indication of the housing conditions of the mice, were they single or group housed? This could have implications on replicability of the study since single vs group housing can affect the results.

We have now specified that mice were group housed, in the Materials and Methods within the EAE and cuprizone model induction sections. As now stated in the methods, female mice were employed to allow for group housing throughout the study. Also for EAE studies, female mice are the ones that are typically used to elicit robust and disease.

Comment 22
In page 31: "The differences observed in our study between the in vitro BTK-dependent transcriptional signature of human and mouse microglia highlight the need to study more complex human models." This statement lacks more references and in-depth discussion. For example, differences in cell culture conditions could have stronger effects than species differences. Microglia change completely when cultured with and without fetal bovine serum, and iPSC cultures are usually defined medium (without FBS) https://doi.org/10.1016/j.neuron.2017.04.043.

We thank the reviewer for their comment and provided reference. We have expanded the discussion on this topic, referencing the above study.

Comment 23
On page 40 Transcriptomic signature: Not enough details were provided, and a preprint from 2021 is referenced here

We have now included the protocol for the generation of the human bulk RNA-seq data set in which we performed Hierarchical clustering (63 genes) of PMS patient lesion signature from that in NAWM using the BTK-dependent signature identified in human microglia *in vitro.* This was first shared in the referenced Biorxv study: Proto *et al.* 2021

Reviewer #4 (Remarks to the Author):

In this manuscript, Gruber et al aim to determine the impact of BTK inhibitors on the response of microglia to several models of neuroinflammation including EAE, cuprizone demyelination, and mouse and iPSC 2D and 3D cultures. Untangling the biology of BTK in microglia is relevant, as most current knowledge has explored it in B cells. Although the authors provide a comprehensive and logically structured series of experiments to prove that a brain penetrant BTK inhibitor can change microglia transcriptomes, there are a number of major points I think should be addressed:

- In the introduction, the authors mention that 'microglia are identified histologically as CD68+ MHCII+ cells". I am not convinced this is a complete definition of these cells, not even at histological level. I suggest rewording it and provide a more comprehensive definition.

The reviewer rightly points out the challenge of differentiating resident vs. infiltrating phagocytes in the CNS. The conclusions of this study apply to both cells, so we have reworded this sentence in the introduction to include both types of cells that also express BTK.

- In the EAE model, it is difficult to separate what are the direct effects on microglia or indirect effects from modulating other immune aspects. I think these set of experiments would greatly benefit from adding a side-by-side non-brain penetrant compound to be able to dissect better the contribution of different compartments. The authors try to partially address that by adding a cuprizone model of demyelination, however the comparison made between the models is very poor. What are the changes in common between these systems? What are the changes in the microglia that can be observed in both models and therefore confidently say that due to direct microglia modulation?

We agree that distinguishing the transcriptional effects in microglia versus other cell types is relevant to the conclusions drawn in this manuscript. We have therefore performed an additional EAE experiment in which we measure the effects on specific cell types in the CNS using single nuclei RNA sequencing (Fig. 1). In this manner, we have distinguished which transcriptional effects occur specifically within microglia, as opposed to monocyte-derived macrophages and intruding adaptive immune cells. In microglia, BTK inhibition induced a robust transcriptional response in both naive and EAE conditions. Moreover, we found that BTKi reversed the EAE-induced increase in multiple disease-associated microglial genes (Fig. 1H).

We have also added a four-way plot which directly compares differential gene expression results across *in vivo* and *in vitro* microglial populations. Specifically, in Fig. 3I, we compare EAE pseudo-bulk microglia differential expression results for EAE + PRN2675 vs. EAE + vehicle (Fig. 1G, right) and *in vitro* mouse microglia differential expression results for IgG + tolebrutinib vs. IgG alone (Fig. 3G). In doing so, we highlight genes which are especially likely to be modulated by BTKi directly in microglia and relevant to pathological neuroinflammation.

- In general, the study has been performed from bulk tissue. Although this could be interesting to assess overall changes in the tissue, it makes it hard to make a point of specific transcriptional regulation of microglia. If the authors want to specifically investigate those, at least some key experiments should be performed in sorted microglia.

As stated in an earlier response to this reviewer, we agree that distinguishing the transcriptional effects in microglia versus other cell types is relevant to the conclusions drawn in this manuscript. We have therefore performed an additional EAE experiment in which we measure the effects on specific cell types in the CNS using single nuclei RNA sequencing (Fig. 1). In this manner, we have distinguished which transcriptional effects occur specifically within microglia, as opposed to monocyte-derived macrophages and intruding adaptive immune cells. In microglia, BTK inhibition induced a robust transcriptional response in both naive and EAE conditions. Moreover, we found that BTKi reversed the EAE-induced increase in multiple disease-associated microglial genes (Fig. 1H).

- I did not find a histological assessment of the cuprizone model. Is the treatment modifying the kinetic of de- or remyelination?

We have added a new figure in which we examine the role of BTK in remyelination. Our data show that acute BTK inhibition does not measurably affect remyelination in the cuprizone model. While 5 days of BTK inhibition is sufficient to alter cuprizone-induced transcriptional changes in the CNS (Fig. 2E), treatment with a BTKi inhibitor doesn't alter the dynamics of remyelination. (Fig. S3).

- The observation of differences between mouse and human need to be further elaborated. The article tries to present a new therapeutic avenue, and the effects observed in human systems become relevant. What are the differences between species in the experiments performed, and what can we learn from them?

We thank the reviewer for their feedback. To more effectively highlight differences and consistencies between models and species, we have now added four-way plots which directly compare differential gene expression results across datasets. Specifically, we compare the following:

- Fig. 3I – EAE pseudo-bulk microglia differential expression results for EAE + PRN2675 vs EAE + vehicle (Fig. 1G, right) and *in vitro* mouse microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 3G).
- Fig. 4E - human iPSC microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 4B) and *in vitro* mouse microglia differential expression results for IgG + tolebrutinib vs. IgG (Fig. 3G).
- Fig. 5E – human iPSC triculture differential expression results for IgG + tolebrutinib vs. IgG (Fig. 5B) and human iPSC microglia monoculture differential expression results for IgG + tolebrutinib vs. IgG (Fig. 4B).

These raw data will also be provided, so that others may pursue additional inquiries.

- The tricultures is certainly very interesting, and again there are clear differences between 2D and 3D systems when it comes to microglia. I think the authors should elaborate on this, and even put more emphasis on those systems they consider relevant. The authors should also perform treatments of cultures lacking microglia (only neurons and astrocytes) to differentiate between direct and indirect effects.

We thank the reviewer for this suggestion. We have therefore repeated the experiment described in Fig. 5C-D, this time quantifying chemokine and cytokine secretion in cultures with and without microglia (i.e. only neurons and astrocytes), using multiplexed panels (Fig. S8). Broadly, we see minimal effects from either IgG stimulation or BTK inhibition in cultures lacking microglia. In doing so, we have further reproduced, in cultures with microglia, the findings described in Fig. 5C-D.

- In figure 2C the authors talk about differences between time points but there is no statistical significance provided. Also in figure 2G, the statistical tests are not clear to me? What test was performed? And what significance is displayed?

We thank the reviewer for pointing out the lack of statistics for Fig. 2C. This figure now includes statistics and the statistical methods have been provided in the figure legend.

Regarding Fig. 2G, the figure legend now states that the p-values were calculated using Array Studio and the statistical tests employed have been specified in the Materials and Methods section, under "Mouse *in vivo* EAE model – Bulk RNA processing and analysis": "p-values were corrected for multiple testing using the Benjamini and Hochberg method, which controls the false discovery rate (FDR). DEGs were

determined using a 1.5-fold-change (FC) threshold and FDR-corrected p-value threshold of 0.05." Displayed in this figure are the FDR-corrected p-values, specified in the figure legend as "P-values are indicated by *≤0.05, **≤0.01, ***≤0.001, and ****≤0.0001."

Minor comments
In the intro reads both Corpus callosum and corpora callosa

All instances of "corpora callosa" have been replaced with "Corpus callosum".

Thank you very much for your feedback and consideration!

We would like to thank all the reviewers for going through our manuscript: NCOMMS-23-22421A: "BTK regulates microglial function and neuroinflammation in multiple sclerosis". We were happy to see the positive comments about our study. The team was very pleased with the reviewer comments. Two reviewers were fully satisfied by our extensive resubmission package. Both reviewers 1 and 4 highlighted a key point around trying to differentiate the effects of a CNS penetrant BTK inhibitor from a non-penetrant molecule to better understand the role of BTK in the CNS versus the periphery. In this current resubmission, we have tried to address these points both experimentally and throughout the text of our study. In this updated version of the manuscript, we have included three additional *in vivo* studies to address the reviewers' concerns. We have added data showing an anti-CD20 depleting antibody, which only targets B-cells, is non-efficacious in this EAE model. Suggesting that the efficacy observed through BTK inhibition is mostly through innate immune cells. Furthermore, we have performed EAE using two non-CNS penetrant molecules, ibrutinib and acalabrutinib, and these molecules are mildly efficacious in this model. While we believe it is extremely challenging to compare different small molecule kinase inhibitors with different potencies, off-target profile, and PK properties, we believe our new data suggests a role for BTK in innate immune cells in driving disease in both the periphery and CNS. We have also further discussed the interpretations of our findings as well as some of the limitations of our study.

We value the suggestions from the reviewers and have further generated a more comprehensive data set that supports our key findings. Furthermore, these data help strengthen the key hypothesis of our study which highlights the importance of BTK as a deleterious inflammatory mediator in innate immune cells, including macrophages and microglia.

Please find below a point-by-point response to each of the reviewer's comments. We sincerely appreciate your time and consideration in revisiting our manuscript and eagerly await your feedback.

Point by point response:

Reviewer comments:

Reviewer #1 (Remarks to the Author):

I recognize that the authors have provided an additional analysis to measure the effects on specific cell types in the CNS using single nuclei RNA sequencing. As the time-point of intervention has not changed, this approach unfortunately fails to answer whether immune cell function is directly altered within the CNS. As stated in my previous review, the authors need to investigate the effect of tolebrutinib in two further EAE settings, namely in a) late-stage fully established EAE, and b) in adoptive transfer (passive) EAE, in which readily primed T cells only require reactivation in the CNS bypassing the periphery. Only a clinical and/or immunological effect in these settings would justify concluding that tolebrutinib exerts direct effect within the CNS.

We thank the reviewer for their comments and recognize that distinguishing the CNS vs. non-CNS effects of BTK inhibition are quite challenging. To better support our conclusions, we have now included data from 3 additional EAE experiments to better resolve the role of BTK inhibition in EAE and potentially MS. We have performed EAE experiments using both a depleting anti-CD20 antibody and using two non-brain penetrant BTK inhibitors, ibrutinib and acalabrutinib. We have also included PK information for these compounds to better support the statement non-brain penetrant.

Additionally, we have expanded the following portion of the discussion text to further highlight the limitations of the experiments/ treatment design and to include directions for future research:

"Because of the somewhat pleiotropic expression of BTK in both B-cells, macrophages, and microglia the cellular target of PRN2675 in this model could be multi-faceted. In particular, B cells, macrophages, and microglia all express BTK and can contribute to EAE disease pathology. However, while targeting B cells by BTKi is therapeutically relevant in MS (as is B cell depletion using anti-CD20 agents), we found that B cell depletion had no effect on disease progression in this EAE model and further note that this approach may not be sufficient to attenuate clinical disease progression.... As it stands, distinguishing the effects of BTKi on innate versus adaptive immunity in the EAE model remains challenging."

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns, thank you.

We thank the reviewer for their evaluation of this manuscript.

Reviewer #3 (Remarks to the Author):

Gruber and colleagues went at length to address the issues raised and added extensive new data and analyses. Key groups were added that solidify the conclusions drawn.
No further comments, a well performed and complete study.

We thank the reviewer for their evaluation of this manuscript.

Reviewer #4 (Remarks to the Author):

Thanks to the authors for the extensive revision. In general, all my concerns have been adequately addressed, with the exception of the two points below:

"In the EAE model, it is difficult to separate what are the direct effects on microglia or indirect effects from modulating other immune aspects. I think these set of experiments would greatly benefit from adding a side-by-side non-brain penetrant compound to be able to dissect better the contribution of different compartments.

The reviewer correctly points out that in the current version we did not include a direct comparison using a non-CNS penetrant molecule. In our experience, it is extremely challenging to do this because each kinase inhibitor has different potencies, off-target profile, and PK properties. Nonetheless, in the current revision, we have included **3 more EAE studies** to better understand the role of BTK in this mouse model of MS. First, in Fig. S2 we use a B-cell targeting antibody to deplete these cells and show that it has no efficacy in this model. Furthermore, in Fig. S1 we show that 2 non-CNS penetrant BTK inhibitors, ibrutinib and acalabrutinib, are mildly efficacious in this model. These data further suggest that BTK inhibition can target non-B cells to elicit a protective effect. This is consistent with previous studies from our group (Hagan *et al.* 2020), where targeting macrophages/microglia in the EAE model attenuates disease severity.

Additionally, as discussed in our response to Reviewer 1, we have expanded portions of the discussion text to further highlight the limitations of the experiments/ treatment design and to include directions for future research.

The authors try to partially address that by adding a cuprizone model of demyelination, however the comparison made between the models is very poor. What are the changes in common between these systems? What are the changes in the microglia that can be observed in both models and therefore confidently say that due to direct microglia modulation?"

As requested by the reviewer, we have now included as Fig. S6 a four-way plot directly comparing the cuprizone and EAE models. Specifically, EAE pseudo-bulk microglia differential expression results for EAE + PRN2675 vs. EAE + vehicle (Fig. 1G, right) are compared to cuprizone bulk spinal cord differential expression results for cuprizone + PRN2675 vs. cuprizone + vehicle (Fig. 2E).

What I meant here is that the effect of the inhibitors directly on the adaptive immune system at systemic level were not controlled. Although the authors provided RNA-seq evidence of transcriptomic effects in the microglia which are very interesting, my point stands that in the current setting, it could well be that the inhibitor is acting systemically, and only having an indirect effect on microglia. Could it be possible to perform at least one experiment with a non-brain penetrant molecule to be able to pull this confounding effects apart?

As discussed in our above response to Reviewer 4's first comment, we have included 2 additional EAE studies with non-CNS penetrant BTK inhibitors. In a separate EAE study, we also deplete B cells and demonstrate that this has no effect on clinical progression. This suggests that the observed efficacy of BTKi in this model is dependent on non-B cell populations.

"In figure 2C the authors talk about differences between time points but there is no statistical significance provided"
I still cannot see the statistics in the figure provided

We have now included a comparison of all the timepoints compared to control

conditions. *Comments on the new data provided:*

Figure 1G – The transcriptomic changes seem to be larger in the naïve than in the EAE mice. The authors should discuss the possibility that BTKi affect the CNS in the absence of pathology, and given that they propose it as a therapeutic tool, at least discuss what are the implications of the changes observed.

We thank the reviewer for pointing this out and have now performed IPA pathway analysis, using the BTKi-induced transcriptomic changes observed in naive mice (Fig. S4). It should be noted that none of the resultant pathways meet our standard threshold of FDR <0.05, so we instead plot their raw p-values.

Figure 1I - The granulocyte degranulation term in pathways analysis is not very informative, given that the transcriptomes come from microglia, not granulocytes. I recommend the authors to look into what genes are in this pathway and perhaps assigned a different name that is more meaningful in this particular context.

We thank the reviewer for their recommendation. However, to maintain the reproducibility of the findings, it is our preference to leave the "neutrophil degranulation" term unchanged, as the term itself will be meaningful to those who use the Ingenuity Pathway Analysis software.

However, we now convey in the results text that several of the genes within this pathway have been associated with neuroinflammation/neurodegeneration (Lgals3, Ftl, Ctsd, Ctsz) and endosomal/lysosomal function (CD68, Ctsd, Ctsz).

Figure 1H – What is the relevance of the genes displayed and why were they selected? It could be relevant if the authors showed also the expression of genes from the homeostatic signature.

The genes displayed in Fig. 1H are disease-associated microglial genes, selected based on review of the relevant literature (citations below):

- Ofengeim D, et al. RIPK1 mediates a disease-associated microglial response in Alzheimer's disease. Proc Natl Acad Sci USA 114, E8788-E8797 (2017).
- Deczkowska A, Weiner A, Amit I. The physiology, pathology, and potential therapeutic applications of the TREM2 signaling pathway. Cell 181, 1207-1217 (2020).
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While we have chosen to use this space to highlight disease-associated microglial genes, we will provide the expression of genes from the homeostatic signature as raw data in the supplement. Furthermore, we have now performed IPA pathway analysis using the homeostatic signature (Fig. S4).

Figure 2G – It could be very relevant to adjust the genes displayed based on the sequencing in 1H, and find common hubs in both EAE and Cuprizone model

The genes displayed in Fig. 2G and Fig. 2H now include those modulated by BTKi in both the EAE and cuprizone models, as displayed in the newly added four-way plot (Fig. S6). *Csf1* expression is also displayed, despite not meeting the four-way plot fold-change thresholds, as it was also modulated by BTKi in the EAE microglia – and, in fact, in every *in vivo* and *in vitro* model examined.

Minor

3I – not clear what the x-axis legend means

For Fig. 3I, we have re-labeled the axes to more clearly communicate their meaning. As described in the figure legend, the x-axis denotes the differential expression results for the EAE single nucleus RNAseq microglia pseudo-bulk comparison EAE + PRN2675 vs EAE + vehicle (Fig. 1G, right).

Thank you very much for your feedback and consideration!