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Single-cell immune repertoire and transcriptome sequencing reveals that clonally expanded and transcriptionally distinct lymphocytes populate the aged central nervous system in mice

Alexander Yermanos, Daniel Neumeier, Ioana Sandu, Mariana Borsa, Ann Cathrin Waindok, Doron Merkler, Annette Oxenius and Sai T. Reddy

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1st revised submission:	11 November 2020
2nd revised submission:	26 January 2021
Final acceptance:	26 January 2021

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2020-1495.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
Yes

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
In this manuscript, Yermanos et al. characterize the immune repertoire and the transcriptome of mice B- and T-Cells from the brain. They investigate mice at different ages, and detected the presence of clonally expanded B and T cells in the central nervous system (CNS) of aged mice with moderate levels of somatic hypermutation.
Based on single-cell RNA-seq they also demonstrate that such clones reveal distinct transcriptional profiles. This lead to the conclusion that clonally related B- and T-Cells in the Central Nervous System of aged mice could be correlated with neuroinflammation and aging disorders.
The manuscript is overall clear, and their main conclusions are overall supported by the data. I have some minor concerns about their experimental design and about the RNA-seq analyses:

1) Why only male mice were selected? How can the author rule out that their findings are not sex-specific?

2) Were the brains from mice of different ages processed on the same days and on the same mixed batched. Otherwise, I would be concerned about potential batch effects, especially in the RNA-seq data.

3) How did the author perform the differential gene expression analysis? The details are lacking from the method section.

4) Why did the authors choose to use Bonferroni for multiple testing correction, rather than the most commonly used Benjamini-Hochberg FDR? Bonferroni's correction is very strict, and this may justify why they found such a little number of differentially expressed genes.

5) The quality of the figures is very poor and hard to read

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Marginal

General interest: Is the paper of sufficient general interest?
Marginal

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors extracted CD19+ B-cells and CD3+ T-cells using FACS from whole brains of mice, aged 3, 12, 18 old months, and profiled BCRs and TCRs together with transcriptome using 10X Genomics VDJ Kit. The analysis of the BCR repertoire suggests that aged mice have clonally expanded B-cells, most of which express IgM.
The observation of the clonal expansion in aged mice is potentially interesting, and might provide an insight into the age-related inflammation. However, in the current form of the manuscript, it is unclear whether the conclusion is fully supported by the results.

The authors conclude the clonal expansion in the aged CNS in Page 5 and Fig.2. However, because the number of B-cells for each sample seems different, it's hard to compare different age groups. For example, 3-months old mice show only 7 expanded clones (Fig.2A top), potentially because the number of B-cells from 3-months mice is the lowest.

The number of T-cells also seems not stable according to Fig.6A, and hard to interpret Fig.2B. For example, 18-months pooled sample shows many expanded cells, potentially due to the high number of T-cells in this library (clearly much more than other samples according to Fig.6A). The authors should re-evaluate clonality considering the number of T-cells and B-cells, for example, sub-sampling the same number of cells per library.

The level of clonal expansion between 18m pool and 18m alone seems very different, suggesting the possibility that the clonal expansion is associated with individual difference rather than aging. In particular, the sample "18m alone" comes from a single animal. The authors should mention this possibility.

The authors consider that the clonal expansion is a feature of aged brains as written in the manuscript.
Titile: "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice"
Page 9 "Our single-cell sequencing approach revealed that lymphocyte CNS infiltration was driven by clonally expanded lineages."

However, the analyzed lymphocytes might not come from brain tissues, but come from circulating blood. Are there any ways to distinguish infiltered lymphocytes and circulating lymphocytes? One possibility is to analyze circulating lymphocytes, and compare the current datasets. The authors should mention this possibility.

The authors should mention a recent single-cell atlas paper, which reported the clonal expansion of B-cells and T-cells in aged mice [PMID: 32669714].

It's better to improve the resolution of figures. Gene names in Figs. 5 and 6 are not readable. Figure legends are missing.

Decision letter (RSPB-2020-1495.R0)

25-Sep-2020

Dear Dr Yermanos:

I am writing to inform you that your manuscript RSPB-2020-1495 entitled "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
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Sincerely,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Two experts in the field have reviewed your manuscript, and both agree that it is potentially an important contribution to the field. However, both have identified some weakness, including the fact that it is unclear whether the results fully support the conclusion. Considering these comments, I cannot recommend the MS for publication in its current status.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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The authors should mention a recent single-cell atlas paper, which reported the clonal expansion of B-cells and T-cells in aged mice [PMID: 32669714].

It's better to improve the resolution of figures. Gene names in Figs. 5 and 6 are not readable.

Figure legends are missing.

Author's Response to Decision Letter for (RSPB-2020-1495.R0)

See Appendix A.

RSPB-2020-2793.R0

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Acceptable

General interest: Is the paper of sufficient general interest?
Acceptable

Quality of the paper: Is the overall quality of the paper suitable?
Acceptable

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have answered most my questions, and I only have one concern.
It is still not easy to understand Fig.2, because the numbers of analyzed B-cells and T-cells are not clearly written. I think the total numbers of analyzed cells are essential information to interpret Fig.2. If you analyze more cells, you have more chance to find duplicated clones.

I would suggest to add the numbers of T-cells and B-cells for each sample in this figure, which help readers understand the plots.

Decision letter (RSPB-2020-2793.R0)

27-Nov-2020

Dear Dr Yermanos

I am pleased to inform you that your manuscript RSPB-2020-2793 entitled "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice" has been accepted for publication in Proceedings B.

The referee has recommended publication, but also suggests some minor revisions to your manuscript. Therefore, I invite you to respond to the referee comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

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2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that

the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

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It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository (https://royalsociety.org/journals/authors/author-guidelines/#data).

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• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
• Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311
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Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Dear Dr Yermanos,

Following the reviewer's comments on the newer version of your manuscript, I happy to recommend it for publication after the implementation of the changes suggested by the reviewer.
Best wishes,
Roberto Feuda

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The authors have answered most my questions, and I only have one concern.
It is still not easy to understand Fig.2, because the numbers of analyzed B-cells and T-cells are not clearly written. I think the total numbers of analyzed cells are essential information to interpret Fig.2. If you analyze more cells, you have more chance to find duplicated clones.
I would suggest to add the numbers of T-cells and B-cells for each sample in this figure, which help readers understand the plots.

Author's Response to Decision Letter for (RSPB-2020-2793.R0)

See Appendix B.

Decision letter (RSPB-2020-2793.R1)

26-Jan-2021

Dear Dr Yermanos

I am pleased to inform you that your manuscript entitled "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Point-by-point reply for Yermanos et al., "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice"
Manuscript ID: RSPB-2020-1495

In this manuscript, Yermanos et al. characterize the immune repertoire and the transcriptome of mice B- and T-Cells from the brain. They investigate mice at different ages, and detected the presence of clonally expanded B and T cells in the central nervous system (CNS) of aged mice with moderate levels of somatic hypermutation.
Based on single-cell RNA-seq they also demonstrate that such clones reveal distinct transcriptional profiles. This lead to the conclusion that clonally related B- and T-Cells in the Central Nervous System of aged mice could be correlated with neuroinflammation and aging disorders.
The manuscript is overall clear, and their main conclusions are overall supported by the data. I have some minor concerns about their experimental design and about the RNA-seq analyses:

We thank R1 for their constructive feedback and we have incorporated all raised concerns into our manuscript. Please find our response to the specific points in bold italics below.

1) Why only male mice were selected? How can the author rule out that their findings are not sex-specific?

Male mice were selected due to the ability of ordering pre-aged mice in a defined housing environment. Ordering 18-month-old aged female mice is not easily accessible at our animal facility without waiting 18 months before receiving the animals. We understand the concern presented by R1 and agree that this is a crucial consideration for the context of our study. We have therefore added this abstract (line 27), and discussion sections (lines 332-337). We have furthermore added another study that found an increased proportion of clonally expanded clones in aged mice when using both males and females

(lines 337-339), thereby suggesting our results would be consistent in both sexes. We do agree with R1, though, that we cannot exclude this.

2) Were the brains from mice of different ages processed on the same days and on the same mixed batched. Otherwise, I would be concerned about potential batch effects, especially in the RNA-seq data.

The brains from the different mice were processed on the same day and on the same 10x reaction chip. Furthermore, all sorting, antibody labeling, capture, libraries prep, and sequencing were done together. We thank R1 for bringing up this point and have added it to the methods section (lines 378-379).

3) How did the author perform the differential gene expression analysis? The details are lacking from the method section.

ler.rather than the most commonly used Benjamini-Hochberg FDR? Bonferroni's correction is very strict, and this may justify why they found such a little number of differentially expressed genes.

We thank R1 for pointing this out and have updated the methods accordingly to mention that we used Seurat's default FindMarkers function to calculate differential gene expression which uses the Wilcoxon Rank Sum test and automatically employs Bonferroni correction. The authors of Seurat explicitly suggest to not use other correction methods (https://www.rdocumentation.org/packages/Seurat/versions/3.1.4/topics/FindMarkers). We agree with R1 that it is worth mentioning that there were other genes differentially upregulated with a more relaxed threshold, so we have included that there were 247 and 137 genes differentially expressed with an unadjusted p value of <0.01 (lines 292-293). Given we do not have biological replicates we would prefer to use a stricter threshold set by the Bonferroni method.

5) The quality of the figures is very poor and hard to read

We thank R1 for pointing this out and have removed the embedded figures from the manuscript and instead supplied them as TIFF files directly to the manuscript editor.

Referee: 2

Comments to the Author(s)
The authors extracted CD19+ B-cells and CD3+ T-cells using FACS from whole brains of mice, aged 3, 12, 18 old months, and profiled BCRs and TCRs together with transcriptome using 10X Genomics VDJ Kit. The analysis of the BCR repertoire suggests that aged mice have clonally expanded B-cells, most of which express IgM.
The observation of the clonal expansion in aged mice is potentially interesting, and might provide an insight into the age-related inflammation. However, in the current form of the manuscript, it is unclear whether the conclusion is fully supported by the results.

We thank R2 for their constructive feedback and we have addressed all points. Please find our response to the specific points in bold italics below.

The authors conclude the clonal expansion in the aged CNS in Page 5 and Fig.2. However, because the number of B-cells for each sample seems different, it's hard to compare different age groups. For example, 3-months old mice show only 7 expanded clones (Fig.2A top), potentially because the number of B-cells from 3-months mice is the lowest.
The number of T-cells also seems not stable according to Fig.6A, and hard to

interpret Fig.2B. For example, 18-months pooled sample shows many expanded cells, potentially due to the high number of T-cells in this library (clearly much more than other samples according to Fig.6A). The authors should re-evaluate clonality considering the number of T-cells and B-cells, for example, sub-sampling the same number of cells per library.

The level of clonal expansion between 18m pool and 18m alone seems very different, suggesting the possibility that the clonal expansion is associated with individual difference rather than aging. In particular, the sample "18m alone" comes from a single animal. The authors should mention this possibility.

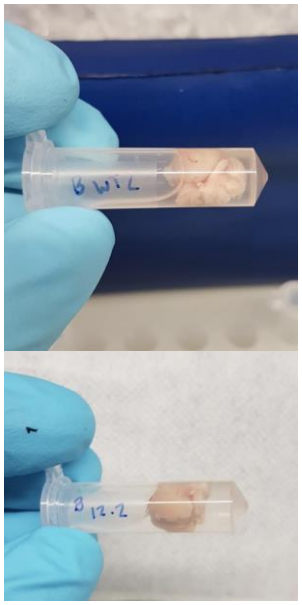
We thank R2 for the suggestion and agree that the differing number of B and T cells across samples present challenges for the analysis. We completely agree with R2's point that the B cells in the 3-month-old mice have less cells and thereby skews the interpretation of clonal expansion. Throughout the manuscript we were hesitant to explicitly compare the clonal expansion levels between the ages and refrained from stating that clonal expansion does not occur in the young brain. We nevertheless included this data in the analysis for reference despite the fewer number of cells. We again agree with R2 that the extent of T cell expansion was not necessarily stable between the aged samples, however, we again tried to simply present the data without focusing too much on explicitly comparing the clonal expansion between the age groups. We hold that the observation of multiple clones supported by two or more cells in both 18month old groups for B and T cells nevertheless supports our claims.
We do agree that more emphasis needs to be added to these experimental considerations and the instability between experimental groups of the pooled vs individual mouse and have therefore added the aforementioned points to the discussion (lines 339-348).

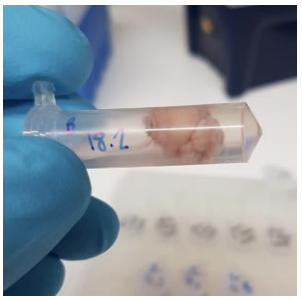
The authors consider that the clonal expansion is a feature of aged brains as written in the manuscript.

Titile: "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice"
Page 9 "Our single-cell sequencing approach revealed that lymphocyte CNS infiltration was driven by clonally expanded lineages."

However, the analyzed lymphocytes might not come from brain tissues, but come from circulating blood. Are there any ways to distinguish infiltered lymphocytes and circulating lymphocytes? One possibility is to analyze circulating lymphocytes, and compare the current datasets. The authors should mention this possibility.

We thank R2 for this point and agree that this point should be explicitly mentioned in the discussion. We have therefore mentioned the possibility that we cannot exclude that these lymphocytes are not blood derived, despite perfusing with PBS for three minutes. We are however, quite confident that the majority of the blood was washed away and that the majority of remaining blood would be very localized in the CNS in small capillaries. We are attaching representative photos demonstrating the brain after extraction had extremely minor traces of blood (e.g. no red in the pellet at all after centrifugation for all samples). We have added these points to lines (348-353) as a further drawback to our study.





The authors should mention a recent single-cell atlas paper, which reported the clonal expansion of B-cells and T-cells in aged mice [PMID: 32669714].

We thank R2 for mentioning this paper and have incorporated this great resource twice into our manuscript, once to refer to clonally expanded B and T cells in aged mice and once to refer that their study included female mice (lines 339, 347).

It's better to improve the resolution of figures. Gene names in Figs. 5 and 6 are not readable.

We thank R2 and have no longer embedded the figures directly in the manuscript but have uploaded individual high-resolution TIFF images.

Figure legends are missing.

We have now ensured that the figure legends are correctly present at the bottom of the text.

Appendix B

Point-by-point reply for Yermanos et al., "Single-cell immune repertoire and transcriptome sequencing reveals clonally expanded lymphocytes populate the aged central nervous system in mice"

Manuscript ID: RSPB-2020-1495

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I would suggest to add the numbers of T-cells and B-cells for each sample in this figure, which help readers understand the plots.

We agree with R2 and have added the requested cell numbers to Figure 2.