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Peer Review Information

Journal: Nature Immunology
Manuscript Title: Neuroinflammation creates an immune regulatory niche at the meningeal lymphatic vasculature near the cribriform plate
Corresponding author name(s): Zsuzsanna Fabry

Editorial Notes:

EA delete any non-applicable rows, and then delete this instruction.

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A32181
Message: 6th Jul 2021

Dear Zsuzsa,

Thank you for providing a point-by-point response to the referees' comments on your manuscript entitled, "Neuroinflammation creates an immune regulatory niche at the meningeal lymphatic vasculature near the cribriform plate". As noted previously, while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version that addresses these concerns along the lines proposed in your rebuttal.

We invite you to submit a substantially revised manuscript, however please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, the revision should include new experiments to address:

1. Co-culture EAE-primed cpLECs with 2D2 T cells with or without PD-L1/PD-1 inhibitors to functionally measure the role of PD-L1 in T cell activation.
2. Compare T cell phenotypes using flow (IL-17, IFN-g, FoxP3) after co-culture with either naïve or EAE-primed cpLECs.

3. Isolate naïve cpLECs, treat with IFN-g, and measure MHC II expression to determine if MHC II upregulation by cpLECs is mediated by IFN-g.
4. Immunohistochemistry/confocal imaging for the following markers:
a. Further characterize the myeloid cell populations with CCR2, CCR7, MHCII, CD11c.
b. IMARIS reconstruction of OVA-GFP+ LECs to confirm their uptake of antigen
c. Include healthy controls showing OVA-GFP uptake by cpLECs

As noted in your rebuttal, please also discuss the novelty concerns posed by referee #3 and the new insights that your study brings in comparison to the recent literature. Please include the additional textual clarifications as indicated in your response letter.

When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after

publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

You may use the link below to submit your revised manuscript and related files:
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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Kind regards,

Laurie

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Referee expertise:

Referee #1: Neuroimmunology

Referee #2: Neuroimmunology

Referee #3: Glymphatic system

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In the present study the authors have characterized the changes in lymphatic endothelial cells of the cribriform plate ,an area they had previously shown to undergo lymphangiogenesis during EAE.
Here they have performed scRNAseq and provide evidence that in EAE, lymphatic endothelial cells close to the cribriform plate – referred to as cpLECs – acquire a phenotypic change that allows for interaction with immune cells – specifically dendritic cells - as previously observed for LECs in lymph nodes. The study provides further evidence that cpLECs may capture CNS antigens in vivo and can present antigen and activate CD4 T cells in vitro. The authors can identify IFN-g as a cytokine that plays a role in regulation of gene expression of some genes in cpLECs, e.g. PD-L1. Last but not least the authors show that cpLECs are in a unique position to sample CNS antigens due to interruptions of the arachnoid barrier at this site.
Taken together the authors conclude that meningeal LECs close to the cribriform plate provide a unique immunoregulatory niche during neuroinflammation.

Overall this is a very relevant study highlighting potentially differential roles of dural lymphatics in neuroinflammation depending on their precise localization in the dura mater and providing further evidence for a role of the cribriform plate in CNS lymphatic drainage which has recently been disputed by many authors. The study is original and provides significant novel insights in the field of lymphatic drainage of the CNS.

Enthusiasm is however dampened as unfortunately, the study as it stands has some shortcomings. The major concern is that it has analyzed a mixture of LECs that are anatomically localized in very different compartments inside and outside of the CNS. In fact the authors analyze cell suspensions derived from the CNS parenchyma, the subarachnoid space, the dura mater and even outside of the skull, e.g. the nasal mucosa. It is not entirely clear why the study did not focus solely on cells from within the cribriform plate, e.g. the brain facing side and thus solely on LECs within the dura mater at the level of the cribriform plate. Analyzing a mixture of LECs from the dura mater and nasal mucosa hampers assignment of the observed changes to the LECs truly localized in the dura mater at the level of the cribriform plate. This shortcoming needs to be addressed.

Also, it would have been very elegant if the authors had aimed at isolating the LECs from the dorsal (close to the sinuses) and ventral aspects of the dura mater at the same time allowing for directly comparison the LEC signatures by scRNAseq during health and EAC. This would further support the localized response of cpLECs in EAE and their important role in CNS drainage.

Additional points:

The authors show that DCs can interact with cpLECs during EAE and propose based on the observed upregulation of PD-L1 in EAE that cpLECs could play a tolerizing role. Although this is a tempting conclusion this does not fit to the observation that these cpLECs have a higher capacity to induce 2D2 proliferation, which would activate autoaggressive T cells.

It would be helpful to see tSNE plots from the entire scRNAseq datasets and to learn how many cells in total were analyzed and how many LECs were analyzed.

Figure 2: The live gate for doublets is too large and catches also dead cells – thus LECs/CD45 doublets with dead cells may be included in the analysis.

The authors propose that the leukocytes they identify to bind to cpLECs derive from the brain parenchyma – but there is no evidence for this in the study. In fact, these cells may be derived from the dura mater, the subarachnoid space or from the nasal mucosa. All these compartments harbor DCs.

The authors also show enhanced AQP1 expression by cpLECs in EAE. Why is upregulated AQP1 expression not seen in cluster 2 of cpLECs in the scRNAseq analysis? Is AQP1 only upregulated in dural lymphatics at the level of the cribriform plate and not in lymphatics of the dorsal and ventral aspects of the brain?

Figure 3 V to Z – is of too low quality.

Figure 5 – it is very hard to appreciate the orientation of the sections as taken

Figure 6 shows higher intensity signal in EAE versus healthy control but rather a signal along the olfactory bulbs and not in between as suggested by the authors. This is why the quantification of Figure 6D is not entirely clear. MRI is done with a very large volume of 10 micoliters injected in 5 minutes into the cisterna magna of the mice.

Reviewer #2:
Remarks to the Author:
In their study, Hsu et al. demonstrate that cribriform plate lymphatic endothelial cells (cpLECs) expand and display an activated phenotype as a consequence of EAE induced neuroinflammation. The activation of cpLECs is characterized by expression of genes and molecules related to antigen processing and presentation as well as leukocyte adhesion and activation. The authors further show uptake of CNS-derived antigens and intimate contact with CD11c+ cells, most likely DCs, and T cells by the cpLECs during EAE. In contrast to cpLECs from healthy mice, cpLECs sorted from EAE animals were further able to activate T cells in an antigen-specific manner in in vitro co-cultures. The presence of an IFN-γ signature in cpLECs during EAE leads to the hypothesis that changes in their expression profile are at least partly mediated through IFN-γ. Indeed, in IFN-γ KO animals, reduced surface expression of PD-L1 and Podoplanin was observed on the cpLECs during EAE. The presence of PD-L1 on activated cpLECs lead the authors to suggest that interaction of DCs and T cells with these cpLECs result in tolerization of DCs in the context of neuroinflammation. This would fit the observation that IFN-γ KO animals display exacerbated EAE severity, potentially due to loss of tolerizing immune interactions.
Overall, these are an intriguing findings. Both, lymphatics in the cribriform plate as well as lymphatic vessels in the dura mater have been shown to drain CNS-derived antigens and have also been suggested to play important roles for the activation of T cells during many neuropathologies, including neuroinflammatory as well as neurodegenerative disorders. The ability of cpLECs to sample CNS-antigens and function as APCs that can locally activate CNS-antigen reactive T cells.

However, several conclusions are thus far mainly hypothesis driven and need further substantiation through experimental evidence. This specifically refers to the claim that the change of the cpLEC phenotype happens through a state of cell proliferation, which is not evidenced by any hard data. Further, the potential activation/tolerization of DCs and T cells through cpLECs needs to be better characterized. Finally, the suggested tolerogenic role of both cpLECs and DCs in later stages of EAE through IFN-γ induced PD-L1 expression is intriguing, but without further data any experimental evidence for this hypothesis is missing.

Main Comments

1. The authors have to further validate their findings from the scRNA-seq analysis on protein levels. Thus far, of all representative genes depicted in Fig. 1, they only demonstrate upregulation of MHCII on cpLECs. This could be either done by additional flow cytometry experiments or, even better, through confocal analysis.
2. The authors state themselves that scRNA-seq revealed expression of many myeloid-associated genes in the cpLECs. It is thus of great importance to perform solid quality control on the sequencing data, including doublet exclusion (see comments on Data and methodology).
3. Visualization of single-cell trajectories through pseudotime infers trajectories based on the available gene expression data. However, this does not mean that it exactly and reliably predicts biological processes that happen in vivo. The authors claim, based on the results from the pseudotime analysis, that all lymphangiogenic cpLECs that derive from steady-state cpLECs have to transition through a stage of cell proliferation. This has to be proven by direct experimental evidence. Through using in vivo proliferation assays such as BrdU incorporation, the authors could directly demonstrate that all cpLECs have undergone recent cell proliferation. If all lymphagiogenic cpLECs are derived from proliferating cpLECs,
4. In Fig.2, the authors should be more conservative with the identification of the myeloid cell subsets. It is well known that CD11b expression can be found on many more cells than only macrophages (monocytes, neutrophils, NK ells etc.) and CD11c expression is not an exclusive marker for DCs, but can also be found on macrophages. If the authors wish to better distinguish the different myeloid cell types, they could include Ly6C, Ly6G, CCR2, CD64, F4/80 or further markers for different DC subsets in their analysis.
5. In Fig.3 A-C, OVA-GFP signal was found close to or even in cpLECs in EAE animals. However, healthy Ctrl animals are missing to demonstrate that this is an EAE specific phenomenon.
6. The representative images in Fig.3 do not allow to judge whether or to which extend OVA-GFP is truly found within cpLECs. It seems that many of the cells depicted with the yellow arrows in Fig.3 D-H (which are supposed to be cpLECs) are actually negative for Podoplanin and are rather lying next to the lymphatic vessels. This would rather suggest them to be MHCII-expressing APCs that are in intimate contact to the lymphatics. Counterstainings with myeloid markers such as CD11b would help to discriminate between cpLECs and APCs. To demonstrate the presence of OVA-GFP within the cpLECs, images with higher magnification are therefore needed. The authors could also use IMARIS based cell reconstruction to show the subcellular location of the OVA-GFP signal. Quantification of the amount of OVA-GFP+ cpLECs in healthy versus EAE mice would also helpful. One option would also be to test whether OVA-GFP signal can be detected in flow cytometry.
7. Similarly, in Fig.3 I it seems that the Podoplanin signal is actually stronger in-between the cells that the authors claim to be MHCII+ OVA-GFP+ cpLECs.
8. In Fig.3 V-Aa, the authors show activation of CD4+ T cells through cpLECs in vitro. It would be very interesting to know which subset of CD4+ T cells is induced through this

interaction. As the EAE model is mainly driven by Th1/Th17 cells, it would be interesting to know whether cpLECs can induce these T cell subsets. Can cpLECs also induce Treg cell subsets? The authors should thus further characterize the in vitro stimulated T cells, e.g. by flow cytometry using more markers (such as Il-17, IFN-γ etc. and transcription factors like Foxp3).
On the other hand, EAE derived cpLECs seem to express high levels PD-L1. Interaction of PD-L1 with PD-1 on T cells usually attenuates T cell activation, which contradicts the observation of strong T cell activation. How do the authors explain this discrepancy.
9. In the same line, it would be interesting to see if and which co-stimulatory molecules are present on cpLECs. Considering that the antigen processing and presentation capabilities of cpLCEs might be regulate through INF-γ signaling, treatment of isolated cpLECs with INF-γ in vitro could be used to assess changes in cpLECS.
10. The authors state that cpLECs still undergo lymphangiogenesis in IFN-γ KO animals during EAE. However, in Supp. Fig. 9 only quantification of cpLEC-CD11c+ cell doublets is shown. This data has to be corrected or else, the statement is wrong.
11. The data in Fig.5 and Fig.6 is very interesting, but also rather descriptive so far. It should be considered to merge the data into a single figure.
Data and methodology
12. Many information regarding methodology of the scRNAseq analysis are missing: Which chemistry has been used? Have doublets been excluded from the analysis? Which exact R packages have been used for the analysis?
Further, the sequencing debts appears to be quite low. Can the authors explain why the targeted read depth was only 7.500? How many genes were detected per cell on average?
13. It has to be clearly stated EAE mice of which score or time point after immunization were used for every experiment. This applies to nearly all experiments throughout the manuscript.
14. The antibody amount and site of injection for the CD45 in vivo labeling has to be clearly mentioned. Also, even though most immune cells in contact with cpLECs are not labeled by the i.v. injection of CD45 three minutes before perfusing the animals, this does not formally allow the conclusion that the immune cells have derived from the CNS parenchyma. They could also have been derived from blood before the three minute incubation, or could have been in the meningeal compartment. In addition, it would be helpful to show the labeling efficiency in blood leukocytes.
15. How many animals were included in the analysis in Fig.5?

Minor comments
1. Why did the authors decide to use tSNE plots for Fig. 1 and not UMAP as in Supp. Fig.4?
2. The exact number of cells from both healthy and EAE mice as well as the total number of cells included in the scRNAseq analysis should be specified
3. Fig.1 G-J: Instead of log fold changes between the clusters, violin blots should be used to show the gene expression levels for all clusters. This would allow unbiased visualization of gene expression on the single-cell level.
4. How many mice/sections have been analyzed in Fig.5. AQP4 expression in dorsal and basal mLVs?
5. General suggestion: Changing the y-axis in histograms from 'counts' to 'modal' scale might improve the readability as the curves for all cell types would then have a similar height.
Some minor mistakes were made in the labeling of graphs or text of the figure legends (non-exhaustive list below).
6. Fig. 4: The labels in the histogram are wrong. It should say 'IFN-γ KO', not 'EAE cpLECS + Leukocytes'

7. Legend of Fig, 6c has a typo: nec ◊ neck
8. Supp. Fig. 4: monAcle3 ◊ monocle3

Reviewer #3:
Remarks to the Author:
This is a manuscript from a group that previously has shown that VEGFR3 triggers lymphangiogenesis of meningeal lymphatics in the vicinity of cribriform plate in experimental autoimmune encephalomyelitis (EAE). In this study, the group shows that EAE changes the phenotype of cribriform plate lymphatic endothelial cells using single-cell RNA sequencing. The genes upregulated are involved in antigen presentation, adhesion and immunoregulatory molecules. The inflamed lymphatic endothelial cells hold on to dendritic cells creating an immune-regulatory niche. Also, discontinuity of the arachnoid membrane near lymphatic endothelial cells allows direct access CSF. The study is a logicfollow-up on the previous publication, but I am not convinced that more than details are added to what we already know using new techniques.

Major critique:

• Venous endothelial cells upregulate podoplanin expression in inflammation. Thus CD31+, podoplanin+ cells are not necessarily lymphatic endothelial cells. Also, venous endothelial cells are significantly affected by inflammation exhibited increased adhesion. Additional validation is needed.
• Inflamed tissue is more edematous and dissociate better. That the authors are capable of isolate a larger number of lymphatic endothelial cells does not prove an increase in their number. Stereology of sections with the appropriate markers are needed
• The authors should clearly indicate how the findings on how lymphatic endothelial cells presentation of antigens to CD4 T cells through MHC II differ from prior publications. The same for leukocyte binding and crosstalk. What is novel?
• A large number of publications have shown that the olfactory bulb serves as an exit route for CSF. The MRI study show absolutely nothing new compared to for example Helene Benveniste MRI studies. Jony Kipnis and other have documented that CSF inflow into brain is reduced in EAE and it subsequently accumulates in the basal cisterns and is shunted out via the olfactory bulb.

Overall, it is a solid study if additional histology is added, but the advances are incremental

Author Rebuttal to Initial comments

Response to Referees

Reviewer #1

(Remarks to the Author)

In the present study the authors have characterized the changes in lymphatic endothelial cells of the cribriform plate ,an area they had previously shown to undergo lymphangiogenesis during EAE.

Here they have performed scRNAseq and provide evidence that in EAE, lymphatic endothelial cells close to the cribriform plate – referred to as cpLECs – acquire a phenotypic change that allows for interaction with immune cells – specifically dendritic cells - as previously observed for LECs in lymph nodes. The study provides further evidence that cpLECs may capture CNS antigens in vivo and can present antigen and activate CD4 T cells in vitro. The authors can identify IFN-γ as a cytokine that plays a role in regulation of gene expression of some genes in cpLECs, e.g. PD-L1. Last but not least the authors show that cpLECs are in a unique position to sample CNS antigens due to interruptions of the arachnoid barrier at this site.

Taken together the authors conclude that meningeal LECs close to the cribriform plate provide a unique immunoregulatory niche during neuroinflammation.

Overall this is a very relevant study highlighting potentially differential roles of dural lymphatics in neuroinflammation depending on their precise localization in the dura mater and providing further evidence for a role of the cribriform plate in CNS lymphatic drainage which has recently been disputed by many authors. The study is original and provides significant novel insights in the field of lymphatic drainage of the CNS.

1. Enthusiasm is however dampened as unfortunately, the study as it stands has some shortcomings. The major concern is that it has analyzed a mixture of LECs that are anatomically localized in very different compartments inside and outside of the CNS. In fact the authors analyze cell suspensions derived from the CNS parenchyma, the subarachnoid space, the dura mater and even outside of the skull, e.g. the nasal mucosa. It is not entirely clear why the study did not focus solely on cells from within the cribriform plate, e.g. the brain facing side and thus solely on LECs within the dura mater at the level of the cribriform plate. Analyzing a mixture of LECs from the dura mater and nasal mucosa hampers assignment of the observed changes to the LECs truly localized in the dura mater at the level of the cribriform plate. This shortcoming needs to be addressed.
    - The reviewer makes a valid point, and we have carefully considered the specificity of our isolation when planning these experiments. There are three potential sources of LECs: 1) meningeal LECs near the cribriform plate, 2) dural meningeal LECs above the olfactory bulbs, and 3) peripheral nasal mucosa LECs. The isolation of the cribriform plate and its associated tissues includes the olfactory bulbs, however the dural meningeal LECs above the olfactory bulbs were carefully removed prior to harvest to exclude the dural meningeal LECs. Removal of these dural meningeal LECs included the meninges above the olfactory bulbs, skull cap, and outside of the skull. For the peripheral nasal mucosa

lymphatics, these exist quite ventrally in the nasal mucosa where we avoided isolation. We have clarified this in more detail in the methods section:
"Mice were terminally anesthetized with isoflurane and transcardially perfused with PBS. Mice heads were removed and the skin was cut dorsal to the midline of the skullcap rostrally to expose the brain. The skullcap was then removed along with the brain and dura after separation from the olfactory bulbs. The cribriform plate and its associated tissues which included the olfactory bulbs, the cribriform plate, and parts of the nasal mucosa adjacent to the cribriform plate were dissected out and placed in a 70-micron strainer submerged in RPMI-1640 in a non-tissue culture treated dish. The tissues were then mechanically dissociated by pushing the tissue through the strainer using a syringe plunger. The mechanically dissociated cells were then spun down, washed, and resuspended in FACS buffer (1% Bovine Serum Albumin in 0.1M PBS) for FACS staining."

- The purity of LECs coming from specifically the cribriform plate region can be seen a couple of ways. First, scRNAseq grouped steady-state LECs into one main cluster, and we would expect the dural meningeal LECs to be unique enough to isolate into a different cluster. This is based on the observation that Jonathan Kipnis's group have compared the dural meningeal LECs (which develop extremely late compared to all other lymphatic networks) to other peripheral lymphatic networks and found that the dural meningeal LECs have a uniquely dysregulated phenotype (reference #6)
- Secondly, it is hypothesized that the peripheral nasal lymphatics are larger collector vessels that drain smaller LEC capillaries near the cribriform plate that directly sample fluid, and consequently the peripheral nasal collector vessels do not express Lyve-1 (Lyve-1 specifically labels lymphatic capillaries, while Podoplanin labels both). Flow cytometry analysis of $CD45^{negative}$ $CD31^{+}$ $Podoplanin^{+}$ LECs reveal that nearly all of these cells express Lyve-1 (Supplementary Figure 2B, C), suggesting that the majority of cells we have isolated are $Lyve\text{-}1^{+}$ LECs near the cribriform plate.

2. Also, it would have been very elegant if the authors had aimed at isolating the LECs from the dorsal (close to the sinuses) and ventral aspects of the dura mater at the same time allowing for directly comparison the LEC signatures by scRNAseq during health and EAE. This would further support the localized response of cpLECs in EAE and their important role in CNS drainage.
    - We agree, and future studies should look at comparing the differences, if any, between the different meningeal lymphatic networks. Other groups have performed bulk RNA sequencing of the dural meningeal lymphatics under different conditions (brain tumor, EAE, Alzheimer's disease) (see references #8, 16, & 68) Interestingly, the dural meningeal lymphatics are quite unique in several aspects: they develop later than any other known lymphatic system, they display an immature phenotype (they require

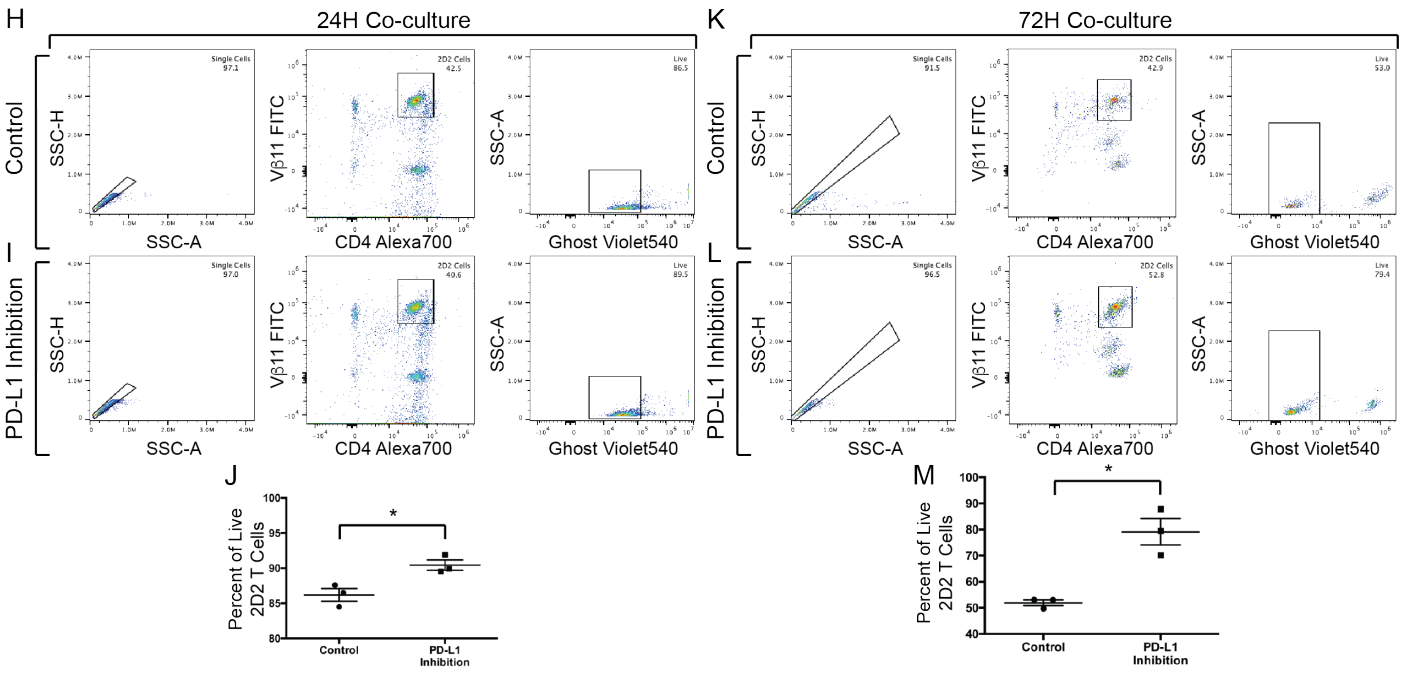
sustained VEGFC-VEGFR3 signaling to maintain baseline levels, whereas other lymphatics no longer require VEGFR3 signaling after development), and consequently they respond differently to neuroinflammation. These potential differences are discussed in the discussion section.

Additional points:

1. The authors show that DCs can interact with cpLECs during EAE and propose based on the observed upregulation of PD-L1 in EAE that cpLECs could play a tolerizing role. Although this is a tempting conclusion this does not fit to the observation that these cpLECs have a higher capacity to induce 2D2 proliferation, which would activate autoaggressive T cells.
    - We agree with the reviewer that the tolerizing role through PD-L1 upregulation and activation of 2D2 T cells needs more consideration. *In vivo* during EAE, cpLECs may interact with naïve T cells, effector T cells that have re-encounterered their cognate antigen either from the CNS or in the meninges, or even exhausted T cells that have encountered excessive antigen. The consequence of both antigen presentation and PD-L1 mediated tolerance by cpLECs may depend on the T cell state. To consolidate the idea that some cpLECs can present antigen and also be regulatory, we applied three different experimental strategies where we co-cultured healthy or EAE cpLECs with either 1) naïve 2D2 T cells, 2) effector T cells from the CNS of EAE, and 3) effector T cells from the CNS of EAE mice that have been pushed towards an exhaustive phenotype:
      In order to test the mechanism of PD-L1 tolerance on naive 2D2 T cells, we co-cultured EAE-primed cpLECs after cell sorting with magnetically purified naïve 2D2 T cells in the presence or absence of PD-L1 blocking antibody. We then measured T cell activation (CD69 expression), proliferation (dilution of Cell Trace), and cell viability (Ghost labeling). After 24H or 72H, inhibition of PD-L1 did not affect T cell expression of CD69 or proliferation. However, there was a significant decrease in cell death after PD-L1 inhibition after 24H, and a significant inhibition of cell death after 72H (approximately 30% reduction; shown below). These data suggest that PD-L1 does not affect the initial activation and proliferation of naïve 2D2 T cells but could induce cell death as previously described (Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, LeibundGut-Landmann S, Garbi N, Reith W, Swartz MA, Hugues S. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific $CD4^+$ T cell tolerance. J Exp Med. 2014 Jun 2;211(6):1153-66. doi: 10.1084/jem.20132000. Epub 2014 May 19. PMID: 24842370; PMCID: PMC4042642.). This is consistent with the expression of the PD-

L1 receptor PD-1 by T cells, where T cell activation by naïve T cells is necessary for the expression of PD-1 and consequently PD-L1/PD-1 mediated tolerance (reference #52). Therefore, it seems that antigen processing/presentation is necessary to activate naïve 2D2 T cells for PD-L1/PD-1 mediated cell death. This experiment has been added to **Figure 4** as panels **H-M**, and the results section has been updated as shown below:

"We next wanted to test how PD-L1 may functionally effect 2D2 T cells in the presence of cpLECs. To do this, we co-cultured EAE-primed cpLECs with naive 2D2 T cells *in vitro* in the presence or absence of PD-L1 blocking antibody **(Figure 4H-M)**. 2D2 T cells expressed CD69 after 24 hours and proliferate after 72 hours, and PD-L1 inhibition had no effect on the expression of CD69 or the frequency and rate of proliferation **(data not shown)**. This is consistent with the observation that naive T cells do not express PD-1 [52], and require activation/proliferation to express PD-1. In contrast, PD-L1 inhibition improved cell viability after 24 hours that was more prominent after 72 hours of co-culture **(Figure 4H-M)**, suggesting that PD-L1 signaling by cpLECs contributes to 2D2 T cell death without influencing the initial activation and proliferation of 2D2 T cells in this system."



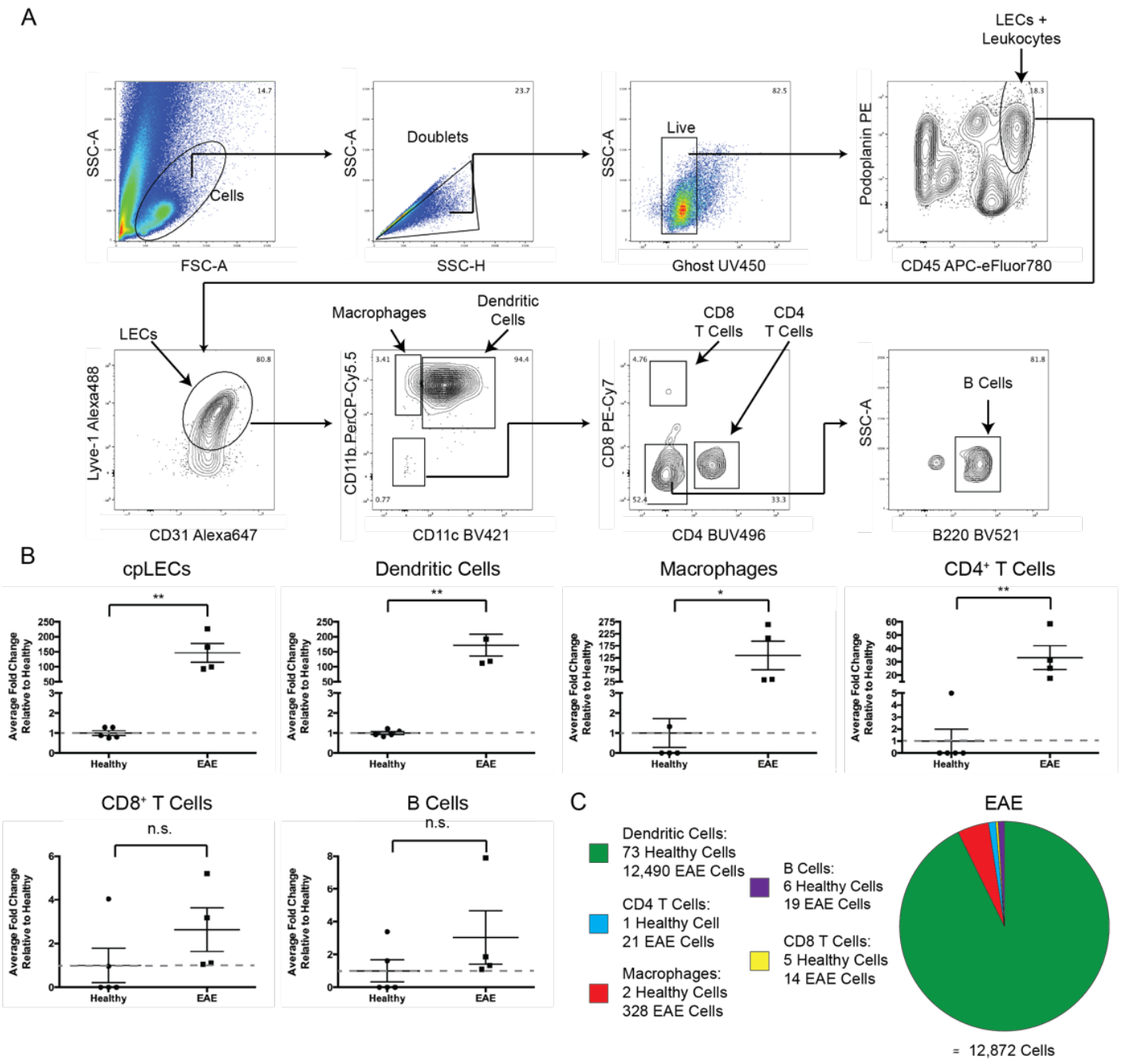
~~o~~ We next looked to see how cpLECs can modify the function of effector T cells by co-culturing healthy or EAE-primed cpLECs with T cells isolated from the

CNS of EAE mice for 24 hours, followed by re-stimulation to visualize the frequency of Foxp3, IL-17, and/or IFN-γ^+ T cells. These T cells should have been initially activated in the draining lymph nodes, have up-regulated PD-1, and differentiated into effector T cells. In line with this, healthy cpLECs + CNS-derived T cells from EAE mice resulted in baseline levels of Th1, Th17, and Treg cell frequencies. When co-cultured with EAE-primed cpLECs, there was a trending but non-significant reduction in the frequency of IL-17 producing Th17 cells (50%), a significant reduction in IFN-γ producing Th1 cells (25%), and no difference in Foxp3 Treg cell frequencies or cell viability.

- Taken together, it is possible that the cpLECs to regulate leukocytes depends on the leukocyte cell type and state. It should also be noted that dendritic cells were found to bind to cpLECs during EAE at high percentages, and other groups have demonstrated the ability of dendritic cells to both gain access to LEC-derived antigens as well as express PD-1. Future studies are currently aimed at investigating the potentially complex interplay between cpLECs, dendritic cells, and T cells.

2. It would be helpful to see tSNE plots from the entire scRNAseq datasets and to learn how many cells in total were analyzed and how many LECs were analyzed.
    - Thank you for the comment. This dataset is available through GEO accession number: GSE175802. It can also be visualized below.
    - A t-SNE plot from the entire scRNAseq dataset before excluding non-LECs is shown below. cpLECs are shown in blue (3,186 cells), orange (1,799 cells), and red (69 cells). A relatively large cluster (1,101 cells) as shown below in green was excluded due to enrichment in genes associated with olfactory sensory neurons and glial cells in this cluster (indicative of olfactory sensory neurons and ensheathing cells). These contaminating cells likely also express Podoplanin at the protein level and are adherent to the cribriform plate, thus making it through the sorting process. Other cells that made it through the cell sort in relatively small numbers include a cluster shown in purple (50 cells, neurons), brown (29 cells, oligodendrocytes), pink (16 cells, endothelial cells), gray (11 cells, epithelial cells), and light green (11 cells, olfactory epithelial support cells).

3. Figure 2: The live gate for doublets is too large and catches also dead cells – thus LECs/CD45 doublets with dead cells may be included in the analysis.
    - The reviewer makes a valid point about the strictness, or lack thereof, of our live/dead gate for the doublet data. We have re-gated our analysis using a stricter gating strategy as shown below. This has reduced the variance in our data creating additional significance with increased fold change from the number of dendritic cells, macrophages, and $CD4^+$ T cells binding to cpLECs.



4. The authors propose that the leukocytes they identify to bind to cpLECs derive from the brain parenchyma – but there is no evidence for this in the study. In fact, these cells may be derived from the dura mater, the subarachnoid space or from the nasal mucosa. All these compartments harbor DCs.

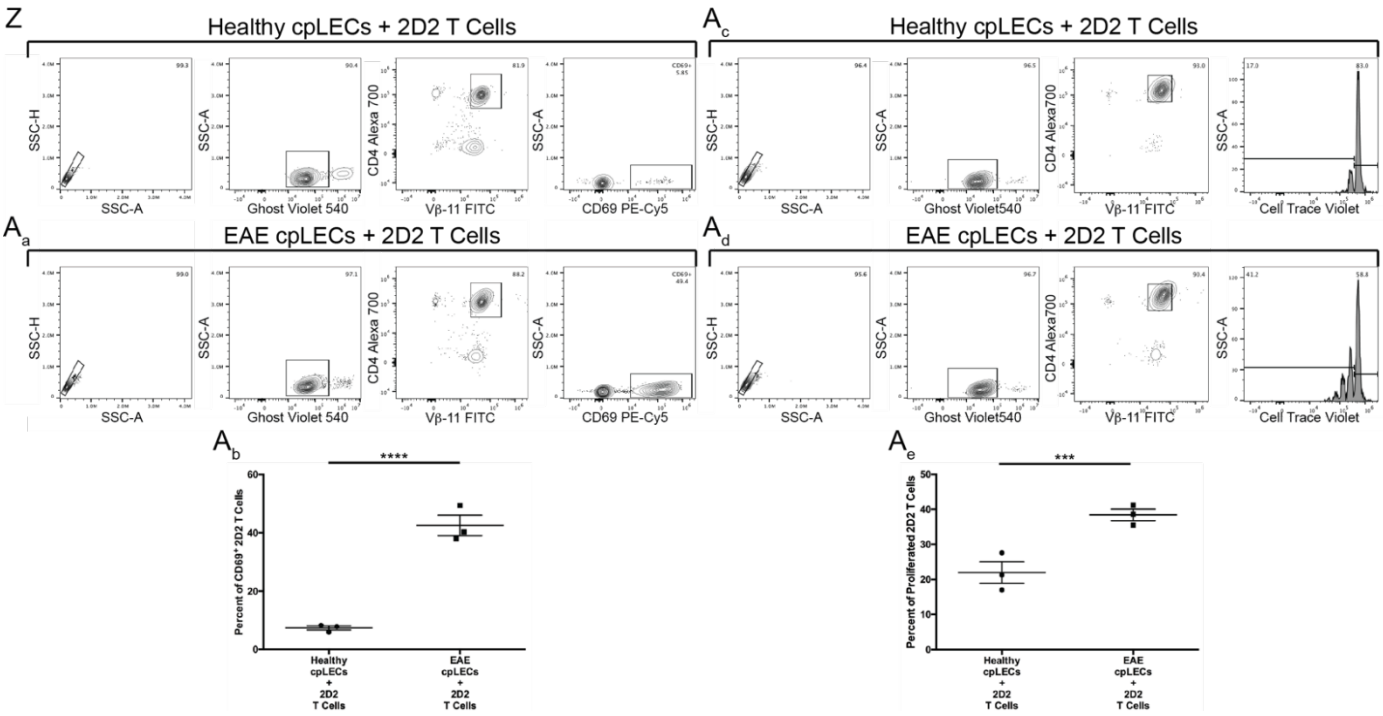
- The reviewer makes a valid point, as we cannot conclusively determine the origin of leukocytes that bind to cpLECs. We removed all and any language from the manuscript suggesting that all of the leukocytes bound to cpLECs came from the CNS parenchyma.
- At the very least, we can only say that a subset of leukocytes that bind to cpLECs came from the CNS. This is based on a previous manuscript where photoconverted cells from the CNS parenchyma can be found within cpLECs (reference #4). Additionally, we hypothesize that at least some of the leukocytes bound to cpLECs came from the CNS parenchyma due to the observation that brain-infiltrating dendritic cells outnumber meningeal/peripheral dendritic cells by a significant amount during EAE.
- An interesting observation is that a subset of DCs in contact with LECs near the cribriform plate contain CNS-derived antigens, indicating that they were CNS-derived (or at the very least have access to CNS-derived antigens). Whether or not the DCs picked up OVA-GFP antigens in the CNS and migrated to the cribriform plate or if OVA-GFP drained to local DCs near the cribriform plate through the CSF is unknown. How exactly CNS-derived antigens (free-floating vs. cell-mediated) drain to lymphatics is the subject of future studies.

5. The authors also show enhanced AQP1 expression by cpLECs in EAE. Why is upregulated AQP1 expression not seen in cluster 2 of cpLECs in the scRNAseq analysis? Is AQP1 only upregulated in dural lymphatics at the level of the cribriform plate and not in lymphatics of the dorsal and ventral aspects of the brain?
    - The reviewer made an important point, and in response we rephrased the language of "upregulation" by AQP-1 in the text. We don't believe that AQP-1 is upregulated by LECs in terms of increased protein expression per cell, but rather that because there is an expansion of LECs, there are more LECs expressing AQP-1. The change in text is quoted below:
      "Immunohistochemistry of the cribriform plate reveals the expression of AQP-1 by cpLECs, highlighting a potential mechanism of fluid transport from the subarachnoid space into cpLECs **(Figure 5D – G)**. Additionally, AQP-1 is expressed throughout the lymphatic vessel, even after lymphangiogenic expansion of cpLECs during EAE **(Figure 5D, F)**. These data suggest that there may be compensatory mechanisms to manage neuroinflammation-induced edema by increasing the numbers of AQP-1 expressing LECs through lymphangiogenesis. The gaps in the arachnoid barrier near cpLECs highlight their ability to sample the CSF compartment, and may explain their unique ability to undergo lymphangiogenesis during neuroinflammation."

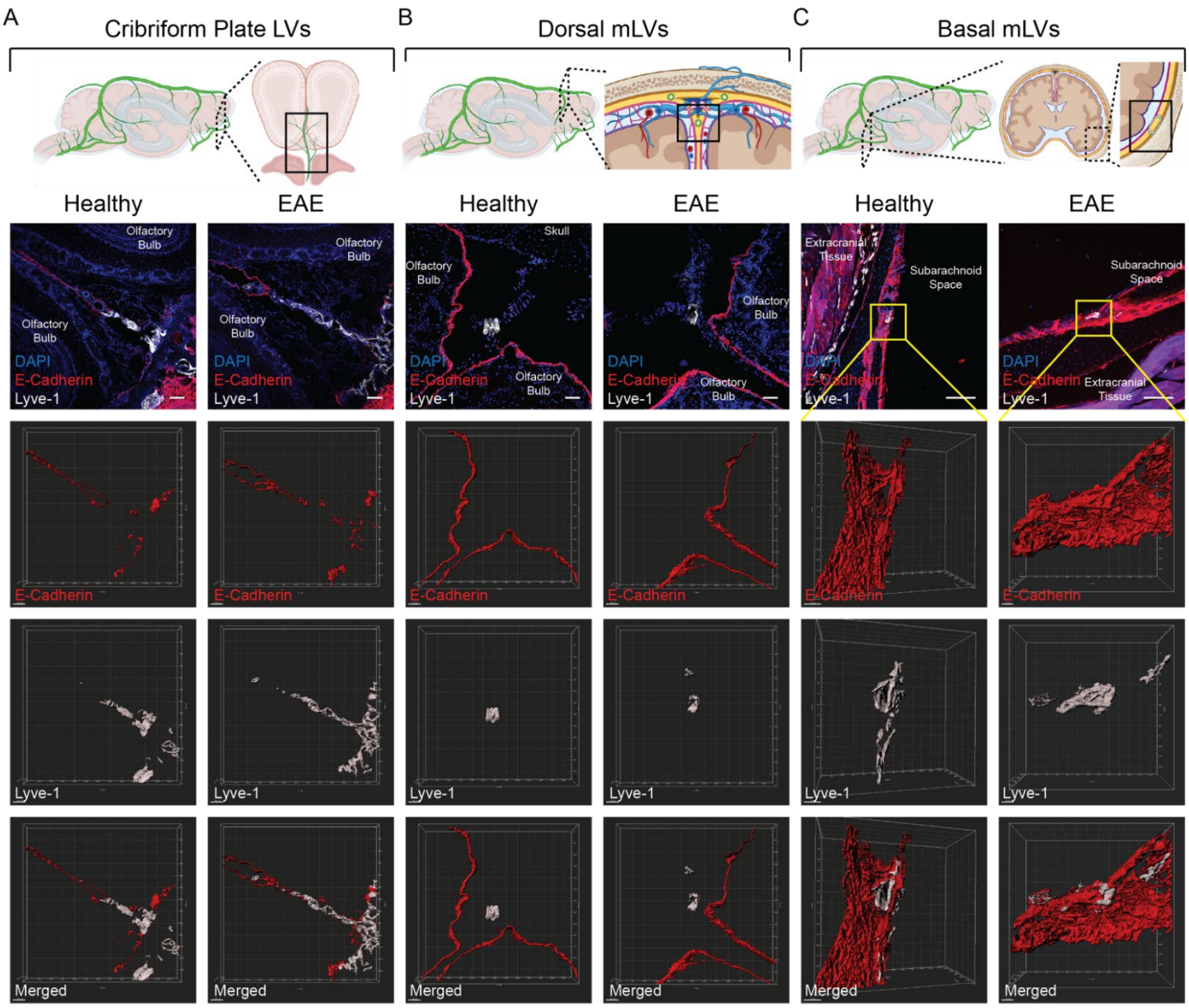
    - Preliminary imaging of the dural lymphatics above the brain reveals AQP-1 expression by these lymphatics as well, suggesting that AQP-1 may be expressed throughout the different meningeal lymphatics to facilitate water exchange. However, the increased surface area of AQP-1 expression by lymphangiogenic LECs and the gaps in the arachnoid barrier is unique to the cribriform plate, as the dural meningeal lymphatics do not undergo lymphangiogenesis during EAE and are separated from the CSF compartment by an uninterrupted arachnoid barrier. How the dural meningeal

lymphatics may bypass the arachnoid barrier to access CSF and utilize AQP-1 is currently unknown but is being investigated by other groups. This is also addressed in the discussion section:

- "In addition to immune surveillance and immune regulation, one of the primary roles of lymphatics is to maintain fluid homeostasis. Because meningeal lymphatics reside outside of the CNS parenchyma, several groups have speculated how meningeal lymphatics may access CSF through the arachnoid barrier, which seems to be more predominant in humans. Anatomically in rodent models, the meningeal lymphatics at the base of the brain are hypothesized to access CSF due to their relatively close location to the subarachnoid space [3], and here we show that there are gaps in the epithelial cells that comprise the arachnoid barrier separating the subarachnoid space and meninges near the cribriform plate as previously reported [53], suggesting direct access by these particular set of lymphatics. Although the dural meningeal lymphatics are more distal from the subarachnoid space, dyes infused into the CSF have identified "hotspots" along the transverse sinuses where CSF is uptaken into the dural meningeal lymphatics relatively early [6,16], suggesting direct uptake of CSF in these regions. Differences in dyes and ideas of how to functionally show CSF drainage (non-invasive imaging, post-mortem analysis, etc.) in animal models have yielded mixed results, where accumulation near the cribriform plate seems to be the most consistent [5]. However, this is further confounded in human imaging studies, where many non-invasive imaging techniques lack the resolution to determine precisely how CSF exits the subarachnoid space. For example, CSF can consistently be found on the CNS-side of the cribriform plate in humans, but whether CSF can exit through the cribriform plate into the nasal mucosa has yielded mixed results [5,15,69]. Nevertheless, we hypothesize that the meningeal lymphatics likely all play a role in CNS homeostasis, and are likely all connected as one large network that can sample from different regions of the subarachnoid space."
- Figure 3 V to Z – is of too low quality.
- We have imported higher resolution flow panels for **Figure 3**. Additionally, to make it easier to visualize the gating strategy, we replaced the dot plots with contour plots as shown below:



6. Figure 5 – it is very hard to appreciate the orientation of the sections as taken
   - We have added an additional row of panels to illustrate where the sections were taken for the cribriform plate meningeal lymphatics, dural meningeal lymphatics, and basal meningeal lymphatics as shown below:



7. Figure 6 shows higher intensity signal in EAE versus healthy control but rather a signal along the olfactory bulbs and not in between as suggested by the authors. This is why the quantification of Figure 6D is not entirely clear. MRI is done with a very large volume of 10 micoliters injected in 5 minutes into the cisterna magna of the mice.
    - We agree with the reviewer that the MRI imaging data shown using a 4.7T machine does not provide sufficient resolution to visualize signals between the olfactory bulbs and specifically within lymphatic vessels, and the signal we are visualizing is actually along the surface of the base of the olfactory bulbs which would indirectly show regions

of CSF accumulation in this area. The rationale for this experiment was to extend data from a previous study by another group showing basal meningeal lymphatics having greater access to CSF compared to the dorsal meningeal lymphatics due to closer proximity to a large CSF reservoir shown by MRI **(Ji Hoon Ahn et al. *Nature* 2019)**; this study did not show any imaging data near the base of the olfactory bulbs near the cribriform plate despite this route being hypothesized to drain the majority of CSF. We reasoned that if cribriform plate lymphatics play a significant role in CSF efflux, they should reside in close proximity to a large volume of CSF as shown with the increased signal intensity along the base of the olfactory bulbs. While other studies have shown CSF accumulation near the cribriform plate and olfactory bulbs in both humans and mice, this is the first to compare CSF in this region between healthy and EAE mice, and between the different meningeal lymphatic networks. Nevertheless, per Reviewer #2's Point #11 and this current point, we are currently considering moving this figure to the Supplementary Data.

- The volume of 10 microliters over 5 minutes (2 microliters per minute) was chosen per previously published results by several groups, whom have independently found that injection of 10 microliters into the cisterna magna at a rate of 2 to 2.5 microliters/minute results in a mild elevation of intracranial pressure that returns to baseline levels within 5 minutes of cessation (see references below). Indeed, the elevation in intracranial pressure during the injection occurs even with smaller volumes (2 microliters), suggesting that elevation in intracranial pressures during the injection is unavoidable (reference #2). Interestingly, infusion of the smaller 2 microliter volume actually results in a decrease in intracranial pressure following cessation over the course of several hours (reference #2), suggesting that higher volumes of 5 to 10 microliters may be ideal to maintain steady-state levels of intracranial pressures similar to baseline.
References:

    - Aditya Raghunandan et al. "Bulk flow of cerebrospinal fluid observed in periarterial spaces is not an artifact of injection." *eLife* 2021.
    - Extended Data Figure 2 from Sandro da Mesquita et al. "Functional aspects of meningeal lymphatics in aging and Alzheimer's disease." *Nature* 2018.

Reviewer #2

(Remarks to the Author)

In their study, Hsu et al. demonstrate that cribriform plate lymphatic endothelial cells (cpLECs) expand and display an activated phenotype as a consequence of EAE induced neuroinflammation. The activation of cpLECs is characterized by expression of genes and molecules related to antigen processing and presentation as well as leukocyte adhesion and activation. The authors further show uptake of CNS-derived antigens and intimate contact with CD11c+ cells, most likely DCs, and T cells by the cpLECs during EAE. In contrast to cpLECs from healthy mice, cpLECs sorted from EAE animals were further able to activate T cells in an antigen-specific manner in in vitro co-cultures. The presence of an IFN-γ signature in cpLECs during EAE leads to the hypothesis that changes in their expression profile are at least partly mediated through IFN-γ. Indeed, in IFN-γ KO animals, reduced surface expression of PD-L1 and Podoplanin was observed on the cpLECs during EAE. The presence of PD-L1 on activated cpLECs lead the authors to suggest that interaction of DCs and T cells with these cpLECs result in tolerization of DCs in the context of neuroinflammation. This would fit the observation that IFN-γ KO animals display exacerbated EAE severity, potentially due to loss of tolerizing immune interactions.

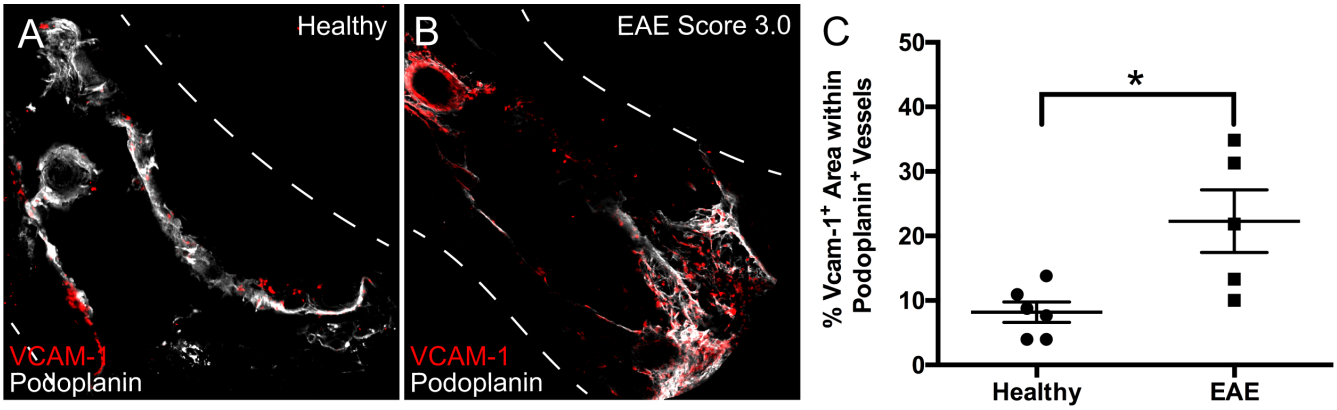
Overall, these are an intriguing findings. Both, lymphatics in the cribriform plate as well as lymphatic vessels in the dura mater have been shown to drain CNS-derived antigens and have also been suggested to play important roles for the activation of T cells during many neuropathologies, including neuroinflammatory as well as neurodegenerative disorders. The ability of cpLECs to sample CNS-antigens and function as APCs that can locally activate CNS-antigen reactive T cells.

However, several conclusions are thus far mainly hypothesis driven and need further substantiation through experimental evidence. This specifically refers to the claim that the change of the cpLEC phenotype happens through a state of cell proliferation, which is not evidenced by any hard data. Further, the potential activation/tolerization of DCs and T cells through cpLECs needs to be better characterized. Finally, the suggested tolerogenic role of both cpLECs and DCs in later stages of EAE through IFN-γ induced PD-L1 expression is intriguing, but without further data any experimental evidence for this hypothesis is missing.

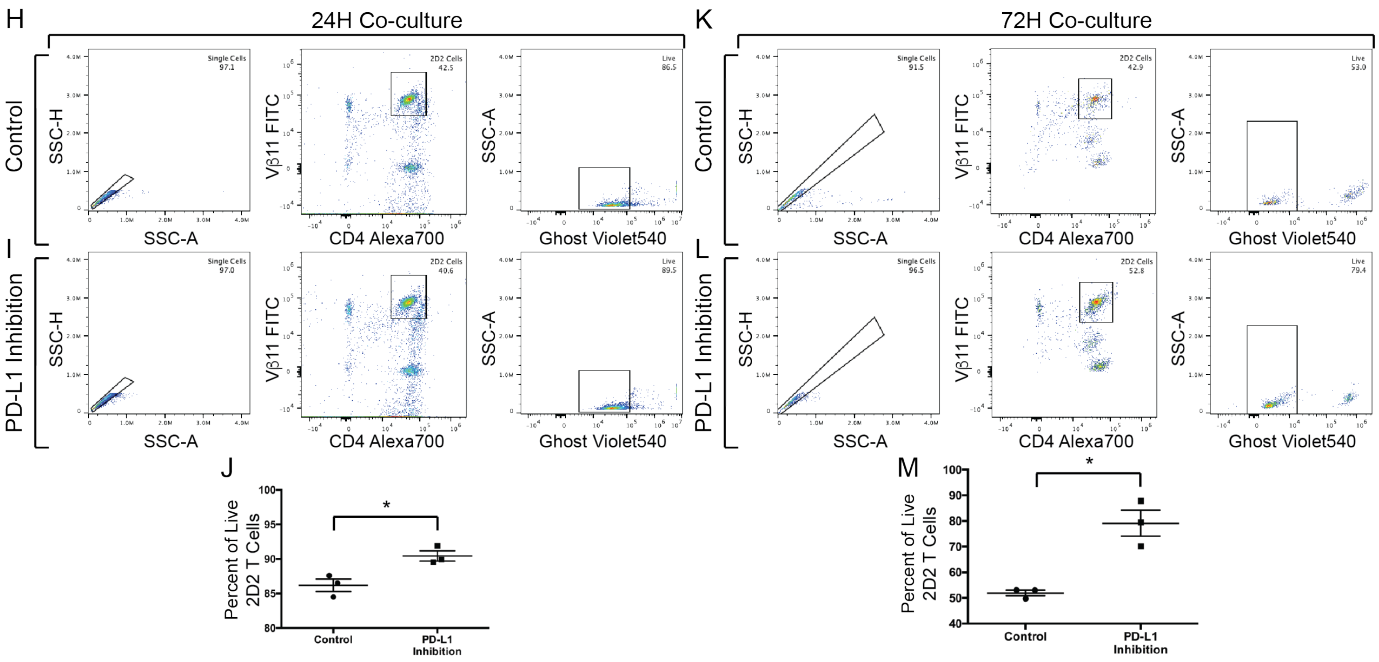
Main Comments

1. The authors have to further validate their findings from the scRNA-seq analysis on protein levels. Thus far, of all representative genes depicted in Fig. 1, they only demonstrate upregulation of MHCII on cpLECs. This could be either done by additional flow cytometry experiments or, even better, through confocal analysis.

- We thank the reviewer for this comment. We categorized the scRNAseq data into 4 main groups to make it easier to study different aspects of cpLEC phenotypes: 1) antigen processing & presentation, 2) adhesion and chemotaxis, 3) IFN-γ response genes, and 4) tolerance.
    - As the reviewer pointed out for antigen processing & presentation, we have confirmed that cpLECs have access to CNS-derived antigens, can express MHC II by immunohistochemistry/confocal imaging and flow cytometry, and can functionally present antigen to both naive antigen-specific 2D2 T cells and OT-II T cells.
    - For adhesion, we agree with the reviewer and have conducted immunohistochemistry/confocal imaging of an additional adhesion molecule that was shown to be upregulated y scRNAseq: Vcam-1 as shown below. This data has been added as an additional supplementary figure in the revised manuscript.



    - For IFN- γ response genes, we confirmed that PD-L1 is downstream of IFN-γ signaling, as IFN-γ deficient mice lack PD-L1 upregulation. This is also true for Podoplanin.
    - We also have shown PD-L1 upregulation by both cpLECs and dendritic cells using flow cytometry, and have also confirmed that PD-L1 expression by both cell types is regulated by IFN-γ. Functionally, we have added an additional *in vitro* experiment co-culturing EAE-primed cpLECs with 2D2 T cells in the presence or absence of PD-L1 inhibition, and found that cpLECs can functionally induce tolerance by mediating cell death through PD-L1 signaling. This data has been added to **Figure 4** as panels **H-M**:



2. The authors state themselves that scRNA-seq revealed expression of many myeloid-associated genes in the cpLECs. It is thus of great importance to perform solid quality control on the sequencing data, including doublet exclusion (see comments on Data and methodology).
    - The reviewer makes a valid point, as doublets in the cell suspension may explain the myeloid cell RNA signature in our scRNAseq dataset. A singlet gate was used to discriminate against doublets during the FACS sort prior to scRNA-seq. Doublets were also further excluded as a secondary measure during scRNA-seq by discriminating against doublet RNA signatures, which has been clarified in greater detail in the methods section as quoted below:
      "Barcodes containing unusually high numbers of detected transcripts indicative of a doublet signature were excluded, and cells that co-express marker genes of distinct cell types were also excluded. The resulting data were then analyzed and explored using the Loupe Cell Browser software and the R packages clusterProlifer and/or monacle3 after excluding non-LEC cells that made it through the FACS-enrichment. In total, 6,272 cells were identified and 1,218 cells were excluded due to non-LEC signatures. The median genes per cell in this analysis was 2,558, and the median UMI counts per cell was 7,480."

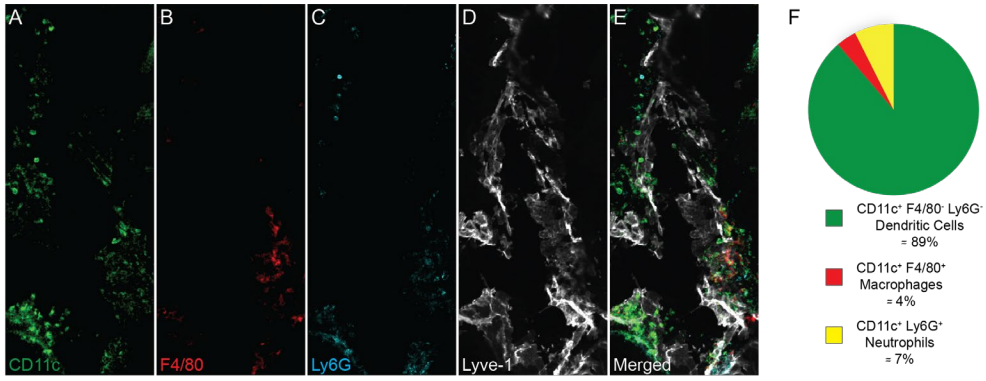
3. Visualization of single-cell trajectories through pseudotime infers trajectories based on the available gene expression data. However, this does not mean that it exactly and reliably predicts biological processes that happen in vivo. The authors claim, based on the results from the pseudotime analysis, that all lymphangiogenic cpLECs that derive from steady-state cpLECs have to transition through a stage of cell proliferation. This has to be proven by direct experimental evidence. Through using in vivo proliferation assays such as BrdU incorporation, the authors

could directly demonstrate that all cpLECs have undergone recent cell proliferation. If all lymphagiogenic cpLECs are derived from proliferating cpLECs.

- The reviewer makes a valid point, and we will alter the language in the text to reflect that although the pseudotime analysis infers proliferation, we cannot exclude the possibility of other processes that may explain lymphangiogenesis such as transdifferentiation. This is clarified in the results section as quoted below:
  "Of note however, scRNA-seq revealed expression of many myeloid-associated genes by cpLECs in addition to LEC genes despite exclusion of $CD45^{intermediate}$ microglia and $CD45^{high}$ leukocytes **(Supplementary Figure 1, 2)** at the protein level while FACS sorting. Therefore, we can confirm that increased proliferation of cpLECs may at least partially account for lymphangiogenesis as shown here and previously through increased Ki67 expression at the protein level [4]. However, we cannot exclude the involvement of myeloid cells in the origin of LECs as previously described and reviewed [34,35]."

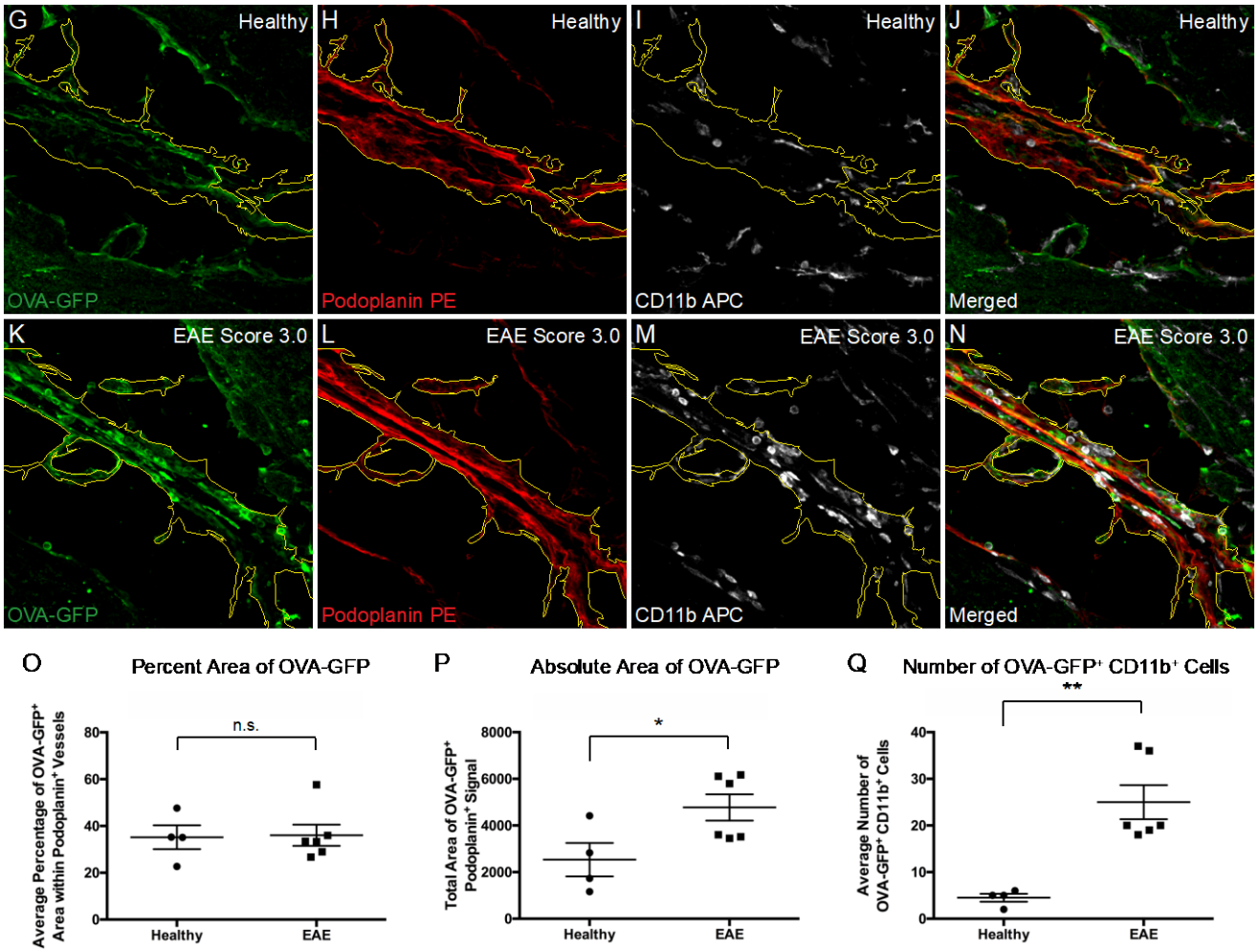
4. In Fig.2, the authors should be more conservative with the identification of the myeloid cell subsets. It is well known that CD11b expression can be found on many more cells than only macrophages (monocytes, neutrophils, NK cells etc.) and CD11c expression is not an exclusive marker for DCs, but can also be found on macrophages. If the authors wish to better distinguish the different myeloid cell types, they could include Ly6C, Ly6G, CCR2, CD64, F4/80 or further markers for different DC subsets in their analysis.
   - We agree with the reviewer that there are other leukocytes that can express CD11c, including activated macrophages. We demonstrate that the majority of $CD11c^{+}$ cells in this region can also express MHCII, and a recent study by Jonathan Kipnis's group has revealed that the meninges can also contain a population of MHCII+ antigen presenting macrophages. However, unlike dendritic cells, these macrophages seem to be unable to egress to the lymph nodes through lymphatics (likely due to the lack of CCR7 expression), but rather locally present antigen to T cells in the meninges **(Justin Rustenhoven et al. *Cell* 2021)**. We have previously demonstrated that the majority of CD11c+ cells near the cribriform plate expresses the migratory chemokine CCR7, indicative of migratory antigen presenting dendritic cells **(see supplementary figure 2, Hsu et al. *Nature Communications* 2018)**. Although CCR7 can be expressed by a subset of T cells, the presence of CD11c in combination with CCR7 should exclude T cells. Nevertheless, we do acknowledge that macrophages can make up a significant population of cells in this region, and after re-gating our flow data looking at leukocyte subtypes bound to cpLECs during EAE per Reviewer's #1 Point #3, there is a significant increase in the number of macrophages binding to cpLECs during EAE. Thus, macrophages have been added to the results section:
     - "Of the leukocytes, a significant increase in the number of $CD11c^{high}$ $CD11b^{+}$ dendritic cells (≈ 173-fold higher than steady-state) and to a lesser extent $CD11b^{+}$ $CD11c^{low}$ myeloid cells (≈ 135-fold higher than steady-state) and $CD4^{+}$ T cells (≈ 21-fold higher than steady-state) were bound to cpLECs **(Figure 2B)**."

- We were also curious about the different myeloid cell populations that may bind to cpLECs, and have consequently performed additional immunohistochemistry/confocal imaging for CD11c, Ly6G, and F4/80 to distinguish dendritic cells ($CD11c^+$, $F4/80^{negative}$), neutrophils ($Ly6G^+$), and macrophages ($F4/80^+$). Indeed, immunolabeling of CD11c+ cells near cpLECs reveal that the majority of them are negative for Ly6G and F4/80, suggesting that most of the $CD11c^+$ cells are dendritic cells. This has been added to the results section, which along with the data is shown below:
"Previous characterization of $CD11c^+$ dendritic cells near cpLECs reveal their expression of CCR7 [4], and further characterization of $CD11c^+$ cells reveal that the majority of these cells are indeed $Ly6G^-$ and $F4/80^-$, excluding $CD11c^+$ expression by neutrophils/macrophages.



5. In Fig.3 A-C, OVA-GFP signal was found close to or even in cpLECs in EAE animals. However, healthy Ctrl animals are missing to demonstrate that this is an EAE specific phenomenon.
   - The reviewer makes a valid point, and we have included healthy control animals to compare the amount of OVA-GFP signal between healthy and EAE. In addition to confocal images, we have also quantified the amount of $OVA\text{-}GFP^+$ area present within cpLECs or within $CD11b^+$ myeloid cells in this region, which revealed a significant increase in $OVA\text{-}GFP^+$ uptake by both cpLECs and $CD11b^+$ monocytes. Quantitation of $OVA\text{-}GFP^+$ uptake by Podoplanin+ LECs specifically is shown both by percent area of $Podoplanin^+$ cpLECs and as total area within $Podoplanin^+$ cpLECs after exclusion of $CD11b^+$ monocytes. This data has been added to the supplementary information as shown below, and the results section have been updated as quoted below:
   - "Supplemental immunolabeling with CD11b confirms that there is indeed OVA-GFP+ expression within Podoplanin+ MHC II+ LECs outside of potential CD11b+ antigen presenting cells that may be migrating through LECs. This is true for both healthy and EAE, consistent with the idea that immune surveillance occurs even during steady-state conditions 41. The average percent area of non-CD11b+ Podoplanin+ LECs containing OVA-GFP did not change between healthy and EAE, likely due to increased Podoplanin+ area due to lymphangiogenesis. Quantifying total area of OVA-GFP within CD11b-

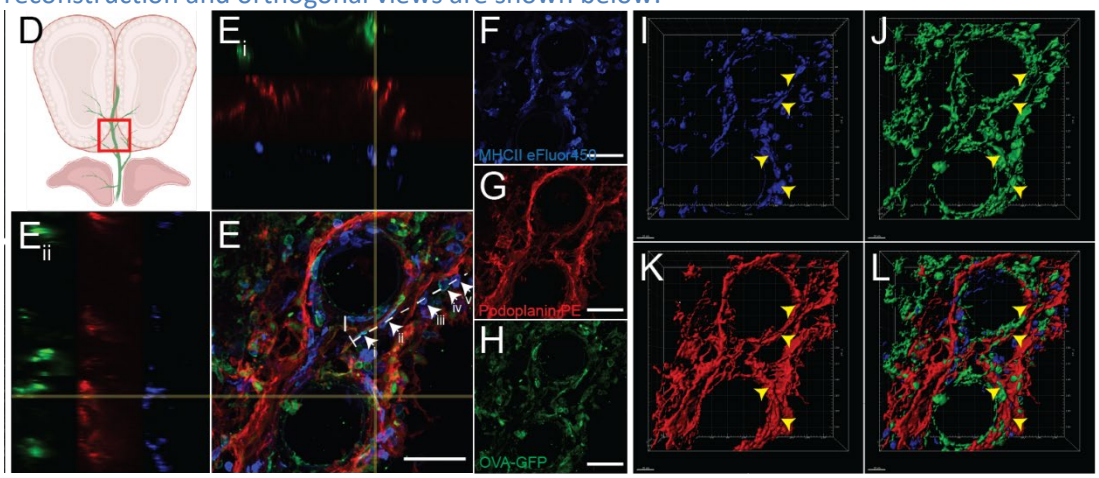
Podoplanin+ LECs during EAE reveal a significant increase relative to healthy controls, suggesting increased CNS-derived antigen accumulation within cpLECs during neuroinflammation. Furthermore, there was also a significant increase in the number of CD11b+ cells containing OVA-GFP within Podoplanin+ LECs during EAE, indicating that additional OVA-GFP accumulation near the cribriform plate may also be picked up by recruited myeloid cells. Alternatively, a subset of CNS-derived OVA-GFP may have been carried to cpLECs by myeloid cells."



6. The representative images in Fig.3 do not allow to judge whether or to which extend OVA-GFP is found within cpLECs. It seems that many of the cells depicted with the yellow arrows in Fig.3 D-H (which are supposed to be cpLECs) are actually negative for Podoplanin and are rather lying next to the lymphatic vessels. This would suggest them to be MHCII-expressing APCs that are in intimate contact to the lymphatics. Counterstaining with myeloid markers such as CD11b would

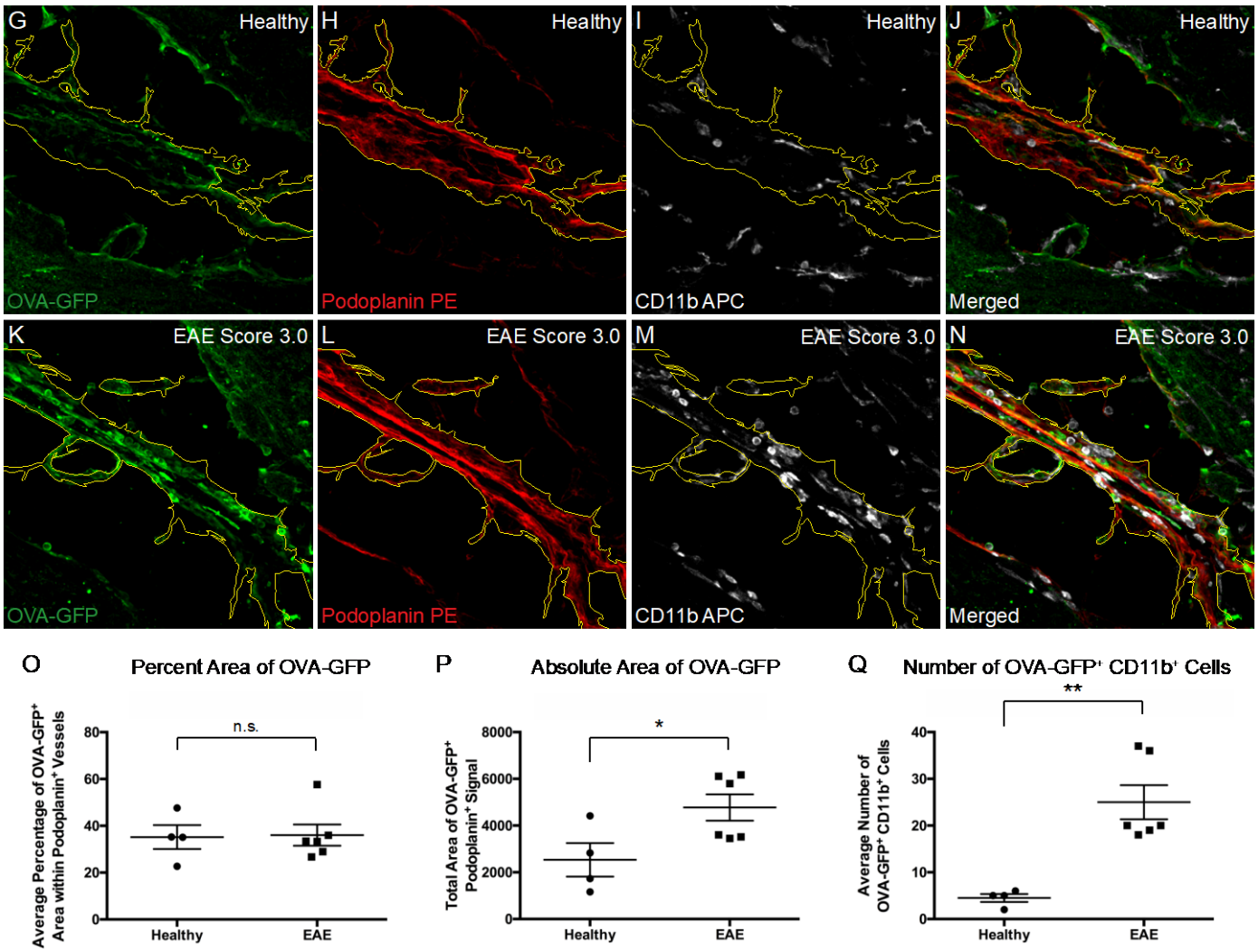
help to discriminate between cpLECs and APCs. To demonstrate the presence of OVA-GFP within the cpLECs, images with higher magnification are therefore needed. The authors could also use IMARIS based cell reconstruction to show the subcellular location of the OVA-GFP signal. Quantification of the amount of OVA-GFP+ cpLECs in healthy versus EAE mice would also helpful. One option would also be to test whether OVA-GFP signal can be detected in flow cytometry.

- We agree that some of the yellow arrowheads point to potentially APCs in contact with LECs, and others point to unclear cells due to the fact that Podoplanin may have heterogenous expression within LECs where it is enriched at the cell membrane to facilitate APC migration (see next point), and have done the following to address this:
    - Instead of showing a zoomed out confocal image followed by higher magnification images, we show the higher magnification confocal images in parallel with IMARIS 3D reconstruction images of each channel separately, with yellow arrowheads showing OVA-GFP/MHCII+ expression by Podoplanin+ cells as determined by 3D reconstruction using IMARIS. Furthermore, we also provided an example of an OVA-GFP+ MHC II+ Podoplanin+ cell using orthogonal views to confirm that a cpLEC expressing Podoplanin can also contain OVA-GFP and MHC II expression in multiple viewing planes. IMARIS 3D reconstruction and orthogonal views are shown below:



    - We also added an additional supplementary figure using CD11b to discriminate between cpLECs and APCs as an additional marker per point #5. We have added healthy control images, followed by quantitation comparing the accumulation of OVA-GFP+ peptides between healthy and EAE in either CD11b$^-$ Lyve-1$^+$ LECs or CD11b$^+$ antigen presenting cells within or in contact with Lyve-1$^+$ LECs (see

response to point #5). The data is also shown again below for the reviewers' convenience:

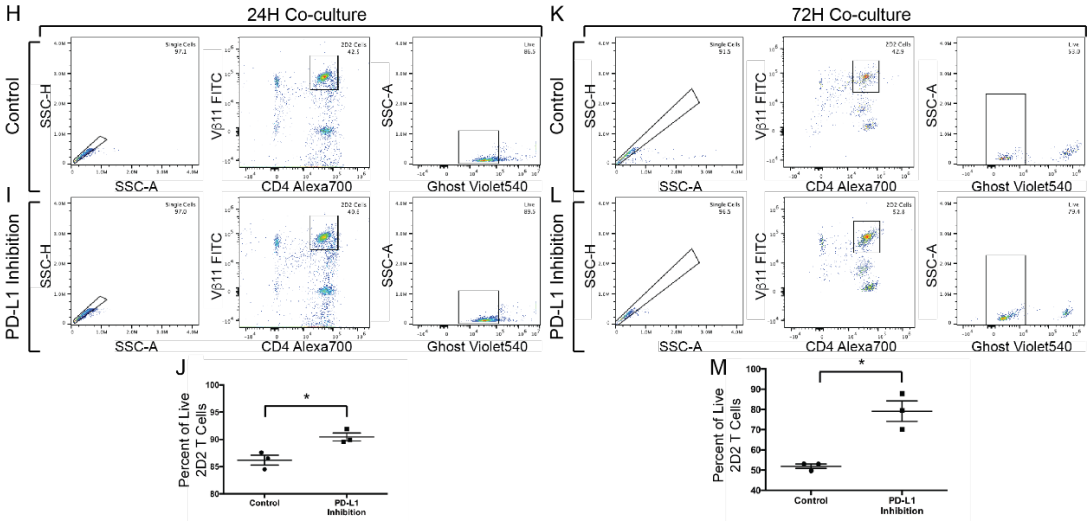


7. Similarly, in Fig.3 I it seems that the Podoplanin signal is actually stronger in-between the cells that the authors claim to be MHCII+ OVA-GFP+ cpLECs.
    - The reviewer makes an interesting point: although Podoplanin is expressed throughout the lymphatic vessel, signal intensity analysis versus distance across a vessel show that in some instances, slightly higher expression of Podoplanin can be seen at the border of cells. We believe that this may be due to the fact that Podoplanin is a membrane-bound protein involved in dendritic cell transmigration, where the membranes between cells (particularly LEC and dendritic cell) contain higher expression of Podoplanin. Indeed,

Podoplanin deficiency causes reduced dendritic cell migration across the LEC endothelium and seems to be important for dendritic cell contact and adhesion or "spreading" to the LEC (Sophie E. Acton et al. "Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2." *Immunity* 2012.

8. In Fig.3 V-Aa, the authors show activation of CD4+ T cells through cpLECs in vitro. It would be very interesting to know which subset of CD4+ T cells is induced through this interaction. As the EAE model is mainly driven by Th1/Th17 cells, it would be interesting to know whether cpLECs can induce these T cell subsets. Can cpLECs also induce Treg cell subsets? The authors should thus further characterize the in vitro stimulated T cells, e.g. by flow cytometry using more markers (such as Il-17, IFN-γ etc. and transcription factors like Foxp3).
    - We agree that the fate or T cell phenotype after its interaction with a LEC is an important and relevant question and is the focus of future studies. However, we also feel that showing functional expression data to validate antigen presentation and/or tolerance through PD-L1 will add significance to this manuscript. We therefore have performed the following experiments to: 1) test if cpLECs can functionally present antigen to naïve 2D2 T cells, 2) skew effector T cell phenotypes, and/or 3) affect T cells functions:
        - In order to test the mechanism of PD-L1 tolerance on naive 2D2 T cells, we co-cultured EAE-primed cpLECs after cell sorting with magnetically purified naïve 2D2 T cells in the presence or absence of PD-L1 blocking antibody. We then measured T cell activation (CD69 expression), proliferation (dilution of Cell Trace), and cell viability (Ghost labeling). After 24H or 72H, inhibition of PD-L1 did not affect T cell expression of CD69 or proliferation. However, there was a significant decrease in cell death after PD-L1 inhibition after 24H, and a significant inhibition of cell death after 72H (approximately 30% reduction; shown below). These data suggest that PD-L1 does not affect the initial activation and proliferation of naïve 2D2 T cells but induces cell death as previously described (reference). This is consistent with the expression of the PD-L1 receptor PD-1 by T cells, where T cell activation by naïve T cells is necessary for the expression of PD-1 and consequently PD-L1/PD-1 mediated tolerance (reference). Therefore, it seems that antigen processing/presentation is necessary to activate naïve 2D2 T cells for PD-L1/PD-1 mediated cell death. This experiment has been added to **Figure 4** as panels **H-M**, and the results section has been updated as shown below:
        - "We next wanted to test how PD-L1 may functionally effect 2D2 T cells in the presence of cpLECs. To do this, we co-cultured EAE-primed cpLECs with naive 2D2 T cells *in vitro* in the presence or absence of PD-L1 blocking antibody **(Figure 4H-M)**. 2D2 T cells were able to sufficiently express CD69 after 24 hours and proliferate after 72 hours as shown before, and PD-L1 inhibition had no effect on the expression of CD69 or the frequency and rate of proliferation **(data not shown)**. This is consistent with the observation that naive T cells do not

express PD-1 [52], and require activation/proliferation to express PD-1. In contrast, PD-L1 inhibition slightly improved cell viability after 24 hours and significantly improved cell viability after 72 hours of co-culture **(Figure 4H-M)**, suggesting that PD-L1 signaling by cpLECs contributes to 2D2 T cell death without influencing the initial activation and proliferation of 2D2 T cells in this system."

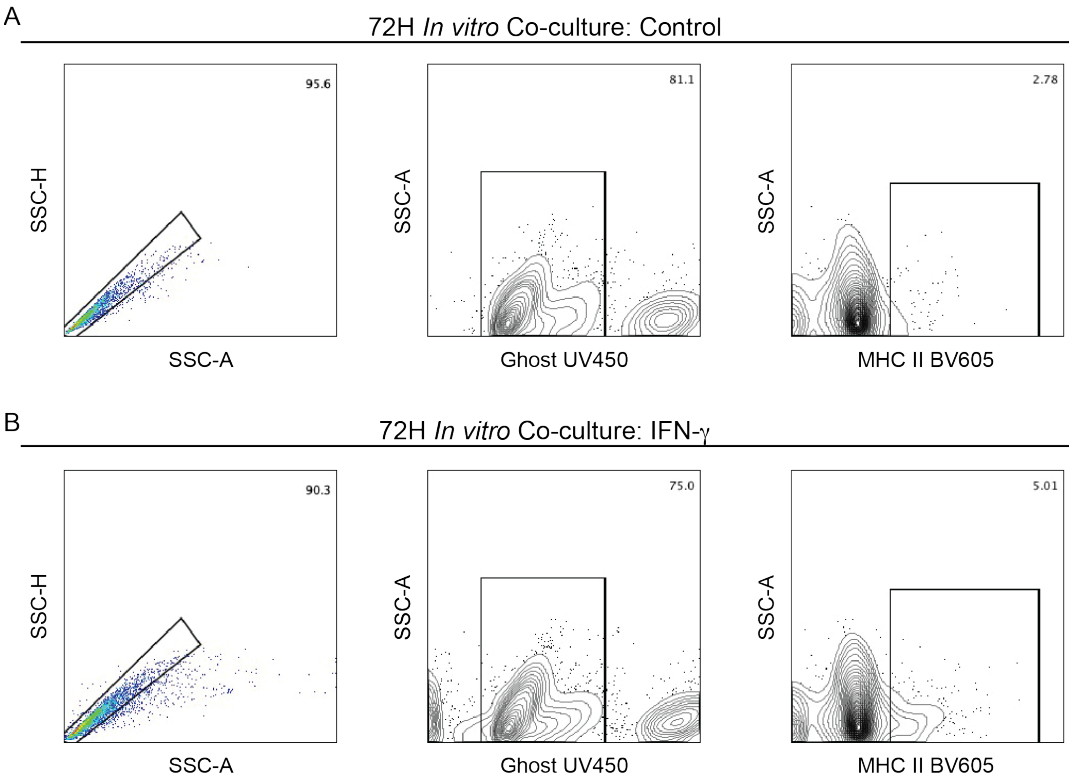


- We next looked to see how cpLECs can influence effector T cells by co-culturing healthy or EAE-primed cpLECs with T cells isolated from the CNS of EAE mice for 24 hours, followed by re-stimulation to visualize the frequency of Foxp3, IL-17, and/or IFN-γ^+ T cells. These T cells should have been initially activated in the draining lymph nodes, have up-regulated PD-1, and differentiated into effector T cells. In line with this, healthy cpLECs + CNS-derived T cells from EAE mice resulted in baseline levels of Th1, Th17, and Treg cell frequencies. When co-cultured with EAE-primed cpLECs, there was a trending but non-significant reduction in the frequency of IL-17 producing Th17 cells (50%), a significant reduction in IFN-γ producing Th1 cells (25%), and no difference in Foxp3 Treg cell frequencies or cell viability. These data suggest that EAE-primed cpLECs can interact with differentiated effector T cells that have already re-encountered their cognate antigen by shunting the Th1/Th17 T cell response.

9. On the other hand, EAE derived cpLECs seem to express high levels PD-L1. Interaction of PD-L1 with PD-1 on T cells usually attenuates T cell activation, which contradicts the observation of strong T cell activation. How do the authors explain this discrepancy.
   - We agree with the reviewer that a tolerizing role through PD-L1 upregulation and activation through antigen presentation seems contradictory. We have attempted to address these concerns in the previous point by performing a series of co-culture experiments using a variety of T cells with or without PD-L1 inhibition, where it seems

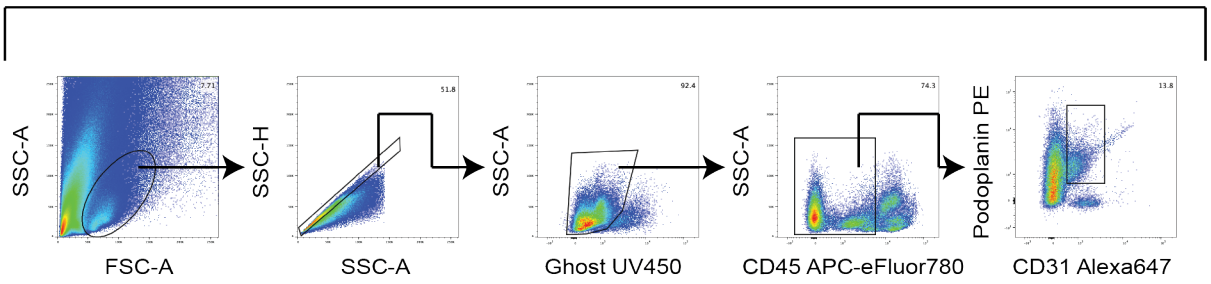
like the ability of cpLECs to effect CD4 T cells depends on the cell state. Naïve T cells that lack PD-1 expression can be activated by EAE cpLECs to upregulate CD69 and undergo proliferation, followed by PD-L1 dependent cell death as activated T cells begin to express PD-1. Effector T cells isolated from the CNS of EAE mice, which likely have already undergone differentiation might be tolerized by reducing Th1 T cell frequencies by EAE cpLECs. Finally, effector T cells isolated from the CNS of EAE mice that have been pushed towards an exhaustive phenotype require PD-L1 by EAE cpLECs to maintain the inhibitory phenotype. It should also be noted that dendritic cells were found to bind to cpLECs during EAE at high percentages, and other groups have demonstrated the ability of dendritic cells to both gain access to LEC-derived antigens as well as express PD-1. Future studies are aimed at investigating the potentially complex interplay between cpLECs, dendritic cells, and T cells.

10. In the same line, it would be interesting to see if and which co-stimulatory molecules are present on cpLECs. Considering that the antigen processing and presentation capabilities of cpLCEs might be regulate through INF-γ signaling, treatment of isolated cpLECs with INF-γ in vitro could be used to assess changes in cpLECS.
    - The reviewer makes an interesting point about co-stimulatory molecules, as their expression or lack of expression can influence T cell activation/tolerance. Mechanisms of tolerance, their influence on leukocyte phenotypes, and their consequences to pathology are the subject of future studies, including the expression or lack of expression of co-stimulatory molecules.
    - The role of IFN-γ in regulating the phenotype of cpLECs is also relevant, as there is substantial evidence in the literature suggesting that MHC II can be regulated by IFN-γ. We have performed the suggested experiment asking the question if IFN-γ is sufficient for MHCII expression: we treated naïve cpLECs with IFN-γ *in vitro* and measured MHC II expression by flow cytometry after 72 hours of culture. Although there is a slight increase in MHC II expression, the difference in frequency from 3% to 5% is negligible compared to our *in vivo* data, suggesting that IFN-γ alone is not sufficient for MHCII expression. One potential explanation is that CIITA transcription may not only require induction by IFN-γ, but may also be suppressed during steady-state conditions and consequently requires both the presence of an inducible cytokine such as IFN-γ and downregulation of a repressor signal for expression. Other regulators of CIITA and MHC II expression are also likely involved, as many other genes are altered during EAE. Another potential explanation is that dendritic cells have been shown to transfer MHCII molecules to non-professional APCs including LECs, which may occur independently of IFN-γ (Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, LeibundGut-Landmann S, Garbi N, Reith W, Swartz MA, Hugues S. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific $CD4^+$ T cell tolerance. J Exp Med. 2014 Jun 2;211(6):1153-66. doi: 10.1084/jem.20132000. Epub 2014 May 19. PMID: 24842370; PMCID: PMC4042642). Indeed, dendritic cells bind to cpLECs during EAE with relatively high frequencies. Assessing the complex interplay between different leukocyte interactions with cpLECs and the consequences of these interactions is the subject of future studies.

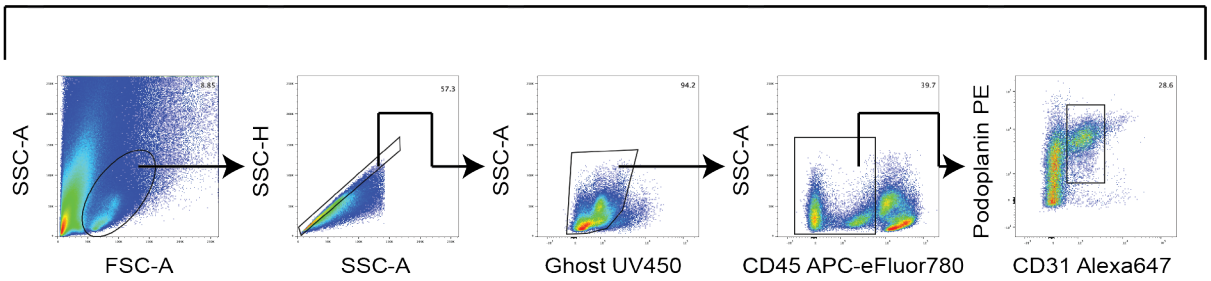


11. The authors state that cpLECs still undergo lymphangiogenesis in IFN-γ KO animals during EAE. However, in Supp. Fig. 9 only quantification of cpLEC-CD11c+ cell doublets is shown. This data has to be corrected or else, the statement is wrong.
    - We believe that there may have been an error in uploading the correct version of this figure. The correct supplementary figure 9 shown below shows LECs being gated from live singlet gates, with the quantitation showing the average number of LECs in the singlet gate per group:

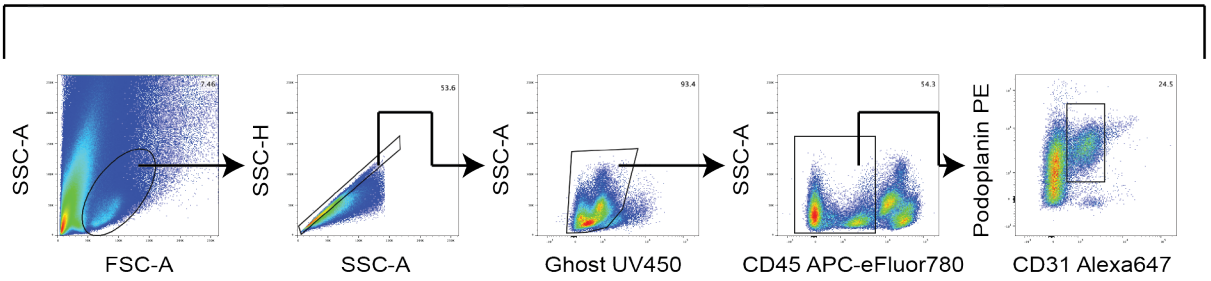
A


Wild-type Healthy
SSC-A
FSC-A
SSC-H
SSC-A
SSC-A
Ghost UV450
SSC-A
CD45 APC-eFluor780
Podoplanin PE
CD31 Alexa647

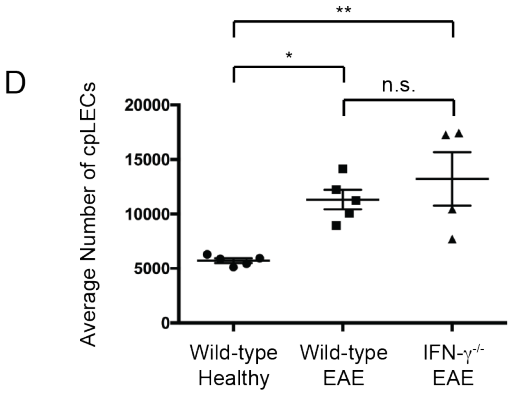
B


Wild-type EAE
SSC-A
FSC-A
SSC-H
SSC-A
SSC-A
Ghost UV450
SSC-A
CD45 APC-eFluor780
Podoplanin PE
CD31 Alexa647

C


IFN-γ-/- EAE
SSC-A
FSC-A
SSC-H
SSC-A
SSC-A
Ghost UV450
SSC-A
CD45 APC-eFluor780
Podoplanin PE
CD31 Alexa647

D


**
*
n.s.
Average Number of cpLECs
20000
15000
10000
5000
0
Wild-type Healthy
Wild-type EAE
IFN-γ-/- EAE

12. The data in Fig.5 and Fig.6 is very interesting, but also rather descriptive so far. It should be considered to merge the data into a single figure.
    - The reviewer makes an interesting point, and we will discuss this in further detail along with the editor.

Data and methodology

13. Many information regarding methodology of the scRNAseq analysis are missing: Which chemistry has been used? Have doublets been excluded from the analysis? Which exact R packages have been used for the analysis? Further, the sequencing debts appears to be quite low. Can the authors explain why the targeted read depth was only 7.500? How many genes were detected per cell on average?
    - We agree with the reviewer and have added additional information into the methods section to elaborate on how the scRNAseq analysis was done as well as the number of genes detected per cell on average as shown below. The R packages used for analysis are added to the scRNAseq subsection in the methods section as shown below, as well as reiterated in the Gene Ontology and Cell Trajectory subsections of the methods section. Lastly, the targeted read depth was actually 48,000 per cell; this was a typo, as we meant the median UMI count per cell was approximately 7,500. This is also fixed in the methods section as shown below.
      "5 healthy mice were pooled for the control group, and 5 EAE mice were pooled for the experimental EAE group. A single cell suspension of the cribriform plate and its associated tissues were generated as described, and cpLECs were FACS sorted for singlets, Ghost$^-$ live cells, CD45^{low}, Podoplanin$^+$, and CD31$^+$ [2,6,8,10,26]. Sorted cpLECs were then provided to the Biotechnology Core facility at the University of Wisconsin Madison for single cell sequencing using the 10x Genomics Chromium Single Cell Gene Expression Assay. A total of 25,102 cells from the control group and 53,224 cells from the experimental group post-sort were provided to the Biotechnology Core for scRNA-seq. Cells were loaded onto a Chromium Controller to generate a single cell + barcoded gel-bead emulsion. Libraries were prepped with the 10X Genomics 3' reagents kit (v3 chemistry). The target recovery rate was 3,000 cells with a targeted read depth of 48,000 per cell. Cells were sequenced on the NovaSeq S1 100-cycle flow cell in collaboration with the University of Wisconsin Biotechnology Center (UWBC) DNA Sequencing Facility. Data was then analyzed using a custom developed single cell data analysis pipeline generated by the UWBC Bioinformatics Resource Center. Briefly, experimental data were demultiplexed using the Cell Ranger Single Cell Software Suite, mkfastq command wrapped around Illumina's bcl2fastq. The MiSeq balancing run was quality controlled using calculations based on UMI-tools. Sample libraries were balanced for the number of estimated reads per cell and ran on Illumina's NovaSeq system. Barcodes containing unusually high numbers of detected transcripts indicative

of a doublet signature were excluded, and cells that co-express marker genes of distinct cell types were also excluded. The resulting data were then analyzed and explored using the Loupe Cell Browser software and the R packages clusterProlifer and/or monacle3 after excluding non-LEC cells that made it through the FACS-enrichment. In total, 6,272 cells were identified, and 1,218 cells were excluded due to non-LEC signatures. The median genes per cell in this analysis was 2,558, and the median UMI counts per cell was 7,480. Cell Ranger software was then used to perform demultiplexing, alignment, filtering, barcode counting, UMI counting, and gene expression estimation for each sample according to 10x Genomics."

14. It has to be clearly stated EAE mice of which score or time point after immunization were used for every experiment. This applies to nearly all experiments throughout the manuscript.

We incorporated this information to the methods section under the EAE immunization subsection and have also included this in all the figure legends where appropriate. An EAE clinical score of 3.0 at day 15 post-immunization was used for all experiments as now mentioned in the methods section under the "EAE Induction" subsection: "An EAE clinical score of 3.0 at day 15 post-immunization was used for all experiments." Additionally, we have added the following statement near the beginning of the results section: "For the EAE group, an EAE clinical score of 3.0 at 15 days post-immunization was used for all experiments."

15. The antibody amount and site of injection for the CD45 in vivo labeling has to be clearly mentioned. Also, even though most immune cells in contact with cpLECs are not labeled by the i.v. injection of CD45 three minutes before perfusing the animals, this does not formally allow the conclusion that the immune cells have derived from the CNS parenchyma. They could also have been derived from blood before the three minute incubation, or could have been in the meningeal compartment. In addition, it would be helpful to show the labeling efficiency in blood leukocytes.

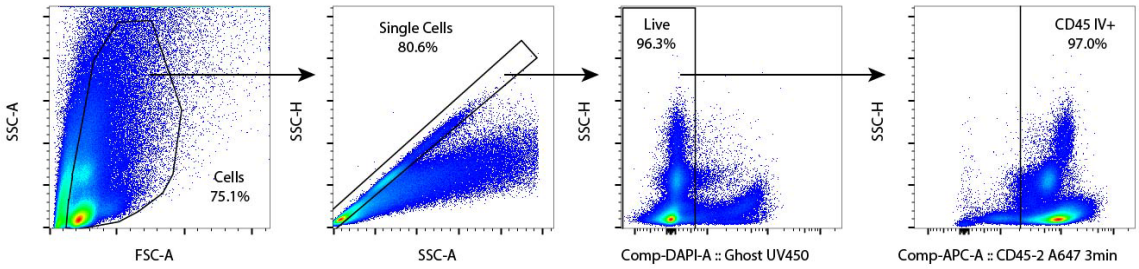
- We have added the CD45 antibody amount and concentration into the methods section as quoted below, along with a citation to the original article describing this technique: "Each mouse received 2.5 µg of CD45 conjugated to Alexa647 (Biolegend, Catalog #: 109818) in 200 µL of PBS as previously described [70]."

  Reference #70: Anderson, K. G. *et al.* Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat. Protoc.* **9**, 209–222 (2014).

- We also agree with the reviewer that the CD45 labeling does not allow for the conclusion of CNS-derived cells, and have removed this statement from the manuscript and simplified our statement to exclude blood-derived cells as shown below: "We excluded the possibility of blood-derived leukocytes binding to cpLECs either *in vivo* through direct access between blood and lymphatic vessels, or *ex vivo* through blood-contamination by intravenously (IV) labeling blood-derived leukocytes with

CD45.2 conjugated to BV711 *in vivo*, 3 minutes before harvest (Supplementary Figure 5). After perfusion, the majority of leukocytes binding to cpLECs were not blood-derived (≈ 96%) (Supplementary Figure 5)."

The efficiency of I.V. labeling blood-derived leukocytes using CD45 antibody has been shown to be quite efficient (>95%), where the antibody remains on the cell for over 3 days. In addition to the cited reference[70], we have also validated the efficiency of our I.V. labeling as greater than 95% as shown below after 3 minutes. Briefly, 2.5 μg of CD45 antibody conjugated to Alexa Fluor 647 in 200 μL of PBS was intravenously injected into wild-type mice, and blood was collected for analysis after 3 minutes after red blood cell lysis. The following statement has also been added to the methods section for clarification: "Unperfused mice were used as positive controls to visualize



blood-derived binding of leukocytes to cpLECs, where intravenous delivery of CD45 antibody labeled at least 95% of blood-derived leukocytes after 3 minutes."

16. How many animals were included in the analysis in Fig.5?
    - We have included this information in the figure legend. Figure 5 shows representative images from 3 sections per mouse spanning different depths of the cribriform plate from 3 mice per group. In total, 9 sections were analyzed per group.

Minor comments

1. Why did the authors decide to use tSNE plots for Fig. 1 and not UMAP as in Supp. Fig.4?
    - The reviewer makes an interesting observation; we intentionally used tSNE plots for the main figures as we felt that this was a more standardized way of visualizing the data. However, we did not intentionally choose UMP for the supplementary figure for any particular reason other than it was the default portrayal of the data after the analysis.
2. The exact number of cells from both healthy and EAE mice as well as the total number of cells included in the scRNAseq analysis should be specified

- We agree with the reviewer, and this information has been clarified in the methods section as shown below:
  "5 healthy mice were pooled for the control group, and 5 EAE mice were pooled for the experimental EAE group. A single cell suspension of the cribriform plate and its associated tissues were generated as described, and cpLECs were FACS sorted for singlets, Ghost^{-} live cells, CD45^{low}, Podoplanin^{+}, and CD31^{+} [2,6,8,10,26]. Sorted cpLECs were then provided to the Biotechnology Core facility at the University of Wisconsin Madison for single cell sequencing using the 10x Genomics Chromium Single Cell Gene Expression Assay. A total of 25,102 cells from the control group and 53,224 cells from the experimental group post-sort were provided to the Biotechnology Core for scRNA-seq. Cells were loaded onto a Chromium Controller to generate a single cell + barcoded gel-bead emulsion. Libraries were prepped with the 10X Genomics 3' reagents kit (v3 chemistry). The target recovery rate was 3,000 cells with a targeted read depth of 48,000 per cell. Cells were sequenced on the NovaSeq S1 100-cycle flow cell in collaboration with the University of Wisconsin Biotechnology Center (UWBC) DNA Sequencing Facility. Data was then analyzed using a custom developed single cell data analysis pipeline generated by the UWBC Bioinformatics Resource Center. Briefly, experimental data were demultiplexed using the Cell Ranger Single Cell Software Suite, mkfastq command wrapped around Illumina's bcl2fastq. The MiSeq balancing run was quality controlled using calculations based on UMI-tools. Sample libraries were balanced for the number of estimated reads per cell and ran on Illumina's NovaSeq system. Barcodes containing unusually high numbers of detected transcripts indicative of a doublet signature were excluded, and cells that co-express marker genes of distinct cell types were also excluded. The resulting data were then analyzed and explored using the Loupe Cell Browser software and the R packages clusterProlifer and/or monacle3 after excluding non-LEC cells that made it through the FACS-enrichment. In total, 6,272 cells were identified, and 1,218 cells were excluded due to non-LEC signatures. The median genes per cell in this analysis was 2,558, and the median UMI counts per cell was 7,480. Cell Ranger software was then used to perform demultiplexing, alignment, filtering, barcode counting, UMI counting, and gene expression estimation for each sample according to 10x Genomics."

3. Fig.1 G-J: Instead of log fold changes between the clusters, violin blots should be used to show the gene expression levels for all clusters. This would allow unbiased visualization of gene expression on the single-cell level.
   - We agree with the reviewer and have changed the bar graphs in Figure 1 to Violin plots.
4. How many mice/sections have been analyzed in Fig.5. AQP4 expression in dorsal and basal mLVs?

- We have included this information in the figure legend. Figure 5 shows representative images from 3 sections spanning different depths of the cribriform plate per mouse from 3 mice per group. In total, 9 sections were analyzed per group.

5. General suggestion: Changing the y-axis in histograms from 'counts' to 'modal' scale might improve the readability as the curves for all cell types would then have a similar height.
   - The reviewer makes an interesting point of whether to plot "count" versus "modal." We would prefer to keep the y-axis in the histograms as "counts", as we feel this allows the reader to better compare general cell numbers between the groups and increases transparency of our data. For example, increased numbers of cpLECs or dendritic cells that express PD-L1 during EAE can be reflected in the histograms compared to steady-state. However, we can change the histograms to "modal" if the reviewer and/or editor requests.
6. Some minor mistakes were made in the labeling of graphs or text of the figure legends (non-exhaustive list below).
   a. Fig. 4: The labels in the histogram are wrong. It should say 'IFN-γ KO', not 'EAE cpLECS + Leukocytes'

   b. Legend of Fig, 6c has a typo: nec neck

   c. Supp. Fig. 4: monAcle3 monocle3

   - We apologize for the mistakes and have corrected the above mistakes as well as additional mistakes.

Reviewer #3

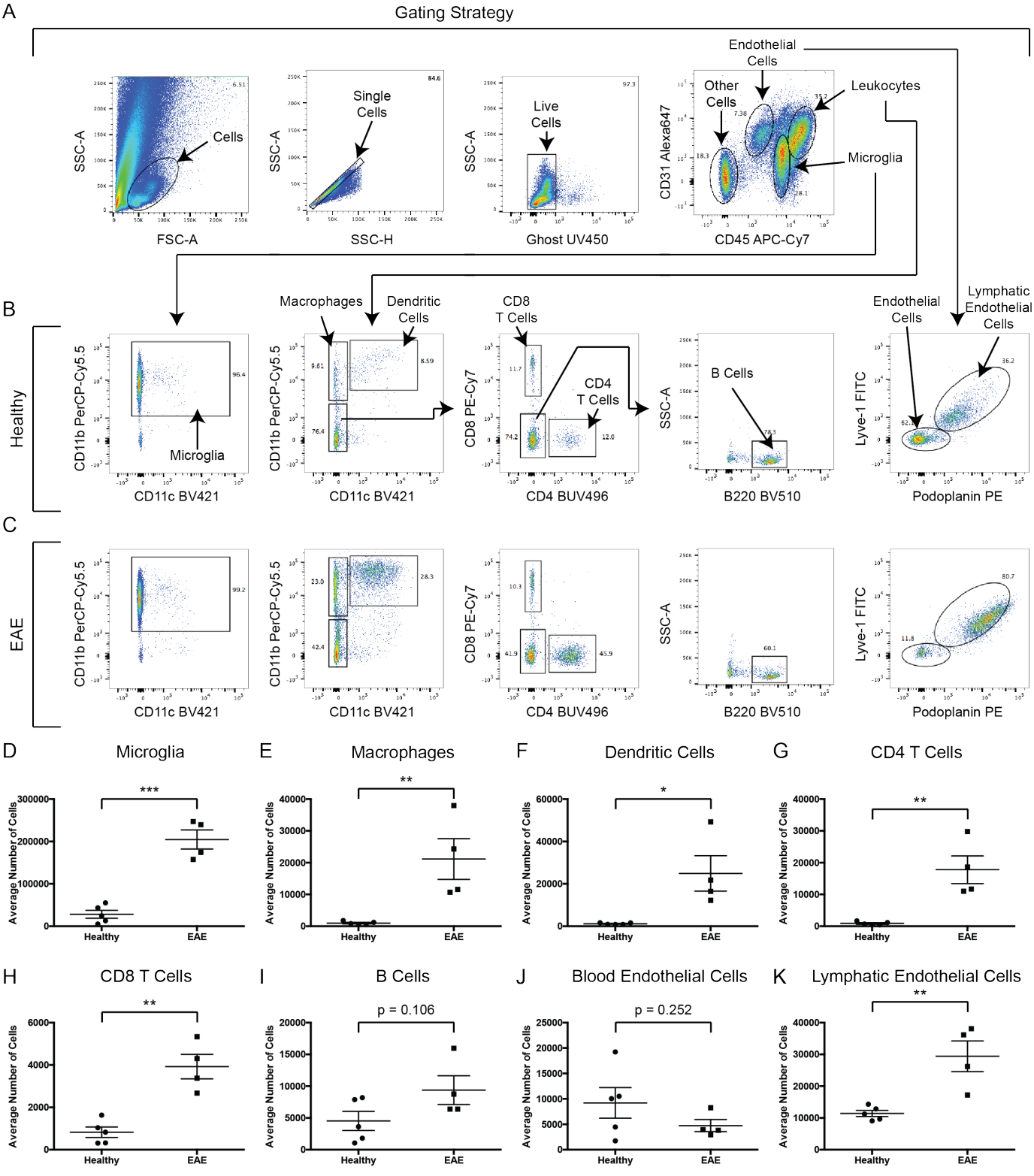
(Remarks to the Author)

This is a manuscript from a group that previously has shown that VEGFR3 triggers lymphangiogenesis of meningeal lymphatics in the vicinity of cribriform plate in experimental autoimmune encephalomyelitis (EAE). In this study, the group shows that EAE changes the phenotype of cribriform plate lymphatic endothelial cells using single-cell RNA sequencing. The genes upregulated are involved in antigen presentation, adhesion and immunoregulatory molecules. The inflamed lymphatic endothelial cells hold on to dendritic cells creating an immune-regulatory niche. Also, discontinuity of the arachnoid membrane near lymphatic endothelial cells allows direct access CSF. The study is a logicfollow-up on the previous publication, but I am not convinced that more than details are added to what we already know using new techniques.

Major critique:

1. Venous endothelial cells upregulate podoplanin expression in inflammation. Thus CD31+, podoplanin+ cells are not necessarily lymphatic endothelial cells. Also, venous endothelial cells are significantly affected by inflammation exhibited increased adhesion. Additional validation is needed.
    - We agree with the reviewer that venous endothelial cells can also express CD31 and Podoplanin under certain inflammatory conditions. Further validation of CD31/Podoplanin by flow cytometry using an additional LEC marker that is not expressed by venous endothelial cells such as Lyve-1 confirms that all of the $CD31^{+}$ $CD45^{negative}$ cells that express Podoplanin also express Lyve-1 **(Supplementary Figure 2, shown below)**. This suggests that venous endothelial cells specifically in this region do not upregulate Podoplanin during EAE. Blood endothelial cells including venous endothelial cells can be found in the $CD31^{+}$ $CD45^{negative}$ gate, where they are $Podoplanin^{negative}$ and $Lyve\text{-}1^{negative}$. We have clarified this in the text with the following sentence:
      "Furthermore, gating on $CD31^{+}$ endothelial cells confirms that lymphatic endothelial cells in this gate express both Podoplanin and Lyve-1, excluding venous endothelial cells."

nature research



2. Inflamed tissue is more edematous and dissociate better. That the authors are capable of isolate a larger number of lymphatic endothelial cells does not prove an increase in their number. Stereology of sections with the appropriate markers are needed
    - The reviewer makes an interesting point about inflamed tissues dissociating easier, which may in part explain the increase in cell numbers during EAE. We have previously shown using serial histology sections along with section matching between healthy and EAE groups that there is indeed an increase in the amount of LEC vessel area across 9 sections spanning almost 2 mm of the cribriform plate. In this same study, we also validated the increased proliferation of LECs through Ki67 expression:
      Reference #4: Martin Hsu et al. " *Nature Communications* 2019.

    - Expansion of lymphatic vessels near the cribriform plate has also been confirmed independently by another group using histology and confocal imaging:
      See Supplementary Figure 6E, from Antoine Louveau et al. "CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature." *Nature Neuroscience* 2019.

    - We hope that the combination of published data showing increased Ki67 expression at the protein level by LECs through immunohistochemistry/confocal imaging and increased lymphatic vessel area across 9 section-matched immunohistochemistry/confocal images during EAE combined with the proposed unpublished studies showing increased LEC cell number by flow cytometry during EAE and elevated numbers of cells in the EAE group being enriched for proliferation genes by scRNAseq are sufficient to show lymphangiogenesis.
3. The authors should clearly indicate how the findings on how lymphatic endothelial cells presentation of antigens to CD4 T cells through MHC II differ from prior publications. The same for leukocyte binding and crosstalk. What is novel?
    - We thank the reviewer for bringing up this important point. This has been discussed briefly in the original discussion section, and in the revision, we elaborate further on the novelty and significance of a lymphatic endothelial cell in the meninges near the cribriform plate being able to engage in leukocyte crosstalk through mechanisms such as antigen processing and presentation. The meninges being involved in leukocyte regulation during steady-state conditions has just recently been appreciated, and the significance of meningeal lymphatics during neuroinflammation is restricted to their role in drainage. The ability of meningeal lymphatic endothelial cells to acquire the ability to directly regulate immunity through leukocyte crosstalk has never been shown, indicating that these meningeal LECs are dynamic cells that can respond to the microenvironment which is novel. This is the first study to characterize and identify a novel role of lymphatics in the meninges as a neuro-immune niche, adding to recent data showing similar roles by more conventional APCs that reside in the meninges. Furthermore, the functional consequence of antigen presentation by cpLECs during an

autoimmune disease may have significant therapeutic relevance, as PD-L1/PD-1 mediated tolerance is a major target for cancer immunotherapy.

4. A large number of publications have shown that the olfactory bulb serves as an exit route for CSF. The MRI study show absolutely nothing new compared to for example Helene Benveniste MRI studies. Jony Kipnis and other have documented that CSF inflow into brain is reduced in EAE and it subsequently accumulates in the basal cisterns and is shunted out via the olfactory bulb.
    - This is an excellent point. We agree with the reviewer that there is indeed ample evidence of CSF exit through the olfactory bulbs, and hypothesize that this is the primary route of CSF efflux (consistent with many published studies by Helen Cserr, Miles Johnston, Roy Weller, etc.) However, there is much debate about the nuances of efflux (lymphatic dependent vs. independent along cranial nerves); furthermore, with the recent rediscovery of meningeal lymphatics surrounding the CNS, it is currently debated as to which lymphatic network serves as the primary route of CSF efflux. The reviewer cites Jonathan Kipnis: interestingly, one of his earlier experiments published in 2015 has added to this debate: dye delivered intranasally and presumably within nasal lymphatics did not drain to the draining lymph nodes suggesting that the olfactory route plays a minor role in drainage to the lymph nodes **(Extended Figure 8, Antoine Louveau et al. *Nature* 2015)**, despite other recent studies showing the opposite result after intranasal delivery of antibodies **(Figure 12A, B, Michelle E. Pizzo et al. *The Journal of Physiology* 2018)**. This debate is further compounded by several publications from Jonathan Kipnis's group highlighting the meningeal lymphatics along the superior sagittal sinus and transverse sinuses as the primary route of efflux. Additionally, this topic is heavily debated in humans where the relative size of the olfactory bulbs compared to the rest of the brain is smaller compared to rodents. Additional citations are included in the discussion section to highlight this, where there is currently conflicting live imaging data in humans questioning the relative contribution(s) of the dorsal meningeal lymphatics versus the olfactory route. We hope that additional citations highlighting the current controversies in this topic will be sufficient, as discussing the full scope of the current controversies between the different meningeal lymphatic networks may be exorbitant for the discussion section. Nevertheless, we feel that it is important to mention in the discussion that all of these routes likely play a role in drainage, and future studies are needed to elucidate the relative contribution(s) of each pathway during steady-state and neuroinflammatory conditions. We have added the following paragraph to the discussion section:
    - "In addition to immune surveillance and immune regulation, one of the primary roles of lymphatics is to maintain fluid homeostasis. Because meningeal lymphatics reside outside of the CNS parenchyma, several groups have speculated how meningeal lymphatics may access CSF through the arachnoid barrier. Anatomically in rodent models, the meningeal lymphatics at the base of the brain are hypothesized to access CSF due to their relatively close location to the subarachnoid space [3], and here we show that there are gaps in the epithelial cells that comprise the arachnoid barrier separating the subarachnoid space and meninges near the cribriform plate as previously reported

[53], suggesting direct access by these particular set of lymphatics. Although the dural meningeal lymphatics are more distal from the subarachnoid space, dyes infused into the CSF have identified "hotspots" along the transverse sinuses where CSF is uptaken into the dural meningeal lymphatics relatively early [6,16], suggesting direct uptake of CSF in these regions. Differences in dyes and ideas of how to functionally show CSF drainage (non-invasive imaging, post-mortem analysis, etc.) in animal models have yielded mixed results, where accumulation near the cribriform plate seems to be the most consistent [5]. However, this is further confounded in human imaging studies, where many non-invasive imaging techniques lack the resolution to determine precisely how CSF exits the subarachnoid space. For example, CSF can consistently be found on the CNS-side of the cribriform plate in humans, but whether CSF can exit through the cribriform plate into the nasal mucosa has yielded mixed results [5,15,69]. Nevertheless, we hypothesize that the meningeal lymphatics likely all play a role in CNS homeostasis, and are likely all connected as one large network that can sample from different regions of the subarachnoid space."

- In terms of the MRI experiments, this is the first study to our knowledge looking at the cribriform plate using MRI comparing healthy and EAE. As the reviewer mentioned, other groups have looked at MRI under different conditions, with Jonathan Kipnis' group looking primarily at the dorsal meningeal lymphatics (interestingly, other groups have conflicted the dorsal meningeal lymphatics as a route of CSF efflux using alternative non-invasive imaging methods; Reference #11: Ma, Q. et al. *Nature Communications* 2017). Similar experiments have looked at the basal meningeal lymphatics without looking at the cribriform plate, and Helene Benveniste's MRI studies look primarily during steady-state conditions. Overall, we feel that this MRI experiment not only validates previous similar experiments using non-invasive imaging of CSF efflux, but ties the location of lymphangiogenic cpLECs to a CSF reservoir that is increased during neuroinflammation. In addition to cpLECs directly interacting with leukocytes during neuroinflammation, we speculate that lymphangiogenesis may help facilitate the drainage of excess fluid during neuroinflammation in a model where the other meningeal lymphatics do not.

5. Overall, it is a solid study if additional histology is added, but the advances are incremental.
   - We thank the reviewer for the insight and suggestions. Additionally, there were several thought-provoking points as discussed above. We hope that clarification on the novelty of identifying a new LEC site in functionally regulating immunity instead of acting as passive drainage conduits have added significance to our study. Currently, studies on meningeal lymphatics in neuroinflammation look to manipulate drainage to shape pathology, and here we propose that meningeal lymphatics may play a direct role in shaping immunity in addition to drainage.

Decision Letter, first revision:

Subject: Your manuscript, NI-A32181A

Message: Our ref: NI-A32181A

10th Jan 2022

Dear Dr. Fabry,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Neuroinflammation creates an immune regulatory niche at the meningeal lymphatic vasculature near the cribriform plate." (NI-A32181A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Neuroinflammation creates an immune regulatory niche at the meningeal lymphatic vasculature near the cribriform plate.". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Immunology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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As you prepare your final files we encourage you to consider whether you have any images or illustrations that may be appropriate for use on the cover of Nature Immunology.

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Please use the following link for uploading these materials: [REDACTED]

If you have any further questions, please feel free to contact me.

Best regards,

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On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
The authors have answered all queries in a very competent manner. They have added additional experiments where needed, downtoned conclusions and explained limitaionts and shortcomings of the methodological approaches.

Some of the explanations about the limitations of the methodologies available at present provided to the Reviewers have not been included in e.g. Material and Methods parts, which would still be an asset. E.g. instead of moving the MRI data to the supplement inclusion of the shortcomings of these experiments leading to transient intracranial pressure changes would be more meaningful if mentioned in the figure legend and in the methods part.

Reviewer #2:
Remarks to the Author:
The authors largely addressed my questions and I have no additional comments.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A32181B

Message: In reply please quote: NI-A32181B

Dear Zsuzsa,

I am delighted to accept your manuscript entitled "Neuroinflammation creates an immune regulatory niche at the meningeal lymphatic vasculature near the cribriform plate." for

publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

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Kind regards,

Laurie

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