nature portfolio

Peer Review File

PD-1 regulates CD4+ T cell-mediated CD8+ T cell responses in the brain to balance viral control and neuroinflammation

Corresponding Author: Dr Aron Lukacher

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study investigates the role of the Programmed Cell Death Protein 1 (PD-1) receptor during central nervous system (CNS) infection by murine polyomavirus (MuPyV), a model for human progressive multifocal leukoencephalopathy (PML)1. The authors explore how PD-1 signaling balances the dual necessity of controlling viral replication and preventing immune-mediated tissue damage (neuroinflammation).
The key strenghts of the manuscript are:
- Novel Mechanistic Discovery: The most significant contribution is the identification of a cell-extrinsic mechanism. While PD-1 is traditionally studied for its intrinsic inhibition of CD8+ T cells, this work demonstrates that the regulation of brain antiviral responses is primarily driven by PD-1 signaling on CD4+ T cells.
-Methodological Rigor: The use of intravascular labeling (IV staining) effectively distinguishes between resident brain-infiltrating T cells and circulating blood cells, proving that the observed immune changes are autonomous to the brain environment.
-Anatomical Contextualization: Through MERFISH spatial transcriptomics, the researchers localized PD-1+ T cells and PD-L1 ligands near the cerebral ventricles, providing a high-resolution map of where immune-viral interactions occur.
- Advanced Characterization: The integration of scRNA-seq with flow cytometry provides a detailed landscape of T cell states, identifying specific "stem-like" (TCF1+) and "effector-like" (CX3CR1+) clusters regulated by the PD-1 pathway.

The manuscript does not require any changes

Reviewer #2

(Remarks to the Author)
Title
PD-1 Regulates $CD4^+$ T Cell-Mediated $CD8^+$ T Cell Responses in the Brain to Balance Viral Control and Neuroinflammation

Review
In this study, the authors investigated antiviral T cell responses in a mouse model in which mice were intracerebrally (via the cerebrospinal fluid: CSF) inoculated with mouse polyomavirus (MuPyV). The role of PD-1 signaling was examined using wild-type and conditional PD-1 knockout mice. Immune responses were analyzed by spatial transcriptomics (MERFISH), flow cytometry, and single-cell RNA sequencing. The results demonstrate that PD-1 is highly expressed on brain-infiltrating $CD4^+$ and $CD8^+$ T cells localized near PD-L1–expressing ependymal cells and myeloid cells. Loss of PD-1, particularly in $CD4^+$ T cells, promoted expansion of virus-specific $CD8^+$ T cells within the brain, resulting in enhanced neuroinflammation, whereas PD-1 deletion in $CD8^+$ T cells alone had minimal effects. The authors therefore conclude that PD-1 signaling in $CD4^+$ T cells plays a central role in balancing antiviral immunity and neuroinflammation. I agree with most of the authors' claim, but there are several concerns as described below.

Major Concerns

1. Distribution of Virus-Infected Cells (viral antigens)
In human brain tissue, JCPyV infects oligodendroglia and replicates within the nuclei of infected cells. When assessing host immune responses in PML, it is critical to consider the spatial distribution of virus-infected cells and immune effector cells, particularly T lymphocytes. Unfortunately, in the current model, viral replication is predominantly described in ependymal cells lining the ventricular system. Therefore, it remains uncertain to what extent this model recapitulates the pathological features of human PML. Nonetheless, due to the lack of an appropriate animal model for PML, progress in this field has been delayed. Thus, I think the data generated in this study are valuable. It would be helpful for the authors to describe the limitations and differences of this model relative to human PML pathophysiology.

2. Functions of macrophages
In human PML brain tissue, numerous M2 macrophages are observed in advanced demyelinated lesions, and PD-L1 expression on these macrophages has been reported (Neuropathology. 2024 Feb;44(1):47–58). This is consistent with the findings in the present mouse model (Figure 1B). From a histopathological perspective, spatial analyses comparing PD-1 and PD-L1 expression at early (e.g., 8 dpi) and chronic (e.g., 30 dpi) stages of infection would be better presented. The viral genome quantity is also necessary for comparing 8 dpi and 30 dpi.

3. Other Points
The experimental systems presented in Figures 2–8 are logically designed, yield reliable results, and are interpreted appropriately. The data convincingly show that $CD4^+$ T cells regulate $CD8^+$ T cell responses during the early phase of infection and that PD-1 signaling is involved in this process. However, while PD-L1 expression on macrophages has been reported in advanced human PML lesions, PD-L1 expression on $CD4^+$ T cells has not been described in human brain tissue. A discussion that clearly addresses both similarities and differences between the animal model and human pathology would be valuable.

Summary:
PML may be associated with a prominent host inflammatory response, and this has emerged as a significant clinical concern (PML-associated immune reconstitution inflammatory syndrome (PML-IRIS) (irAE-like state). Pathologically, PML-IRIS is characterized by excessive $CD8^+$ T cell infiltration, but the lack of suitable animal models has long hindered PML research. In humans, however, JCPyV reaches the brain via blood vessels and tends to form initial lesions in regions with abundant blood supply, such as the corticomedullary junction. Differences in infection routes inevitably affect antigen distribution and impose limitations on spatial analyses in this model. A precise description of both the commonalities and limitations of the animal model relative to human disease would further enhance the manuscript's impact and clarity.

Reviewer #3

(Remarks to the Author)
Reviewer Notes

Summary:
While the overall quality of the study and the data presented is strong, the current manuscript would benefit from refinement to improve the precision and alignment between claims in the text and the evidence shown in the figures. In addition, the phenotypic assignments (e.g., effector T cells, tissue-resident memory) often rely on one or two markers, which may be insufficient for robust classification and could lead to oversimplified interpretations. Finally, the manuscript draws parallels to human JCPyV infection, but the data do not directly support such extrapolation in its current form.

The comments below follow the section of the paper.

1. T cells are the major cell type expressing PD-1 in the MuPyV-infected brain

In this section, the authors compared two conditions: sham and MuPyV-infected mice. They then used MERFISH to assess the expression of Pdcd1 and Cd274 in brain sections.
However, it is unclear whether these two conditions can be meaningfully compared. Do sham mice exhibit a similar amount of CD8+ T-cell infiltration as infected mice at 8 days post-infection? The absence of Pdcd1 expression in uninfected mice could reflect a true lack of gene expression, but it could also simply result from the absence (or low number) of infiltrating specific CD8+ T cells capable of expressing Pdcd1. This distinction seems important for the interpretation of the data and should be clarified. Units in the violin plots in Fig S1 are missing.

Line: 99-101
The authors present an analysis of Pdcd1 expression on the ependyma. However, the definition and explanation of the ependymal layer are only provided later (lines 105-106). It would be clearer to define the ependyma at its first mention, before describing or interpreting its associated gene expression.

Line 136-137:
The authors claim cell clustering of CD4+ and CD8+ T cells in proximity to PD-L1 expressing ependymal cells, activated microglia and macrophages. However, CD4 T cells could not be analysed with MERFISH (line 102) and no proximity

analysis has been presented to substantiate this claim that PD1 expressing T cells are indeed closer to virus infected PD-L1 expressing cells. Could such analysis be done using classical immunofluorescence stainings?

Supplementary 2:
Typo in legend hinders understanding: WT mice were infected with MuPyV i.c., and at 8 and 30 dpi. Cells were…

2. PD-1 alters the magnitude, function, and differentiation of brain T cells and limits viral control

The authors demonstrates that loss of PD-1 does not lead to an increase in IFN producing CD8+ T cells (Fig. 2G). Do the authors have additional functional readouts supporting this conclusion, such as TNF , IL2, CD107a expression?

Line 144:
Clarify “Cre-negative Rosa-Cre” mouse line. Have these been bred back using WT mice?

Line 164-165: Immediately before euthanasia
The authors state that analyses were performed “immediately before euthanasia.” It is unclear whether this timing is relevant for assessing infiltrating immune cells. Would similar results be expected if peripheral T cells were depleted 5-7 days before euthanasia? This would help distinguish recently recruited circulating cells from long-term tissue-resident populations.

Line 179: The authors ask whether PD-1 loss during the late stage of infection leads to T-cell clonal expansion and analyse at day 50 post infection (Fig 3A). However, earlier in the manuscript, day 30 post-infection is defined as the late stage. The time until the mice are sacrificed after tamoxifen treatment differs greatly between Fig2A and Fig 3A, 3 days vs 23 days, respectively. Why has day 50 been chosen, could the authors be more explicit on why they are choosing this later time point. For instance, is ongoing proliferation anticipated, as assessed by Ki-67? Alternative metrics of clonal persistence or expansion being considered? Demonstrate that the measured populations are indeed PD1-/- in Fig 3B and S8D,F.
Overall, the experiment in S8 is preferred to the one presented in Fig 3.
Has this experiment also been done with depleting peripheral CD4 cells instead of CD8 cells?

3. Transcriptomic analysis of T cells from MuPyV-infected brains of WT and PD-1-deficient mice

In this section, the authors compared expression of several genes in antigen-specific CD4 and CD8 T cells in PD-1 competent and PD-1 deficient mice at 15 dpi.
The analysis enabled visualization by UMAP (Line 207: text mentions t-SNE?) and identified 11 distinct clusters. When comparing PD-1 deficient and PD-1 sufficient antigen-specific CD8+ T cells, the authors observed a reduced proportion of cells in cluster 0 and an increased proportion in cluster 1. This suggests that PD-1 regulates the balance between effector-like and resident-memory precursor $CD8^+$ T cells versus late-differentiated effector $CD8^+$ T cells.
However, the expression of canonical cytotoxic effector markers such as Gzmb, Prf1, and Cx3cr1, as well as markers associated with effector and resident memory populations (Gzmb, Prf1, Itgae), remains weak. It would therefore be informative to further refine the classification of these clusters using well-established effector markers such as Klrg1, Tbx21, and CD127low, and resident-memory markers such as CD69 and Klf2^low in addition to Itgae.
Furthermore, Fig 4 would greatly benefit from labelled clusters (in parallel with the text) and the numbers should be added to the text e.g. for clusters common to both populations (7,9 and 10). Fig 4H is a nice summary of the findings, but it is difficult for the reader to extract the key differences.

4. PD-1 deficiency shifts toward effector-like and resident-memory-like CD8+ T cell differentiation

Line 271-272
Again, it seems weak to conclude that loss of PD-1 results in expansion of effector and resident memory-like CD8+ T cells only on the basis of CD103 as marker of residency, TCF-1 as marker of memory and Perforin and Granzyme as marker of effector cells.
Can findings be validated in-situ using IHC?

Figure 5E: The authors show plots of perforin- and granzyme B–expressing virus-specific $CD8^+$ T cells across three subsets (CD103+TCF1-, TCF1+CD103-, and TCF1-CD103-). For an accurate comparison between these subsets, could the authors perform the analysis using identical gating strategies?

5. PD-1 loss intrinsic to brain CD8+ T cells does not drive T cell expansion or enhance antiviral control

Effect of PD1 is timing depended. Can the authors speculate what would happen if tamoxifen treatment would be done as in Fig 3A?

6. PD-1 on brain-infiltrating CD4+ T cells inhibits CD4+ and CD8+ T cell proliferation and CD8+ T cell differentiation

Line 304-305
The authors compared both the proportion and the absolute number of $CD103^+$ $CD8^+$ T cells and conclude that both are

increased. While an increase in absolute numbers can be seen, the conclusion regarding the proportion lacks an appropriate statistical analysis (Fig 7E) Same for Fig. 7F: The proportion doesn't look maintained with the loss of PD-1(Line 306)

7. PD-1 on CD4+ T cells regulates MuPyV-associated neuroinflammation

It would add value to the study to include the gating strategy used to identify microglia (Fig 8).
The authors assess neuroinflammation primarily by measuring MHC II expression. While increased MHC II expression is indeed associated with microglial activation and neuroinflammation, a more comprehensive characterization would strengthen this conclusion. For instance, additional activation markers such as CD86, or pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, could provide a more precise assessment of microglial activation status.
Furthermore, complementary histological analyses on brain sections would substantially reinforce the findings. Quantification of Iba1+ cells across conditions, as well as the assessment of axonal injury markers (APP) and demyelination (such as Luxol fast blue staining), could provide important spatial and pathological context to support the neuroinflammatory phenotype. A discussion of the consequences of the increased neuroinflammation is lacking.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper is acceptable for publication. The authors answered to all the issues

Reviewer #2

(Remarks to the Author)
The revised manuscript presents a more balanced interpretation of the animal model and describes commonalities and differences in relevance to human disease. I believe that the authors have satisfactorily addressed the concerns raised in the initial review, and I have no further major comments.

Reviewer #3

(Remarks to the Author)
The authors have addressed all points of the original review.
I have no further comments.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response to Referees

We are deeply appreciative of the four Reviewers many positive comments on our manuscript. Thank you for your feedback, which was insightful and valuable in strengthening our manuscript and clarifying its data presentation. We have addressed each point below. Each Reviewer's comments are italicized, and our responses are non-italicized.

All changes in the manuscript are highlighted in yellow.

Reviewer #1 (Remarks to the Author):

This study investigates the role of the Programmed Cell Death Protein 1 (PD-1) receptor during central nervous system (CNS) infection by murine polyomavirus (MuPyV), a model for human progressive multifocal leukoencephalopathy (PML)1. The authors explore how PD-1 signaling balances the dual necessity of controlling viral replication and preventing immune-mediated tissue damage (neuroinflammation).

The key strenghts of the manuscript are:

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-Methodological Rigor: The use of intravascular labeling (IV staining) effectively distinguishes between resident brain-infiltrating T cells and circulating blood cells, proving that the observed immune changes are autonomous to the brain environment.

-Anatomical Contextualization: Through MERFISH spatial transcriptomics, the researchers

localized PD-1+ T cells and PD-L1 ligands near the cerebral ventricles, providing a high resolution map of where immune-viral interactions occur.

- Advanced Characterization: The integration of scRNA-seq with flow cytometry provides a detailed landscape of T cell states, identifying specific "stem-like" (TCF1+) and "effector-like" (CX3CR1+) clusters regulated by the PD-1 pathway.

The manuscript does not require any changes

We thank the reviewer for his appreciation of the strengths of our manuscript, including its novel mechanistic discovery that PD-1 signaling on $CD4^+$ T cells "balances the dual necessity of controlling viral replication and preventing immune-mediated tissue damage (neuroinflammation)."

Reviewer #2 (Remarks to the Author):

Title

PD-1 Regulates $CD4^+$ T Cell-Mediated $CD8^+$ T Cell Responses in the Brain to Balance Viral Control and Neuroinflammation

Review

In this study, the authors investigated antiviral T cell responses in a mouse model in which mice were intracerebrally (via the cerebrospinal fluid: CSF) inoculated with mouse polyomavirus (MuPyV). The role of PD-1 signaling was examined using wild-type and conditional PD-1 knockout mice. Immune responses were analyzed by spatial transcriptomics (MERFISH), flow cytometry, and single-cell RNA sequencing. The results demonstrate that PD-1 is highly expressed on brain-infiltrating $CD4^+$ and $CD8^+$ T cells localized near PD-L1–expressing

ependymal cells and myeloid cells. Loss of PD-1, particularly in CD4⁺ T cells, promoted expansion of virus-specific CD8⁺ T cells within the brain, resulting in enhanced neuroinflammation, whereas PD-1 deletion in CD8⁺ T cells alone had minimal effects. The authors therefore conclude that PD-1 signaling in CD4⁺ T cells plays a central role in balancing antiviral immunity and neuroinflammation. I agree with most of the authors' claim, but there are several concerns as described below.

Major Concerns

1. Distribution of Virus-Infected Cells (viral antigens)

In human brain tissue, JCPyV infects oligodendroglia and replicates within the nuclei of infected cells. When assessing host immune responses in PML, it is critical to consider the spatial distribution of virus-infected cells and immune effector cells, particularly T lymphocytes. Unfortunately, in the current model, viral replication is predominantly described in ependymal cells lining the ventricular system. Therefore, it remains uncertain to what extent this model recapitulates the pathological features of human PML. Nonetheless, due to the lack of an appropriate animal model for PML, progress in this field has been delayed. Thus, I think the data generated in this study are valuable. It would be helpful for the authors to describe the limitations and differences of this model relative to human PML pathophysiology.

We added a paragraph in the Discussion detailing limitations and differences of this model compared to PML pathophysiology in humans. Briefly, the viruses may target different cells in their respective natural hosts. JCPyV has been shown to infect oligodendrocytes and astrocytes in humans. We previously demonstrated that MuPyV CNS infection results in large T antigen transcripts in astrocytes, microglia, oligodendrocytes, and macrophages, although significantly less LT mRNA was detected in oligodendrocytes (Shwetank et al., 2019 PMCID:

PMC6499176). Immunodeficient shiverer mice engrafted with human astrocytes and oligodendrocytes supported productive JCPyV replication in astrocytes, but apparently abortive replication in oligodendrocytes (Kondo et al., 2014 PMCID: PMC4348956). Whether mouse oligodendrocytes *in vivo* support MuPyV productive or nonproductive infection remains to be determined. While the dominant histopathology of PML is multifocal demyelination, healthy mice inoculated i.c. with MuPyV develop ventriculomegaly and cortical atrophy, which are accentuated under conditions of immunodeficiency. The MuPyV mouse model allows us to probe early events of polyomavirus CNS infection prior to development of irreversible neuropathology, as PML is diagnosed when symptoms manifest and MRI scans reveal foci of demyelination indicative of advanced JCPyV CNS infection.

2. Functions of macrophages

In human PML brain tissue, numerous M2 macrophages are observed in advanced demyelinated lesions, and PD-L1 expression on these macrophages has been reported (Neuropathology. 2024 Feb;44(1):47–58). This is consistent with the findings in the present mouse model (Figure 1B). From a histopathological perspective, spatial analyses comparing PD-1 and PD-L1 expression at early (e.g., 8 dpi) and chronic (e.g., 30 dpi) stages of infection would be better presented. The viral genome quantity is also necessary for comparing 8 dpi and 30 dpi.

Thank you for the relevant article. We have added this finding and citation to the Discussion. Unfortunately, we are unable to perform additional spatial analyses using MERFISH for the 30 dpi timepoint due to logistical (as well as financial) limitations. This data was derived from a MERFISH analysis we published last year (Alexander et al. 2025 PMCID: PMC12205503) using brains of MuPyV-infected mice only at 8 dpi. Regarding histopathological staining of 8 and 30 dpi stages of infection, we previously showed that at 8

dpi, $CD3^+$ T cells localize along the vimentin-positive ependyma lining the ventricles (Figure 3G in Alexander et al., 2025 PMCID: PMC12205503). We have also previously shown that $CD4^+$ and $CD8^+$ T cells co-localize in the subventricular zone of the ependyma at 15 dpi (Figure 1H and Supplementary S1J in Ren et al., 2020 PMCID: PMC7721466).

Regarding viral genome quantities, we have previously demonstrated that viral genome quantities gradually decrease from 8 days to 30 days post-infection in MuPyV i.c.-infected mice (Fig. 1A in Frost et al., 2015 PMCID: PMC4592826; Fig. 1B in Shwetank et al., 2017 PMCID: PMC5698165). Although virus levels decrease, T cells retain high PD-1 expression in the brain.

3. Other Points

The experimental systems presented in Figures 2–8 are logically designed, yield reliable results, and are interpreted appropriately. The data convincingly show that $CD4^+$ T cells regulate $CD8^+$ T cell responses during the early phase of infection and that PD-1 signaling is involved in this process. However, while PD-L1 expression on macrophages has been reported in advanced human PML lesions, PD-L1 expression on $CD4^+$ T cells has not been described in human brain tissue. A discussion that clearly addresses both similarities and differences between the animal model and human pathology would be valuable.

We have added a section to the Discussion describing similarities and differences between the mouse MuPyV CNS infection model and JCPyV-PML pathogenesis. Although PD-L1 expression on $CD4^+$ T cells in brain tissue to our knowledge has not been reported, one publication in 2021 describes PD-L1 expression on human activated effector $CD4^+$ T cells, an observation that we have added to the Discussion (Mazerolles et al., 2021 PMCID: PMC8601581).

Summary:

PML may be associated with a prominent host inflammatory response, and this has emerged as a significant clinical concern (PML-associated immune reconstitution inflammatory syndrome (PML-IRIS) (irAE-like state). Pathologically, PML-IRIS is characterized by excessive $CD8^+$ T cell infiltration, but the lack of suitable animal models has long hindered PML research. In humans, however, JCPyV reaches the brain via blood vessels and tends to form initial lesions in regions with abundant blood supply, such as the corticomedullary junction. Differences in infection routes inevitably affect antigen distribution and impose limitations on spatial analyses in this model. A precise description of both the commonalities and limitations of the animal model relative to human disease would further enhance the manuscript's impact and clarity.

We thank the reviewer for these comments and have incorporated them into the Discussion.

Reviewer #3 (Remarks to the Author)

Reviewer Notes

Summary:

While the overall quality of the study and the data presented is strong, the current manuscript would benefit from refinement to improve the precision and alignment between claims in the text and the evidence shown in the figures. In addition, the phenotypic assignments (e.g., effector T cells, tissue-resident memory) often rely on one or two markers, which may be insufficient for robust classification and could lead to oversimplified interpretations. Finally, the manuscript draws parallels to human JCPyV infection, but the data do not directly support such extrapolation in its current form.

The comments below follow the section of the paper.

1. T cells are the major cell type expressing PD-1 in the MuPyV-infected brain

In this section, the authors compared two conditions: sham and MuPyV-infected mice. They then used MERFISH to assess the expression of Pdcd1 and Cd274 in brain sections.
However, it is unclear whether these two conditions can be meaningfully compared. Do sham mice exhibit a similar amount of CD8+ T-cell infiltration as infected mice at 8 days post-infection? The absence of Pdcd1 expression in uninfected mice could reflect a true lack of gene expression, but it could also simply result from the absence (or low number) of infiltrating specific CD8+ T cells capable of expressing Pdcd1. This distinction seems important for the interpretation of the data and should be clarified. Units in the violin plots in Fig S1 are missing.

- *Do sham mice exhibit a similar amount of CD8+ T-cell infiltration as infected mice at 8 days post-infection?* Sham mice do not exhibit a similar amount of CD8+ T cell-infiltration as infected mice at 8 dpi. We clarified this point in the descriptive text in the results for Supplementary 1A (line 99-101).
- *Units in the violin plots in Fig S1 are missing*. The numbers on the violin plots refer to the log-normalized expression values of Pdcd1 and Cd274. This is now clarified in the legend for fig. S1.

Line: 99-101

The authors present an analysis of Pdcd1 expression on the ependyma. However, the definition and explanation of the ependymal layer are only provided later (lines 105-106). It would be

clearer to define the ependyma at its first mention, before describing or interpreting its associated gene expression.

The ependyma has been defined when first introduced, as suggested (line 103).

Line 136-137:

The authors claim cell clustering of CD4+ and CD8+ T cells in proximity to PD-L1 expressing ependymal cells, activated microglia and macrophages. However, CD4 T cells could not be analysed with MERFISH (line 102) and no proximity analysis has been presented to substantiate this claim that PD1 expressing T cells are indeed closer to virus infected PD-L1 expressing cells. Could such analysis be done using classical immunofluorescence stainings?

We previously showed that $CD3^+$ T cells localize along the vimentin-positive ependyma lining the ventricles (Figure 3G in Alexander et al., 2025 PMCID: PMC12205503 and Figure 2C and 2E in Lauver et al., 2020 PMCID: PMC7541085; Supplementary S1J in Ren et al., 2020 PMCID: PMC7721466).

Supplementary 2:

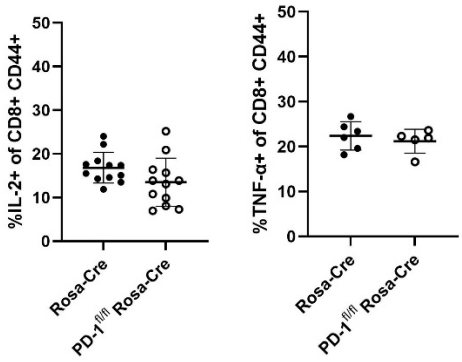
Typo in legend hinders understanding: WT mice were infected with MuPyV i.c., and at 8 and 30 dpi. Cells were…

We reworded this legend to make it clearer. Thanks.

2. PD-1 alters the magnitude, function, and differentiation of brain T cells and limits viral control

The authors demonstrates that loss of PD-1 does not lead to an increase in IFNγ producing CD8+ T cells (Fig. 2G). Do the authors have additional functional readouts supporting this conclusion, such as TNFα, IL2, CD107a expression?

Yes, staining for TNFα and IL2 was performed for these mice, as shown by the following graphs. The frequencies of TNFα and IL2-positive CD8 T cells do not change with loss of PD-1. We have added these functional readouts to Figure 2.



Line 144:

Clarify "Cre-negative Rosa-Cre" mouse line. Have these been bred back using WT mice?

This was an error on our part, as the Rosa-Cre mice are Cre-positive. We have corrected this text. Thanks.

Line 164-165: Immediately before euthanasia

The authors state that analyses were performed "immediately before euthanasia." It is unclear whether this timing is relevant for assessing infiltrating immune cells. Would similar results be

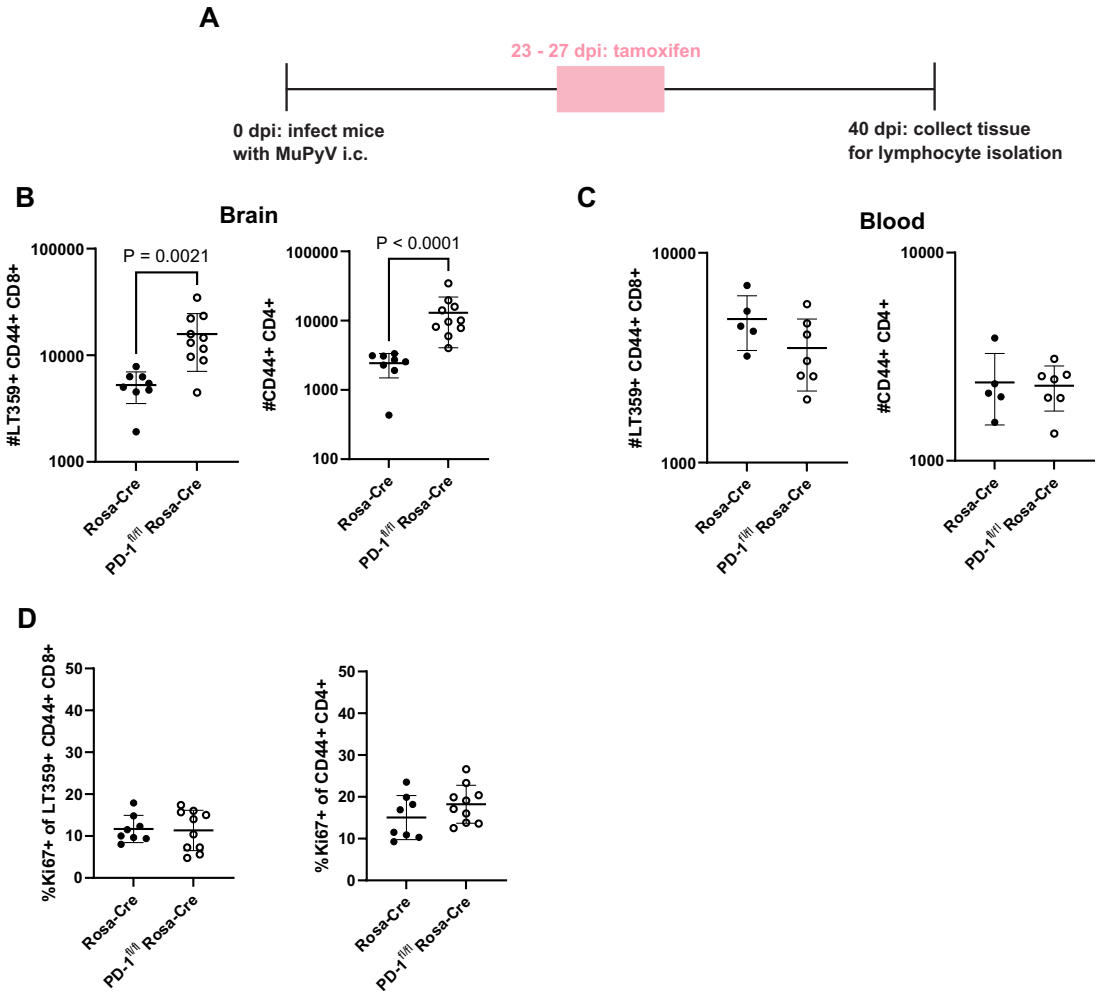
expected if peripheral T cells were depleted 5-7 days before euthanasia? This would help distinguish recently recruited circulating cells from long-term tissue-resident populations.

We retro-orbitally injected mice with fluorophore-conjugated CD45 antibody and waited three minutes before euthanasia to allow sufficient time for the antibody to circulate and label circulating cells as per Anderson et al., 2014 (PMCID: PMC4428344). Unfortunately, depleting only peripheral T cells 5-7 days before euthanasia at 15 dpi is not possible as the blood-brain barrier is leaky at this post-MuPyV infection timepoint (PMCID: PMC7108847), such that exogenously administered antibodies will enter the brain.

Line 179: The authors ask whether PD-1 loss during the late stage of infection leads to T-cell clonal expansion and analyse at day 50 post infection (Fig 3A). However, earlier in the manuscript, day 30 post-infection is defined as the late stage. The time until the mice are sacrificed after tamoxifen treatment differs greatly between Fig2A and Fig 3A, 3 days vs 23 days, respectively. Why has day 50 been chosen, could the authors be more explicit on why they are choosing this later time point. For instance, is ongoing proliferation anticipated, as assessed by Ki-67? Alternative metrics of clonal persistence or expansion being considered? Demonstrate that the measured populations are indeed PD1-/- in Fig 3B and S8D,F.
Overall, the experiment in S8 is preferred to the one presented in Fig 3.
Has this experiment also been done with depleting peripheral CD4 cells instead of CD8 cells?

- *Why has day 50 been chosen, could the authors be more explicit on why they are choosing this later time point?* We consider 30 dpi as the beginning of late-stage infection, so any timepoint thereafter is relatively arbitrary. When testing the effects of later tamoxifen administration, we initially chose 30 dpi as the endpoint and administered 23 to 27 dpi based

on the information that when giving tamoxifen 8 to 12 dpi and with an endpoint of 15 dpi, a 3-day time period was sufficient to induce floxed PD-1 recombination. At 30 dpi, this time period did not induce sufficient floxed PD-1 recombination. Therefore, we opted for a later endpoint. We have repeated this experiment with an endpoint of 40 dpi and found the same increase in T cell counts (shown in B in the following figure).



- *For instance, is ongoing proliferation anticipated, as assessed by Ki-67?* At 50 dpi, Ki67 expression is unchanged between the two strains. We hypothesized that we may have missed the period of proliferation—by 50 dpi, the T cells may have ceased proliferating, translating to a lack of change. As such, we assessed the proportion of $Ki67^+$ T cells at 40 dpi

(experimental scheme in A shown above) but found that although $CD4^+$ and virus-specific $CD8^+$ T cell numbers are increased in number in the brain at this timepoint (shown in B above), no change in $Ki67^+$ of either $CD4^+$ or $CD8^+$ T cells (D). This increase in T cell numbers is not seen in the blood (C), suggesting that T cells are not entering or leaving the brain at significantly different rates with loss of PD-1. The T cell depletion experiment demonstrates that at the very least, virus-specific CD8 T cells are proliferating in the brain without recruitment from the periphery. At 40 dpi and later, we may have missed this period of proliferation, during which more cells are in S phase and consequently express more Ki67.

- *Alternative metrics of clonal persistence or expansion being considered?* Assessing TCR clonal diversity by scRNAseq of paired TCR genes with loss of PD-1 is an interesting question and one we intend to investigate in a separate study.
- *Demonstrate that the measured populations are indeed PD1-/- in Fig 3B and S8D, F.* New graphs have been added to Supplementary Figure 8, showing that the analyzed populations are $PD\text{-}1^{-/-}$ for Fig. 3B and Supplementary 8D and F.
- *Overall, the experiment in S8 is preferred to the one presented in Fig 3.*

  Figure 3 has been merged with Supplemental 8.
- *Has this experiment also been done with depleting peripheral CD4 cells instead of CD8 cells?* We have shown that depleting peripheral $CD4^+$ T cells depletes $CD4^+$ T cells in the brain after the blood-brain barrier in i.c. MuPyV-infected mice has recovered integrity; in marked contrast, peripheral CD8 mAb-mediated depletion doesn't change numbers of CNS-nonvascular CD8+ T cells (PMCID: PMC4592826 , PMCID: PMC6224182). From these data, we previously posited that ongoing replenishment of brain $CD4^+$ T cells from the peripheral $CD4^+$ T cell compartment regulates maintenance of brain-localized $CD8^+$ T cells.

In a broader sense, we think that the results in this manuscript point to PD-1 as another facet by which $CD4^+$ T cells in the brain interact with $CD8^+$ T cells during MuPyV CNS infection.

3. Transcriptomic analysis of T cells from MuPyV-infected brains of WT and PD-1-deficient mice

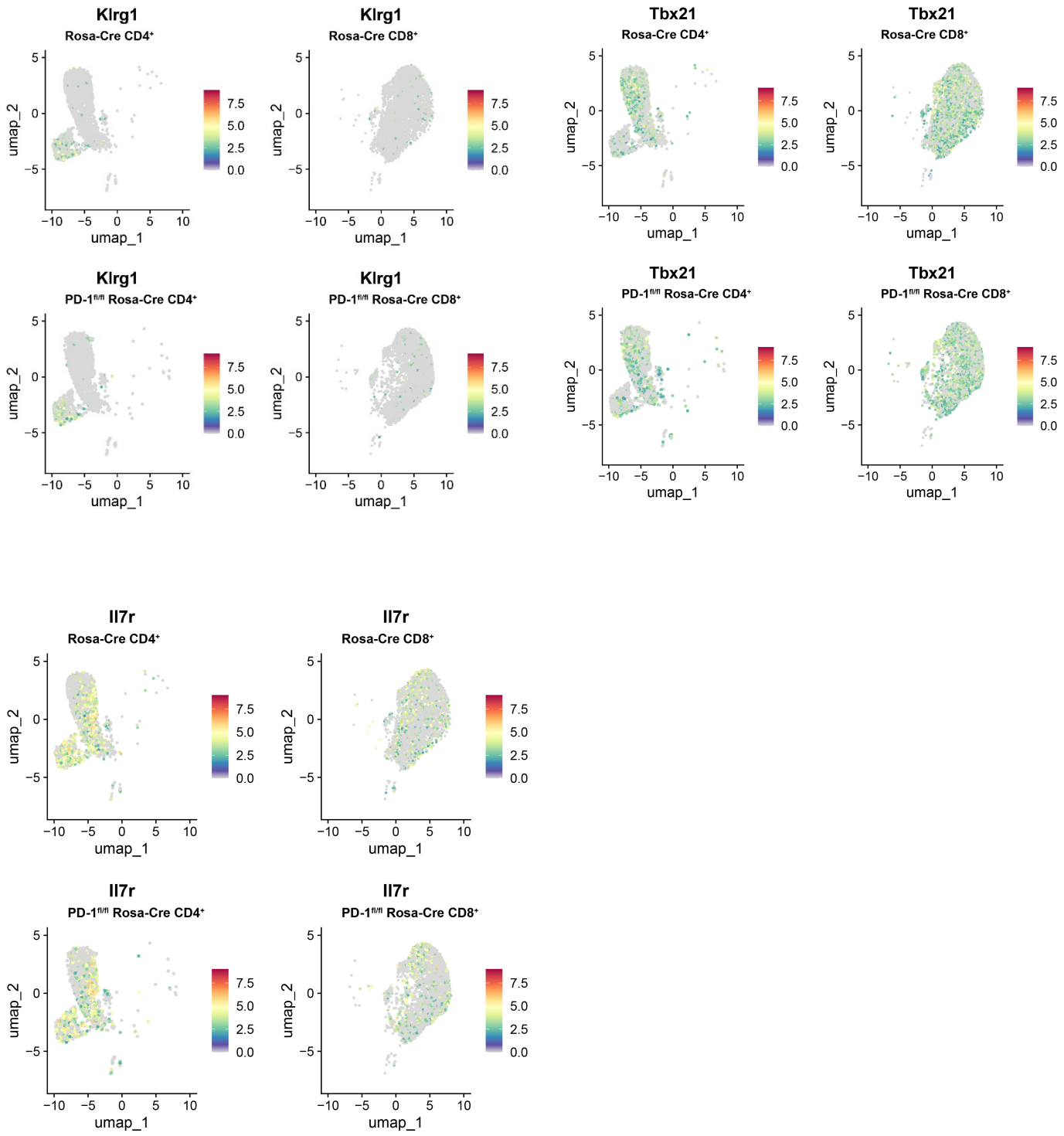
In this section, the authors compared expression of several genes in antigen-specific CD4 and CD8 T cells in PD-1 competent and PD-1 deficient mice at 15 dpi.

The analysis enabled visualization by UMAP (Line 207: text mentions t-SNE?) and identified 11 distinct clusters. When comparing PD-1 deficient and PD-1 sufficient antigen-specific CD8+ T cells, the authors observed a reduced proportion of cells in cluster 0 and an increased proportion in cluster 1. This suggests that PD-1 regulates the balance between effector-like and resident-memory precursor $CD8^+$ T cells versus late-differentiated effector $CD8^+$ T cells.

However, the expression of canonical cytotoxic effector markers such as Gzmb, Prf1, and Cx3cr1, as well as markers associated with effector and resident memory populations (Gzmb, Prf1, Itgae), remains weak. It would therefore be informative to further refine the classification of these clusters using well-established effector markers such as Klrg1, Tbx21, and CD127low, and resident-memory markers such as CD69 and Klf2^low in addition to Itgae. Furthermore, Fig 4 would greatly benefit from labelled clusters (in parallel with the text) and the numbers should be added to the text e.g. for clusters common to both populations (7,9 and 10). Fig 4H is a nice summary of the findings, but it is difficult for the reader to extract the key differences.

We removed the wording related to t-SNE, which was erroneously included (line 213). We understand the importance of further classification using other effector markers. We have

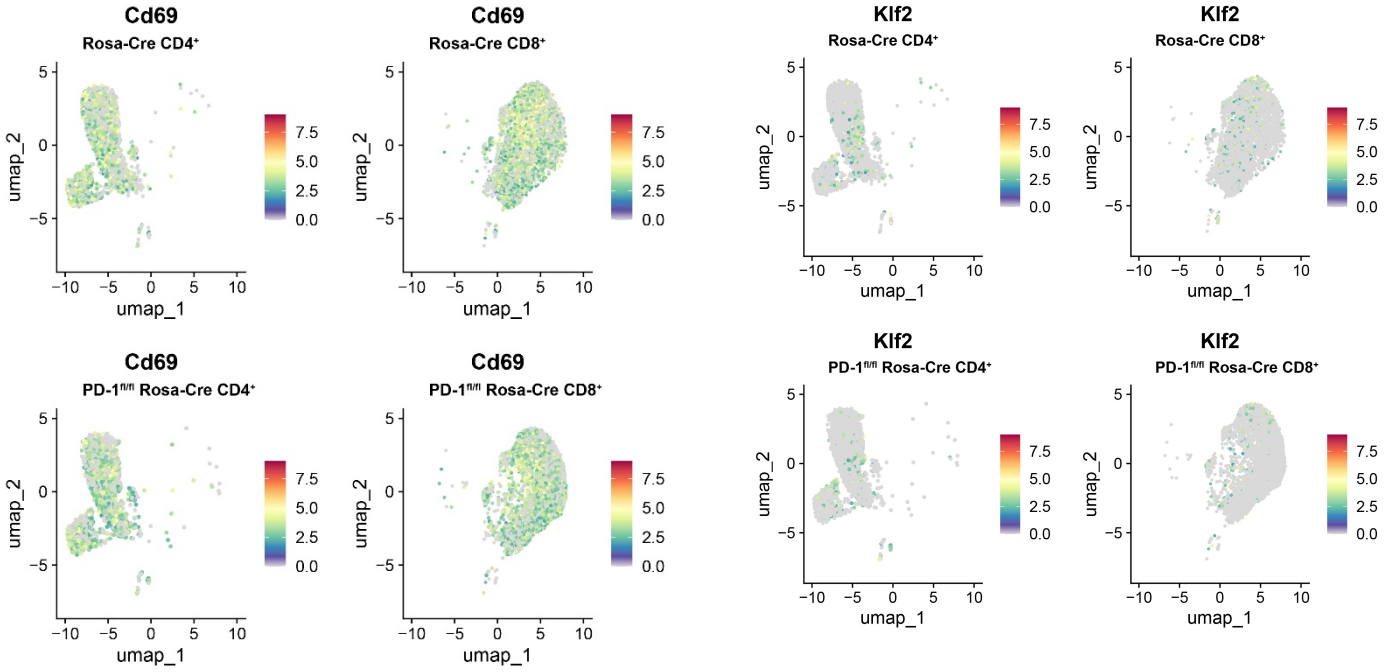
generated feature plots for the transcripts mentioned, but the transcripts for these genes are either low in number or are ubiquitously expressed, making these genes difficult to use for categorizing differentiation state as shown below for the feature plots for *Klrg1, Tbx21,* and *Il7r* (or *Cd127*):



As seen above, *Klrg1* expression is low in the CD8$^+$ T cells, with expression only evident in one CD4$^+$ T cell cluster. *Tbx21* expression does not appear restricted to a particular CD8$^+$ T

cell cluster. *Il7r* expression appears to overlap with *Tcf7* in both $CD4^+$ and $CD8^+$ T cells, supporting a memory precursor profile.

The following feature plots show *Cd69* and *Klf2*. *Cd69* is not expressed in a cluster-specific manner in either $CD8^+$ or $CD4^+$ T cells. Expression of *Klf2* does not overlap with *Itgae* in either $CD8^+$ or $CD4^+$ T cells, making *Itgae*-expressing cells *Klf2*^{low}, with few cells overall expressing *Klf2*.



We have added labelled clusters to Fig. 4 and added numbers to the text for clusters common to both populations. We appreciate the reviewer's comment on Figure 4H. We wrestled with the optimal way of presenting this data. We opted for the format as shown, where we designate groups of expressed genes by molecular identity to simplify readability. In addition, we wrote the corresponding text to draw the reader's attention to expression of transcripts that were

most relevant to appreciating the differences between $CD4^+$ and $CD8^+$ T cells with and without PD-1.

4. PD-1 deficiency shifts toward effector-like and resident-memory-like CD8+ T cell differentiation

Line 271-272

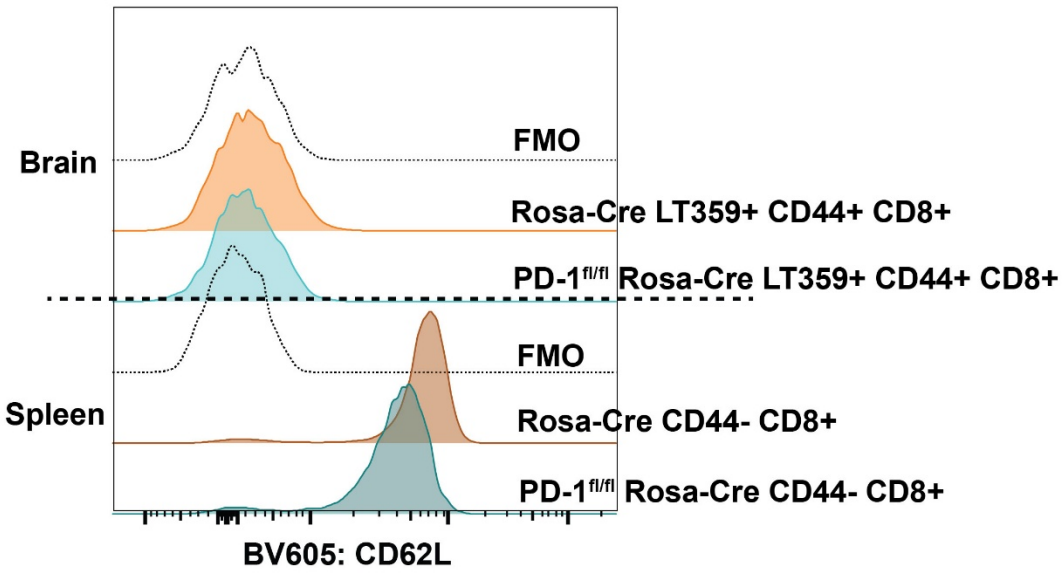
Again, it seems weak to conclude that loss of PD-1 results in expansion of effector and resident memory-like CD8+ T cells only on the basis of CD103 as marker of residency, TCF-1 as marker of memory and Perforin and Granzyme as marker of effector cells.

Can findings be validated in-situ using IHC?

To support CD103 as a marker of residency, we stained virus-specific $CD8^+$ T cells from Rosa-Cre and PD-$1^{fl/fl}$ Rosa-Cre mice with CD69, another commonly used marker of tissue-resident T cells, and CD103. We show that all virus-specific $CD103^+$ $CD8^+$ T cells express CD69. We also added data showing that CXCR6 expression is highest in $CD103^+$ $CD69^+$ $CD8^+$ T cells relative to the $CD103^-$ $CD8^+$ T cell subsets. CXCR6 has been implicated in maintenance of resident memory T cells (Wein et al., 2019 PMCID: PMC6888981, Ganesan et al., 2019 PMCID: PMC6036910). Furthermore, we have previously shown that compared to $CD103^-$ $CD8^+$ T cells, brain-infiltrating virus-specific $CD103^+$ $CD8^+$ T cells show higher expression of B lymphocyte-induced maturation protein-1 (BLIMP1) and lower expression of Eomesodermin (EOMES), a profile associated with tissue residency (Shwetank et al., 2017 PMCID: PMC5698165; Mackay et al., 2016, PMID: 27102484; Parga-Vidal et al., 2021 PMID: 34417257).

Identifying resident-memory T cells is complex and is context-dependent (e.g., nature of infection – type of pathogen, acute vs. chronic pattern of infection, host cell tropism, tissue

localization, etc.). A recent paper from Masopust and colleagues that integrated multiple techniques (parabiosis, intravascular labeling, multiorgan analysis) sought to identify a universal resident-memory signature (Scott et al., 2025, PMCID: PMC11852946). From their comprehensive analyses, the authors concluded that a single gene signature or flow cytometry panel cannot explicitly identify all resident-memory T cells. However, they found that the most encompassing phenotype for identifying $CD8^+$ resident memory T cells was $CX3CR1^-$ $CD62L^-$ $CD69^+$ or $CD103^+$. Although we have not stained for CX3CR1 through flow cytometry, our transcriptomic results indicate that *Cx3cr1* transcripts and *Itgae* transcripts are found in separate clusters (Figure 4E and Supplementary 9C). We do not include this in our manuscript, but flow cytometry analysis indicates that all brain-infiltrating virus-specific $CD8^+$ $CD44^+$ T cells do not express CD62L (shown below in a Rosa-Cre mouse and PD-$1^{fl/fl}$ Rosa-Cre mouse given tamoxifen 8 to 12 dpi with an endpoint of 15 dpi). As a positive control, CD62L expression for naïve $CD44^-$ $CD8^+$ T cells in the spleen (all T cells in the brain are $CD44^+$) is shown as well.

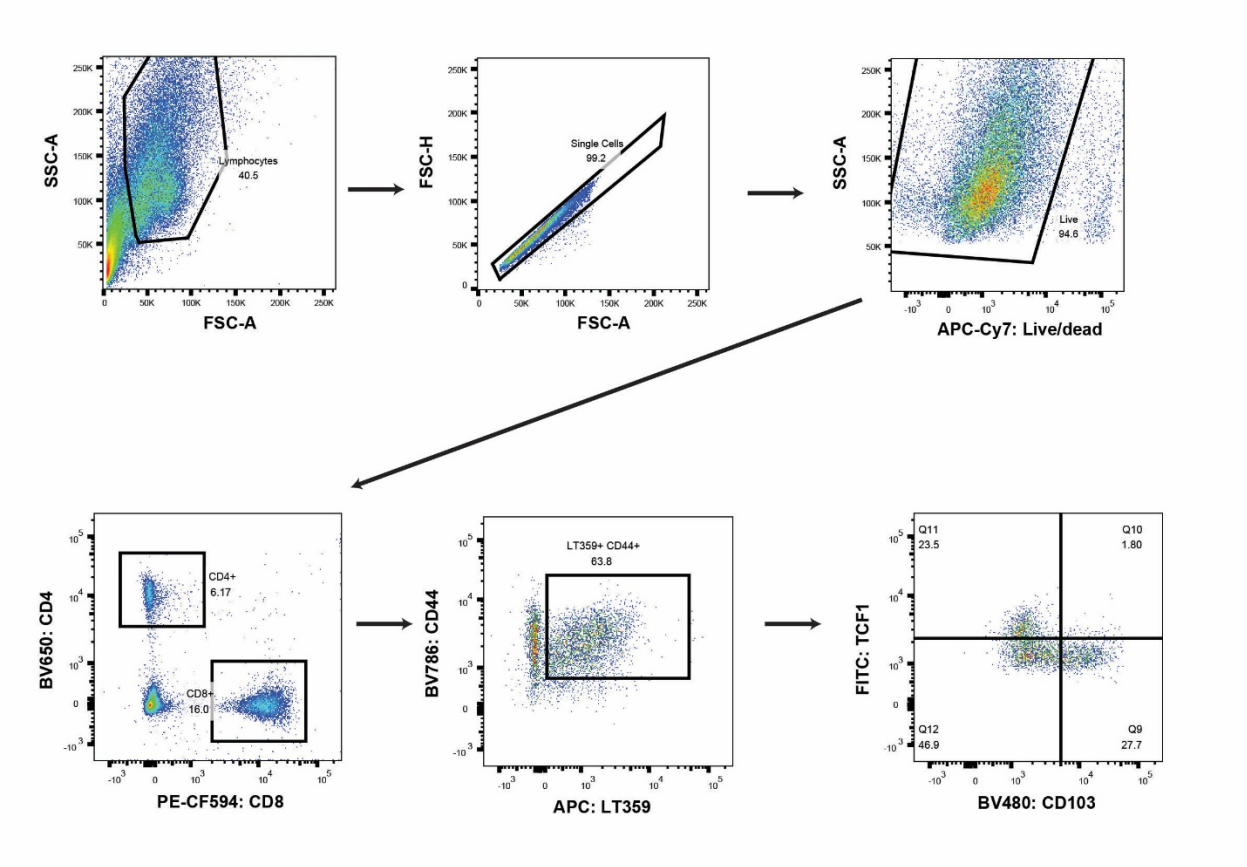


Perforin and granzyme b are commonly used markers of effector $CD8^+$ T cells. We use TCF1 not as a marker of memory, but as a marker of stem-like cells, which are seen in chronic viral infections. The persistent nature of MuPyV complicates this distinction. In chronic viral infection, it is well-established that activated $CD8^+$ T cells differentiate into $TCF1^+$ and $TCF1^-$ subsets. $TCF1^-$ cells are largely terminally differentiated effectors that express granzyme and perforin (Snell et al., 2018, PMCID: PMC8060917; Im et al., 2016 PMCID: PMC5297183; Utzschneider et al., 2016 PMID: 27533016). $TCF1^+$ cells express decreased effector function, seen in our data showing low expression of perforin and granzyme B in $TCF1^+$ $CD8^+$ T cells (Figure 5E). In acute viral infections, TCF1 marks memory precursor T cells. However, MuPyV is neither an acute infection nor an actively chronic infection. It is silent and persistent, which prompts a heterogenous T cell response and may thus stymy a clean characterization of T cells. Despite the persistent nature of MuPyV, denoting TCF1 as a marker of stem-like cells may be accurate, as $TCF1^+$ $CD8^+$ T cells were found to pre-emptively form during early viral infection, independently of whether antigen is acutely cleared or remains present (Chu et al., 2025, PMCID: PMC12003159; McManus et al., 2025, PMID: PMID: 39778710). These cells include memory precursors as well as cells that resemble precursor exhausted T cells.

Figure 5E: The authors show plots of perforin- and granzyme B–expressing virus-specific $CD8^+$ T cells across three subsets (CD103+TCF1-, TCF1+CD103-, and TCF1-CD103-). For an accurate comparison between these subsets, could the authors perform the analysis using identical gating strategies?

All three subsets show cells that are gated the same way up to CD103 and TCF1 co-expression. Lymphocytes were gated the same way to identify virus-specific $CD8^+$ T cells. As

shown below, lymphocytes were gated via FSC and SSC. From the lymphocyte gate, single cells were gated. Live cells were then gated via negative expression for fixable viability dye, then gated for CD8 and CD4. $CD8^+$ cells were gated for positive expression of tetramer and CD44. Tetramer-positive $CD44^+$ cells were then assessed for expression of CD103 and TCF1, resulting in the representative plots in Fig. 5A. The three relevant quadrants were then each assessed for perforin and granzyme B expression.



5. PD-1 loss intrinsic to brain $CD8^+$ T cells does not drive T cell expansion or enhance antiviral control

Effect of PD1 is timing depended. Can the authors speculate what would happen if tamoxifen treatment would be done as in Fig 3A?

If tamoxifen treatment were done as in Fig. 3A in these PD-1$^{fl/fl}$ E8i-Cre mice, based on our findings in Figure 6, we would expect no change in T cell expansion and antiviral control.

6. PD-1 on brain-infiltrating CD4+ T cells inhibits CD4+ and CD8+ T cell proliferation and CD8+ T cell differentiation

Line 304-305

The authors compared both the proportion and the absolute number of CD103$^+$ CD8$^+$ T cells and conclude that both are increased. While an increase in absolute numbers can be seen, the conclusion regarding the proportion lacks an appropriate statistical analysis (Fig 7E) Same for Fig. 7F: The proportion doesn't look maintained with the loss of PD-1(Line 306)

We have edited the text to accurately describe the increase in CD103$^+$ CD8$^+$ T cell numbers. The statistical analysis for the proportion is appropriate; there is just no statistically significant difference. The proportion of TCF1$^+$ CD103$^-$ CD8$^+$ T cells is reduced in Figure 7F, which we describe in the text (lines 327-328).

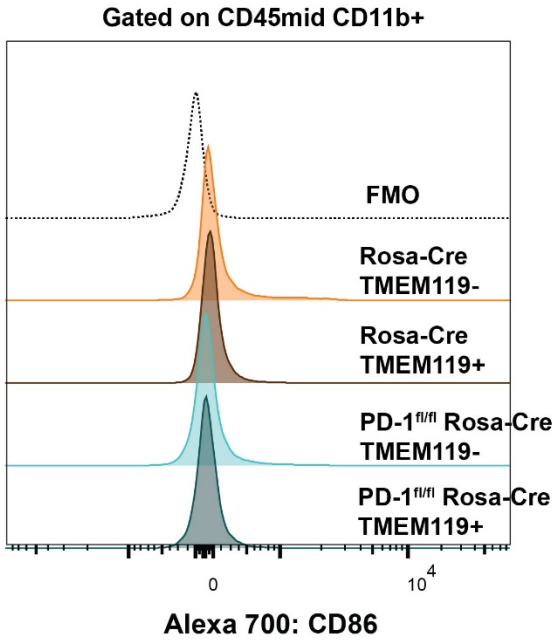
7. PD-1 on CD4+ T cells regulates MuPyV-associated neuroinflammation

It would add value to the study to include the gating strategy used to identify microglia (Fig 8). The authors assess neuroinflammation primarily by measuring MHC II expression. While increased MHC II expression is indeed associated with microglial activation and

neuroinflammation, a more comprehensive characterization would strengthen this conclusion. For instance, additional activation markers such as CD86, or pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, could provide a more precise assessment of microglial activation status.

Furthermore, complementary histological analyses on brain sections would substantially reinforce the findings. Quantification of Iba1+ cells across conditions, as well as the assessment of axonal injury markers (APP) and demyelination (such as Luxol fast blue staining), could provide important spatial and pathological context to support the neuroinflammatory phenotype. A discussion of the consequences of the increased neuroinflammation is lacking.

The gating for microglia was added as a supplementary figure (Supplementary 14). We infected Rosa-Cre and PD-1$^{fl/fl}$ Rosa-Cre mice and, at 15 dpi, performed CD86 staining for CD45mid CD11b+ microglia. We added TMEM119 as an additional marker for homeostatic microglia and found that microglia did not express CD86 under these conditions.



We fully agree that understanding how neuroinflammation drives neural injury is an important, and complex, process with many potential variables that could affect the location and type of lesions. How neuroinflammation is induced (viral, microbial, EAE, trauma), its duration and location in the CNS, nature of interactions between infiltrating lymphoid and myeloid cells with CNS-resident cells, and sex of the host, will result in a panoply of cytokines/chemokines/growth factors being up-/down-regulated that come into play to affect the neuropathologic picture. In fact, we were motivated by this comment to initiate a collaboration with Dr. Elizabeth Proctor at UVA, a systems biologist with expertise using Luminex to model neuroinflammation in AD mouse models. We collected brain tissue homogenates from CD4-Cre and PD-$1^{fl/fl}$ CD4-Cre mice for the Luminex data analysis and found evidence of increased inflammatory cytokine milieu in the setting of PD-1 loss on $CD4^+$ T cells. We have added this data as an additional main figure (Figure 9). As we continue this collaboration, we plan on carrying out more detailed histopathologic studies along the lines suggested by Reviewer 3 that we hope will fill in blanks about defined components of the neuroinflammatory cascade induced by MuPyV upon PD-1 loss and how these factors induce CNS damage. Although this line of investigation well exceeds the scope of this manuscript, it's one we think is important in developing a mechanistic connection between inflammation and injury in the brain to polyomavirus CNS infection.

As suggested, we added a prediction of the consequences of increased neuroinflammation to the new paragraph in the Discussion acknowledging similarities and differences between MuPyV CNS infection and JCPyV-PML. Among these differences is the absence of demyelination (assessed by LFB/PAS staining) in i.c. MuPyV-inoculated mice even under conditions of profound immunodeficiency (i.e., $CD8^+$ T cell depletion of STAT1 KO mice; doi:

10.1128/JVI.02038-19). We left open the possibility that demyelination might manifest with temporal deletion of PD-1 but feel it's unlikely given our earlier study.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for co-reviewing our manuscript as part of this important initiative in peer review training for researchers in their early career stage.