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Peer Review File

Immune landscape of oncohistone-mutant gliomas reveals diverse myeloid populations and tumor-promoting function

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their work, the authors comprehensively characterize the immune tumor microenvironment of pediatric high grade glioma carrying the H3.3K27M and G34R/V mutations. They use state of the art technology including single cell RNAseq, single nuclear RNAseq, imaging mass cytometry and flow cytometry/CyTOF. They take into account batch effects and report their implications. They further used known mouse models and established new ones with similar mutations and confirmed the immune composition phenotypes seen in their human datasets. Moreover, they attempted to modulate the myeloid compartment in some of the animal models using combinatorial treatment regimens with potential translational potential.

Overall, the manuscripts represents a laborious work which brings together multidimensional information on the studied tumor entities (pediatric high grade glioma). The analysis are performed by state-of-the art methodology, and the authors also implemented novel animal models to showcase their findings in an in vivo setting. Serial engraftments again represent a significant experimental workload which I would like to commend the authors on.

I have some comments/suggestions on the CSF1R inhibitor experiments presented towards the end of the article that would need modification/additional data:

- CSF1R inhibition alone was not effective in providing tumor control - other literature reports very strong therapeutic effects of CSF1R inhibition (eg. Pyonteck et al, 2013). How do the authors explain this in the context of their tumor model? Any speculations on epigenetic differences? I would also important to discuss the potential blood-brain barrier penetrance of the applied CSF1r inhibitor in comparison to BLZ945. Can the authors confirm CSF1r antibody levels within the treated tumors? Further, the team sees an increase of T cell infiltration upon myeloid depletion. Even if this might be beyond the scope of the manuscript, it would be worth testing or at least discussing T cell depletion paradigms and characterization of those TILS in terms of tumor cell killing capacity and their immune

checkpoint profiling (e.g. by flow). Furthermore, it would be worth characterizing the myeloid activation status upon CSF1R treatment more in detail (in regard of myeloid checkpoints or general activation markers within myeloid cells, since it is not mere depletion but rather a 'modulation', compare also PMID 37467314).

Figure 4 panels H: the statistical tests in N=2 in the CSF1R+PD1 condition is questionable. Would it be possible to increase the n? Also, the histology in panel G could be quantified by counting multiple high power fields (should be done in different biological replicates). I appreciate this is done in Suppl. Fig. 5 but increase of animal number to faithfully come to this conclusion would be advisable.

Minor comments:
Figure 2 C-E: scale bars are missing
Suppl Fig A: maybe provide higher magnification insert and H&E overview. E: scale bars missing.
Figure 3D: this means both ligand and receptor are expressed? What would be sender, what the receiver cell type? Maybe the caption needs further explanation here, or ligand-receptor pairs need to be outlined.
Suppl Figure B, C, E: scale bars missing
Recent single cell atlas data of pediatric high grade glioma need be cited: https://doi.org/10.1093/neuonc/noad207

Reviewer #2 (Remarks to the Author):
The manuscript presented by Andrade et al. offers a comprehensive analysis of the immune tumor microenvironment (TME) in H3.3K27M and G34R/V-mutant gliomas. This study is notably significant for its depth in exploring the intricate dynamics within the TME, utilizing a blend of transcriptomic and proteomic methods, including advanced spatial single-cell approaches.

Strengths
Immune Lineage Resolution in H3-mutant Gliomas: The study effectively resolves immune

lineages within these gliomas, revealing a high infiltration of diverse myeloid populations. This finding is pivotal, as it underscores the complexity of the immune landscape in these tumors.

Expression of Immune Checkpoints and Lymphoid Cell Scarcity: The high-level expression of immune checkpoints, coupled with the scarce presence of lymphoid cells, is a critical observation. This aspect of the TME can significantly impact the development and progression of these gliomas.

Uniformity Across H3.3K27M Models: The reproduction of these findings across all tested H3.3K27M models adds a layer of robustness to the study, ensuring that the observations are not model-specific but rather inherent characteristics of these gliomas.

Myeloid Populations and Tumor Interaction: The elucidation of the interactions between myeloid populations and H3-mutant cells is a notable advancement. The manuscript convincingly demonstrates that these interactions are conducive to immunosuppression, thereby facilitating tumor formation and maintenance.

Therapeutic Implications: Perhaps most importantly, the study explores the therapeutic potential of dual inhibition of myeloid cells and immune checkpoints. The significant benefits observed in pre-clinical syngeneic models open new avenues for targeted therapy in H3-mutant gliomas.

Weaknesses:

In 103 their sentence suggests that a subset of ATRT tumors have shown response to ICI which is iis misleading as the reference they cite shows ICI response in a flank (non-orthotopic) syngeneic mouse model.

Fig F1E: The authors label this as a comparison of propotion of immune types in the total immune population. What is their definition of total immune population. Are they all CD45+ ells.

The imaging mass cytometry study is commendable and gives rise to fascinating insights in their study. However this also requires validation investigations. From the IMC studies the authors infer that different myeloid subsets are found to interact with H3-mutant cells.

Especially the fact that Classical BMDMs showed strong interactions with both H3.3K27M- and G34R-mutant cells while monocyte subsets tended to interact mainly with H3.3G34R cells.
This needs to be validates using classical Immuno-Flourescence studies using high resolution confocal microscopy using appropriate controls. As presented it is difficult to verify their very interesting observations. Similarly with their observations with endothelial cells in K27M tumors

In Line 248 the authors point out CD47/LGALS9 as potential mechanisms of immune suppression. However LGALS9 is usually expressed on lymphoid cells which are in low numbers. Is this aberrant expression. The cell-cell interactions shown here need to be verified using alternative techniques otherwise the data remains un-corroborated.

I commend the authors on their efforts to corroborate their patient sample findings in additional mouse models.

In line 272 for Fig 4A, the authors talk about 2 syngeneic models but do not describe them here. Are the left and right panels from their 4 hit and 2 hit. The right panel also has PDGFR-OE, which should not be there according to their 2-hit model.

Are there any survival differences between the 2 hit and 4 hit
Classical flow cytometry analysis of the TME between the 2 hit and 4 hit in the de-novo setting and analysis of the draining lymph nodes would be extremely useful and would significantly add to the value of this manuscript.

In Line 343, the authors summarize that "These results suggest that the more aggressive and faster-growing tumors in serial engraftments are associated with an expansion of myeloid cells, including newly recruited 345 BMDM, at the expense of other immune populations." There is an argument to be made that the lack of lymphoid cells could be due to the short life span of tumor growth that occurs. Were the tumors from Egraftments 1 and 3 harvested at the same time?

In their pre-clinical studies some key data is missing

1. The authors need to show the relative tumor sizes when treatment starts and the decrease in tumor burden over time.
2. What is the change in the myeloid compartment after the individual therapies combined
3. Are the CD3 cells in the combination treatment active?
4. In the mice that survived, analysis of the lymph nodes would be very informative
5. What is the difference in the G34R-PAD model with respect to ICI and anti-CSF1R blockade
6. Is the TME dependent on the location of the tumor or the mutation it harbors?
Not sure what is the purpose of the humanized mouse model since it is not used in any functional or mechanistic study.

Reviewer #3 (Remarks to the Author):
The study by Andrade et al describes the characterization of the immune microenvironment of
pediatric Histone H3-mutant gliomas. The authors begin by examination of the immune landscape of
H3.3K27M and G34R/V-mutant gliomas using single cell technologies. Utilizing data derived from both human patient tumors as well as glioma mouse models, they infer that the predominant immune cell population in these tumors are of myeloid origin. They show that
these cells promote immunosuppression and that while blockade of CSF1R or PD-1 individually did
not alter tumor burden or survival, combined blockade of these proteins improved survival in glioma
bearing mice which correlated with increased T cell infiltration. Overall, while the manuscript
represents a large immune landscape study for pediatric Histone H3-mutant gliomas, the lack of any
novel findings that emerge from the analyses dampens the enthusiasm of the reviewer.

Furthermore, despite the use of various advanced technologies (CyTOF, scRNA-seq etc.) the data
provided does not seem to advance apriori knowledge (e.g., myeloid cell abundance in pediatric
gliomas, immunosuppression and targeting CSF1R/PD1, all of which are widely established paradigms). Lastly, the data analyses and interpretation, especially of the scRNA-seq should be
improved to obtain meaningful results. Specific comments are shown below.

1) There is no information provided on single cell dissociation of resected tissues for the human gliomas. How were the samples processed? If enzymatic digestion was used to separate the tissues, more details are needed. One concern is the estimate of the different cell populations as presented may not be accurate. Different methods can significantly affect the yield of immune and non-immune cells as discussed in Sarcocic et al (PMID 36339814). The authors must provide a detailed methodology for the sample preparation and acknowledge the effects of the method on cell yield.

2) The sample numbers, although at first read appears adequate for statistical comparisons, is not sufficient to make meaningful inferences. For example, only two tumors were analyzed for G34R group. Broad inferences cannot be made with n=2.

3) The lack of batch effect correction on scRNA-seq is worrisome. There are many methods for batch correction, and these are evolving. One example is the study by Yu et al (PMID 36810607) where different methods are compared. The authors must apply robust batch correction measures and show evidence of lower rejection rates before and after the correction.

4) The study exemplifies a missed opportunity on deeply analyzing single cell RNA-seq data and especially the myeloid cells in pHGG. There is hardly any information on the number of cells analyzed, immune versus non-immune, sequencing depth etc. How many cells were isolated from each sample? The authors can further strengthen the cell type analyses by sub-clustering of myeloid populations from the scRNA-seq data since this is the strength of the manuscript. More than 70% of the immune populations are myeloid cells, but beyond classifying them as microglia or macrophages, there is not much inference on the cell states. Gene ontology or publicly available resources such as M-VERSE (https://macroverse.gustaveroussy.fr/2021_M-VERSE) can further strengthen the study with

respect the myeloid cell types. This is even more crucial given that the authors claim that myeloid cells are immunosuppressive, but if the identification of CSF1R as a main targetable pathway is the conclusion of the manuscript, there was no need for single cell sequencing, since CSF1R is already shown to play an immunosuppressive role in pediatric and adult gliomas.

5) Many references of previous literature that have shed light on the immune cell landscape on pHGG have not been cited. The authors must (in the discussion section) compare the results from the current study to Plant et al., J Neuroonc, 2021, Griesinger et al, J Immunol, 2013 and the more recent DeSisto et al, Neuro-onc, 2023. How does this study present any advance from these previous reports.

6) Fig. 1B describes the actual number of cells processed for scRNA-seq (n=140743). What is the split for cancer and immune cell contributions?

7) Why is the data only from K27M, but not G34 R/V utilized for comparisons in Fig. 1F and 1G?

8) The data shown in Supp Fig. 1D shows that the myeloid cell populations in pediatric gliomas are mainly macrophage, but not microglia. This contrasts with data shown in scRNA-seq related Fig. 1D, where microglia is the major population. How do the authors explain these differences?

9) Fig. 2 generally validates the scRNA-seq findings that myeloid cells are abundant in gliomas. The data presented in Fig. 2G conveys that microglia and classical monocytes are differential between H3.3K27M- and G34R tumors. This again does not align with the findings from scRNA-seq or scRNA-seq.

10) Fig. 3 is descriptive and no spatial statistics is provided.

11) The rest of the manuscript does not provide any new findings beyond previously reported efficacy or lack thereof of CSF1R and PD1 treatments. The combination therapy however is promising, but it is not clear what is the mechanism of such a response. The authors show a general increase in T cell infiltration in CSF1R + PD1 inhibition group vs vehicle (Fig. 5G and 5H), but the data on individual inhibitors is not shown. Furthermore, while 5G shows increased CD3+ T cells in the picture, this is not corroborated with any significant increases in 5H.

Minor points:

12) What does this mean “Classical BMDMs showed strong interactions with both

H3.3K27M- and G34R-mutant cells 230 while monocyte subsets tended to interact mainly with H3.3G34R cells”

13) There are typos in the manuscript and will benefit from language editing. One example being “which may atively” in line 381.

Point by point response to reviewers' comments:

Reviewer #1: In their work, the authors comprehensively characterize the immune tumor microenvironment of pediatric high grade glioma carrying the H3.3K27M and G34R/V mutations. They use state of the art technology including single cell RNAseq, single nuclear RNAseq, imaging mass cytometry and flow cytometry/CyTOF. They take into account batch effects and report their implications. They further used known mouse models and established new ones with similar mutations and confirmed the immune composition phenotypes seen in their human datasets. Moreover, they attempted to modulate the myeloid compartment in some of the animal models using combinatorial treatment regimens with potential translational potential. Overall, the manuscript represents a laborious work which brings together multidimensional information on the studied tumor entities (pediatric high grade glioma). The analysis are performed by state-of-the art methodology, and the authors also implemented novel animal models to showcase their findings in an in vivo setting. Serial engraftments again represent a significant experimental workload which I would like to commend the authors on.

We would like to thank the reviewer for recognizing the amount of work and various controls we have performed and for their feedback below. We appreciate their constructive suggestions to clarify and strengthen the claims we make in this manuscript.

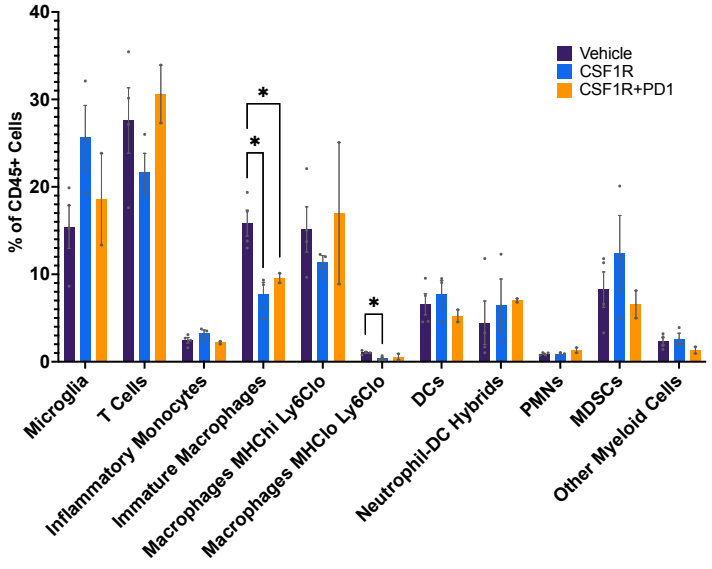
1. I have some comments/suggestions on the CSF1R inhibitor experiments presented towards the end of the article that would need modification/additional data: CSF1R inhibition alone was not effective in providing tumor control - other literature reports very strong therapeutic effects of CSF1R inhibition (eg. Pyonteck et al, 2013). How do the authors explain this in the context of their tumor model? Any speculations on epigenetic differences? I would also important to discuss the potential blood-brain barrier penetrance of the applied CSF1r inhibitor in comparison to BLZ945. Can the authors confirm CSF1r antibody levels within the treated tumors?

We thank the reviewer for their comment. We were also intrigued by the difference observed in the results of our H3K27M model compared to findings in adult mouse models of high-grade gliomas reported in the literature. For instance, Pyonteck et al. successfully utilized BLZ945, a brain-penetrant compound targeting CSFR1, in their induced hemispheric glioma model. Similarly, Gangoso et al. (2021) used an anti-CSFR1 antibody, the same as in our study, in a transgenic mouse model of HGG and observed significant survival improvement (Gangoso et al., Cell 2021, https://doi.org/10.1016/j.cell.2021.03.023).

Several factors may contribute to the varying response to CSFR1 inhibition alone. Despite using the same inhibitor as Gangoso et al. (2021) and similar dosing schedule, we did not observe a survival effect. This suggests that brain penetrance on its own will not account for the varying anti-CSFR1 responses. Differences in molecular drivers of tumor formation may lead to distinct tumor microenvironments (TMEs), as suggested by the reviewer. H3K27M, an epigenetic driver that stalls development and further differentiation of oligodendroglial progenitors, may promote a TME with more immunosuppressive myeloid populations, as indicated by our work. This stands in contrast to other mouse models, which are driven by canonical growth factors and/or loss of suppressors of oncogenic signaling pathways. This may also influence initial levels of T cell infiltration which vary between models, and in ours, only a severely limited number of T cells infiltrated tumors. Moreover, the location of the tumor within the brain may influence the TME, H3K27M tumors in the midline brain may have different TMEs than the hemispheric tumors

obtained in these other studies. Last, as we obtained a moderate decrease in the myeloid population in tumors harvested at endpoint (see CyTOF data below, also provided in Fig6H of our revised manuscript) and based on results from the Gargoso et al. study that used the same inhibitor, we did not assess the antibody level in tumors. Notably, similarly to Pyonteck et al (2013), we have observed persistent myeloid infiltration after treatment with CSF1R inhibitor. This could result from repopulation by the myeloid cells of tumor sites following the suspension of antibody therapy after 4 weeks, as we did not maintain delivery after this time, the low BBB penetrance of the inhibitor we used or, as reported by this group, glioma-supplied factors promoting TAM survival in the presence of a CSF1R inhibitor.

We would like to stress that we have performed our *in vivo* studies as an initial proof-of-principle to show that myeloid cells can be targeted therapeutically. In all, additional complexities including differences in the composition of the TMEs based on the nature of the oncogenic driver and its crosstalk with tumor cells, the brain location, and the brain penetrance of the inhibitor need to be further investigated to address these differences and findings. Further investigations are warranted to better understand the lack of therapeutic effects when CSF1R inhibitors are used on their own and whether similar results will be observed when using brain penetrant CSFR1 inhibitors, the optimal scheduling and combination of inhibitors and to further dissect the crosstalk between tumor cells and these myeloid population. We had alluded to these issues in our initial manuscript and have revised our discussion to address them more explicitly in the text.



2. Further, the team sees an increase of T cell infiltration upon myeloid depletion. Even if this might be beyond the scope of the manuscript, it would be worth testing or at least discussing T cell depletion paradigms and characterization of those TILS in terms of tumor cell killing capacity and their immune checkpoint profiling (e.g. by flow).

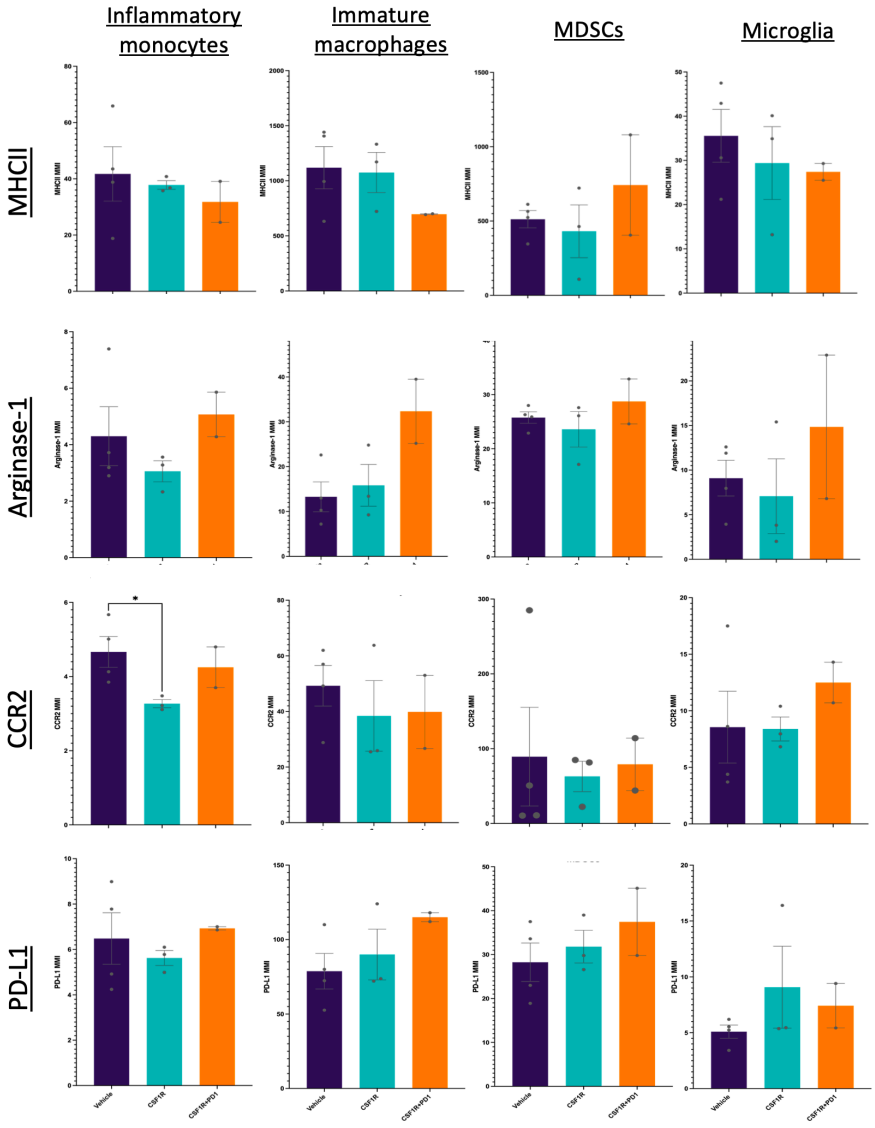
We thank the reviewer for this comment and agree this finding deserves future investigations to characterize the nature of these tumor infiltrating lymphocytes (CD4+, CD8+ other T cell subgroups) and their relation to improved survival, if any. As also noted by the reviewer, this is beyond the scope of this manuscript and we had previously avoided undue speculation on their nature in our original manuscript.

3. Furthermore, it would be worth characterizing the myeloid activation status upon CSF1R treatment more in detail (in regard of myeloid checkpoints or general activation markers within myeloid cells, since it is not mere depletion but rather a 'modulation', compare also PMID 37467314).

We fully agree with the reviewer that altering rather than eliminating myeloid and other immune cells within the TME may explain therapeutic responses. The TME can be manipulated by tumor cells to suppress immune responses, as demonstrated by various groups, including the study referenced by the reviewer. In this study, in a GBM model, hyper-sialylation of tumor cell surface masked them from the immune system and induced tolerance in tumor-associated macrophages and microglia. Conversely, targeting this oncogenic interaction can lead to therapeutic responses, as demonstrated by this group, when combining sialic acid inhibition with immune checkpoint blockade. Therefore, our strategies for therapeutically targeting the TME in H3K27M high-grade gliomas may also likely work through immune modulation rather than simply depleting specific immune subtypes.

We analyzed myeloid activation and immune checkpoint markers using CyTOF in tumors harvested from mice treated with CSF1R inhibition alone and in combination with PD1 blockade. Apart from reduced CCR2 expression in inflammatory monocytes from mice treated with CSF1R alone, we did not observe significant differences in myeloid profiles compared to the vehicle-treated group. We are providing below the results for the reviewer's benefit. More comprehensive studies conducted at various timepoints before and during treatment initiation are necessary to complement the data generated at the study endpoint, when mice needed to be euthanized based on clinical symptoms.

In response to the reviewer's feedback, we have revised our manuscript to further emphasize that modulating myeloid cells rather than simply depleting them may account for the therapeutic benefit observed. We highlight the need for additional studies to characterize in-depth how the TME, particularly the myeloid population, and how it evolves in real-time during therapy.

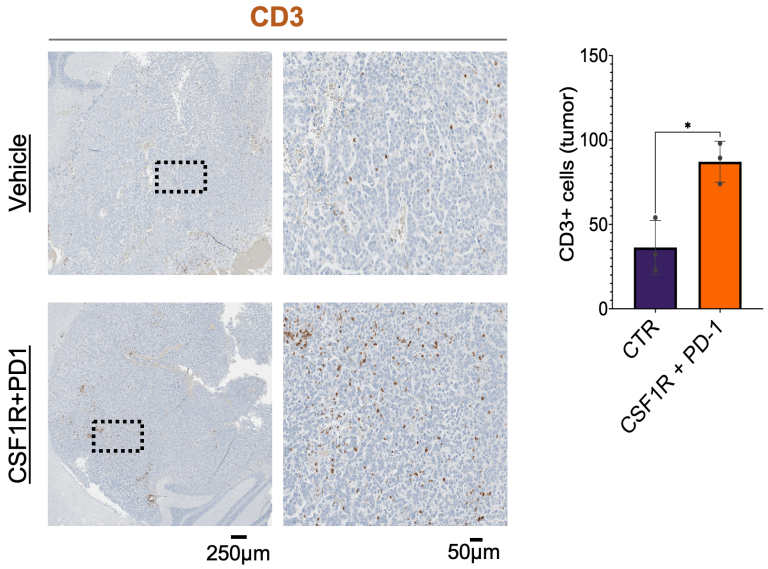


4. Figure 4 panels H: the statistical tests in N=2 in the CSF1R+PD1 condition is questionable. Would it be possible to increase the n? Also, the histology in panel G could be quantified by counting multiple high power fields (should be done in different biological replicates). I appreciate this is done in Suppl. Fig. 5 but increase of animal number to faithfully come to this conclusion would be advisable.

Out of the seven mice used in our study, tumors were observed in only three (with four showing no disease symptoms or tumors). Unfortunately, one of these mice was found dead, resulting in poor tissue quality, which limited further studies. Therefore, we were only able to utilize samples from two mice for IHC and CyTOF analysis. The quantification in former Suppl. Fig5F (now Suppl. Fig6G) had been obtained using multiple high-power fields (at 50µm), as suggested by the reviewer. We would like to apologize for not detailing this in the previous version of this manuscript. The full description of the methodology is now found in the M&M section.

To validate T cell infiltration and increase the number of mice analyzed, we conducted IHC using tumors from the experiment depicted in Fig6E (former Fig5E), where mice were treated with

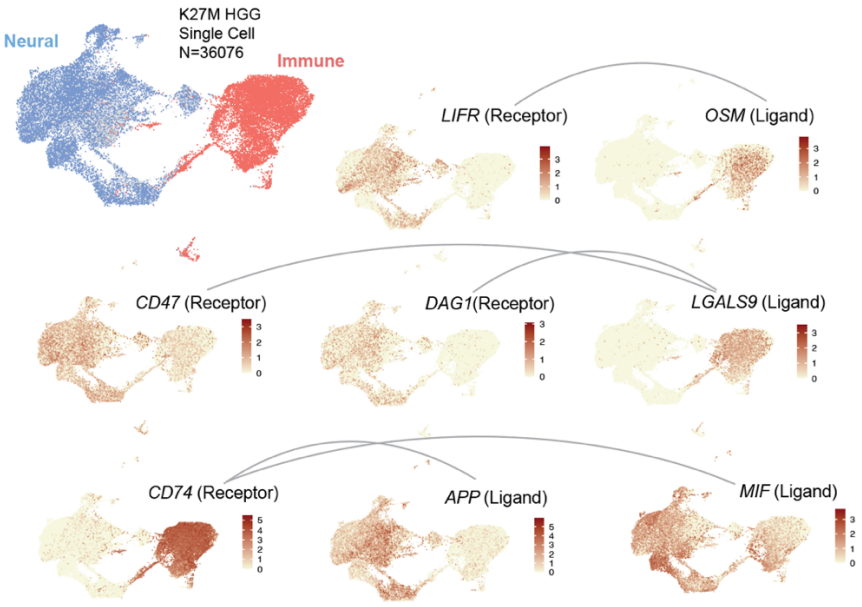
CSF1R for one week and PD-1 for four weeks. Despite the shorter duration of anti-CSFR1 treatment, we observed an increase in CD3 infiltration in tumors with this therapeutic combination. We have included these findings in Suppl Fig6E of our revised manuscript and hope this will address the reviewer's concern.



Minor comments:

5. Figure 2 C-E: scale bars are missing; Suppl Fig A: maybe provide higher magnification insert and H&E overview; E: scale bars missing.
We apologize for this omission. We have updated the figures by adding the scale bars and higher magnification inserts.

6. Figure 3D: this means both ligand and receptor are expressed? What would be sender, what the receiver cell type? Maybe the caption needs further explanation here, or ligand-receptor pairs need to be outlined.
We thank the reviewer for the suggestion. We have revised the figure to clearly label ligand and receptor pairs and updated Supplementary Table S2G, which now includes receptor and ligand information from each interaction as well as the cell types involved.



7. Suppl Figure B, C, E: scale bars missing
The scale bars were added to the figures.

8. Recent single cell atlas data of pediatric high-grade glioma need be cited: https://doi.org/10.1093/neuonc/noad207
Thank you for the suggestion; this study was published after our manuscript was sent for review. The citation has been now added in our revised manuscript. Please see also our response to Reviewer 3, point 5, for a discussion of the quality of this atlas in comparison with the one we report here.

Reviewer #2: The manuscript presented by Andrade et al. offers a comprehensive analysis of the immune tumor microenvironment (TME) in H3.3K27M and G34R/V-mutant gliomas. This study is notably significant for its depth in exploring the intricate dynamics within the TME, utilizing a blend of transcriptomic and proteomic methods, including advanced spatial single-cell approaches.
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demonstrates that these interactions are conducive to immunosuppression, thereby facilitating tumor formation and maintenance.
Therapeutic Implications: Perhaps most importantly, the study explores the therapeutic potential of dual inhibition of myeloid cells and immune checkpoints. The significant benefits observed in pre-clinical syngeneic models open new avenues for targeted therapy in H3-mutant gliomas.
We would like to sincerely thank the reviewer for their laudatory review of our work, and for recognizing its strengths and relevance.

Weaknesses:
1. In 103 their sentence suggests that a subset of ATRT tumors have shown response to ICI which is iis misleading as the reference they cite shows ICI response in a flank (non-orthotopic) syngeneic mouse model.
The mentioned study also used PD-1/PD-L1 blockade to treat their inducible model (Smarcb1$^{flox/flox}$;Rosa26-Cre^{ERT2}), also demonstrating an increased survival (please refer to Figure S5E from their study).

2. Fig F1E: The authors label this as a comparison of propotion of immune types in the total immune population. What is their definition of total immune population. Are they all CD45+ cells.
We defined the total immune population based on a reference mapping approach, performed at the single-cell level. Briefly, we used a consensus of up to 10 machine-learning classifiers that were trained on single-cell atlases to classify each individual cell. First, a murine brain developmental atlas (Jessa et al, Nat Genet 2022, https://doi.org/10.1038/s41588-022-01205-w) was used to identify broad cell types, and a glioblastoma immune atlas (Pombo Antunes et al, Nat Neuroscience 2021, https://doi.org/10.1038/s41593-020-00789-y) was used next to identify immune types with high granularity.

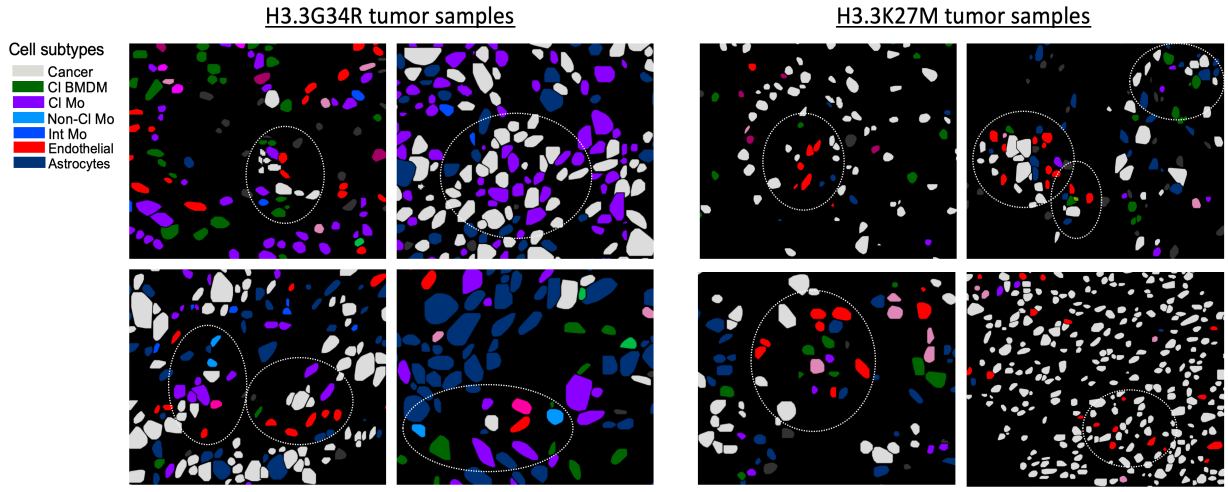
This approach overcomes some of the main limitations of single-cell data. First, it is performed cell by cell and does not rely on clustering, which makes it robust to batch effects. Second, it uses the entire expression profile of each cell instead of relying on a single gene (such as CD45), and thus overcomes the problem of high dropout rate in this data type. We have clarified this definition in the revised version. Furthermore, prompted by the reviewer's question, we quantified CD45 across immune populations, and we found a very high detection rate in all immune types, consistent with the expected dropout levels (as a reference, GAPDH, one of the highest expressed ubiquitous genes, has an average detection rate of 91% in this dataset):

Cell Type	**CD45 (*PTPRC*) % Expressed**
Macrophages	76.6
Microglia	64.2
Monocytes	77.2
Dendritic Cells	82.5
Proliferating Myeloid	76.3
T Cells	80.1
NK Cells	74.5
Average All Immune	75.9

3. The imaging mass cytometry study is commendable and gives rise to fascinating insights in their study. However, this also requires validation investigations. From the IMC studies the authors infer that different myeloid subsets are found to interact with H3-mutant cells. Especially the fact that Classical BMDMs showed strong interactions with both H3.3K27M- and G34R-mutant cells while monocyte subsets tended to interact mainly with H3.3G34R cells. This needs to be validates using classical Immuno-Flourescence studies using high resolution confocal microscopy using appropriate controls. As presented it is difficult to verify their very interesting observations. Similarly, with their observations with endothelial cells in K27M tumors

We thank the reviewer for their constructive feedback on the validation of our IMC findings. In preparation for our IMC studies, we ensured the antibodies were first validated using immunofluorescence on a broad range of control tissues, including spleen, tonsil, lung, GBM, brain metastasis, amongst others. This preliminary validation was critical to establish the specificity and sensitivity of each antibody, ensuring their appropriate application in IMC for accurate and reliable detection of protein expression in our samples. Further details on the validation process, including images from these controls, are extensively documented in our previous publication: Karimi et al., Nature 2023, https://doi.org/10.1038/s41586-022-05680-3. It includes hundreds of quality control images that demonstrate the robustness of the antibody validation process. These QC images serve as a foundational element for the credibility of the antibodies used in our current IMC analysis.

To address concerns regarding the validation of our IMC data using high-resolution confocal microscopy, we have included several high magnification images that highlight some of our findings. Our segmentation pipeline and strategy are robust, and we believe that the accompanying images, along with our rigorous quality control procedures, should provide satisfactory support for our claims. These elements collectively demonstrate the reliability and accuracy of our data. Once again, we thank the reviewer for this comment.



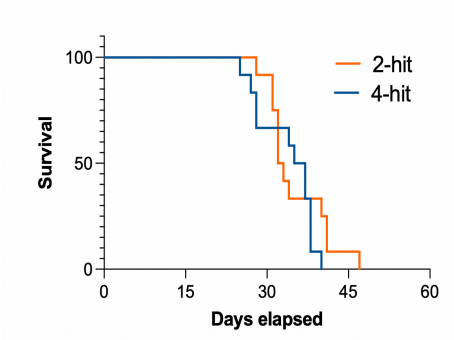
4. In Line 248 the authors point out CD47/LGALS9 as potential mechanisms of immune suppression. However, LGALS9 is usually expressed on lymphoid cells which are in low numbers. Is this aberrant expression. The cell-cell interactions shown here need to be verified using alternative techniques otherwise the data remains uncorroborated.

Recent studies have shown the expression and role of LGALS9 on myeloid cells (Yang et al., Nat Commun 2021, https://doi.org/10.1038/s41467-021-21099-2 and Lee et al. Can Res 2023, https://doi.org/10.1158/1538-7445.AM2023-2882). Yang et al. (2021) suggested that Gal-9 is expressed in APCs (B cells, dendritic cells, and tumor-associated macrophages) and cancer cells and its secretion from these cells dampen antitumor response by inducing T cell death. Regardless, we have edited the text to include more details about the caveats of our study. Specifically, we acknowledge that their expression and role (ligands-receptors identified by scRNAseq) in H3-mutant tumors should be further investigated.

I commend the authors on their efforts to corroborate their patient sample findings in additional mouse models.
We would like to thank the reviewer for recognizing the vast amount of work we undertook prior to reaching the conclusions we included in this manuscript.

5. In line 272 for Fig 4A, the authors talk about 2 syngeneic models but do not describe them here. Are the left and right panels from their 4 hit and 2 hit. The right panel also has PDGFR-OE, which should not be there according to their 2-hit model.
We apologize about the confusion; we used multiple different syngeneic models to corroborate our results on immune cells in the TME. The two syngeneic models referred in Fig4A are two other H3K27M models that were previously published (Pathania et al., Cancer Cell 2017, https://doi.org/10.1016/j.ccell.2017.09.014 and Golbourn et al., Nat Cancer 2022, , https://doi.org/10.1038/s43018-022-00348-3). We have now updated the legend.

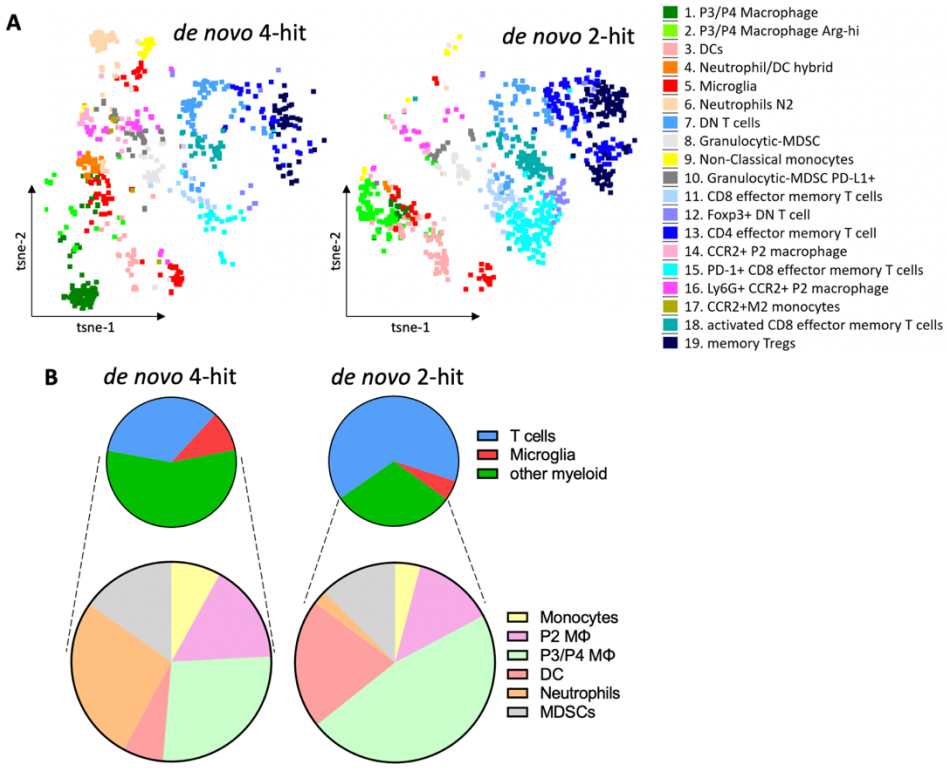
6. Are there any survival differences between the 2 hit and 4 hit
There is no significant survival difference between both. In the 2-hit model, tumor symptoms arise around 32 days, and in the 4-hit model around 36 days.



7. Classical flow cytometry analysis of the TME between the 2 hit and 4 hit in the de-novo setting and analysis of the draining lymph nodes would be extremely useful and would significantly add to the value of this manuscript.
We appreciate the reviewer's suggestion. Regrettably, we did not harvest the draining lymph nodes in the meninges as our focus was on the brain tumor site. However, we intend to include this assessment in our future experiments. In response to the reviewer's recommendation, we conducted flow CyTOF analysis to profile the TME in both the *de novo* K27M 2- and 4-hit models, as presented below and provided in Suppl Fig 5D-E of our revised manuscript.

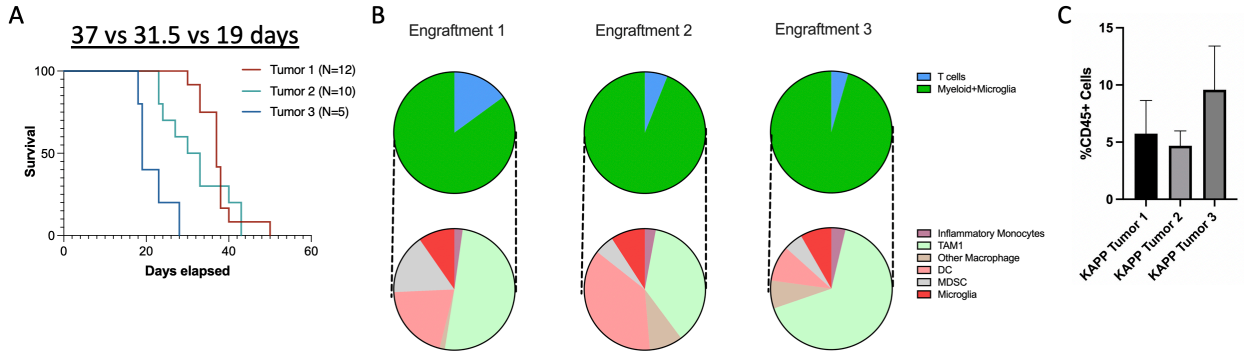
The results are intriguing. We found that the *de novo* 4-hit model exhibited significant myeloid infiltration and minimal lymphoid infiltration, similar to the engrafted 4-hit tumors and to what is observed in human tumors. This myeloid infiltration was primarily comprised of P2 and P3/P4 macrophages, along with myeloid-derived suppressor cells (MDSCs). Conversely, the *de novo* 2-hit model showed higher T cell infiltration, with the myeloid compartment dominated by P2 and P3/P4 macrophages and dendritic cells. Interestingly, upon engraftment of the established 2-hit cell line in C57Bl6 mice, myeloid infiltration became predominant, resembling the pattern seen in 4-hit tumors and human patient samples (Suppl. Fig. 5H).

Given that the majority of patients present with at least three hits and rarely with only two hits, we cannot conclusively demonstrate that these findings reflect the natural course of the disease and the evolving TME and its interaction with tumor cells. Therefore, we have included these findings in our revised manuscript without making definitive conclusions. We suggest that further investigations on patient samples, particularly in the very rare cases where the H3K27M mutation is identified alone or with only TP53 alterations, is necessary to validate whether increased lymphoid infiltration can be observed at earlier disease stages, before the acquisition of other genetic alterations that may further contribute to the development of a more immunosuppressive TME. We have added these new data in our revised version of the manuscript (Suppl Fig 5D-E).



8. In Line 343, the authors summarize that "These results suggest that the more aggressive and faster-growing tumors in serial engraftments are associated with an expansion of myeloid cells, including newly recruited 345 BMDM, at the expense of other immune populations." There is an argument to be made that the lack of lymphoid cells could be due to the short life span of tumor growth that occurs. Were the tumors from Egraftments 1 and 3 harvested at the same time?

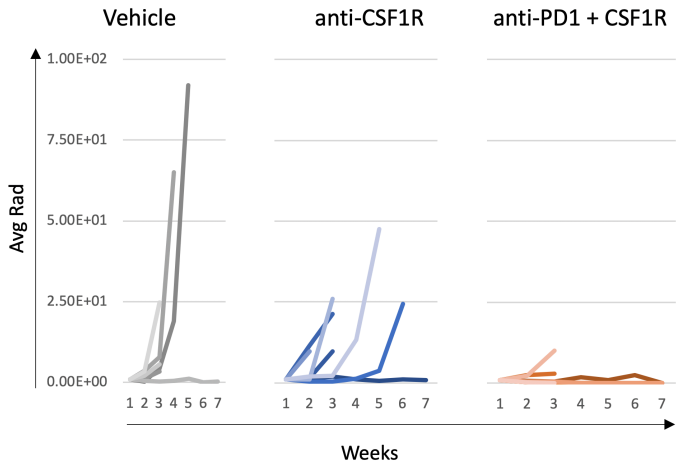
Only the tumors from Engraftment 3 showed different growth kinetics. Engraftment 1 and 2 tumors were collected after an average of 37 and 31.5 days, respectively. Tumors from Engraftment 3 were collected after around 19 days. Remarkably, when we compare tumors with similar growth kinetics (from Engraftment 1 and 2), we already observe a reduction of T cell infiltration at Engraftment 2, and this difference is kept at Engraftment 3. Additionally, Engraftment 3 showed an increased CD45+ infiltration. While we cannot complete exclude the possibility of different kinetics accounting for the lack of lymphoid cells, we do not think this is the case for this model.



In their pre-clinical studies some key data is missing

9. The authors need to show the relative tumor sizes when treatment starts and the decrease in tumor burden over time.

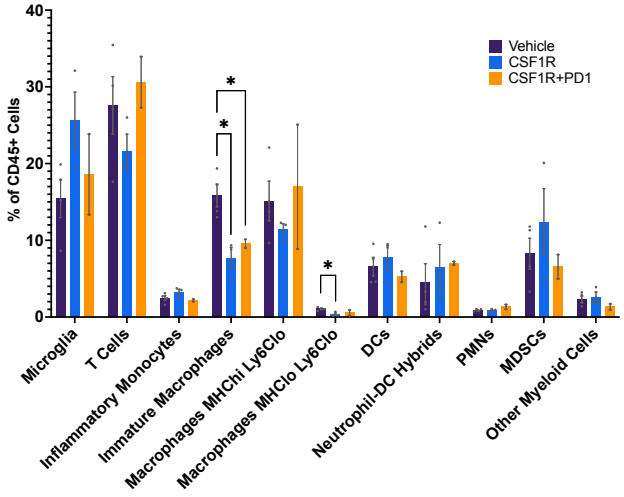
To address the reviewer's concern, we have added below the bioluminescence values (Akaluc/AkaLumine) for the different arms. We have regularly imaged the mice in all arms during 8 weeks to account for tumor engraftment and any therapeutic effect of the different experimental arms. We observed decrease signal only for anti-PD1+CSF1R, suggesting impairment of tumor growth in this arm.



10. What is the change in the myeloid compartment after the individual therapies combined

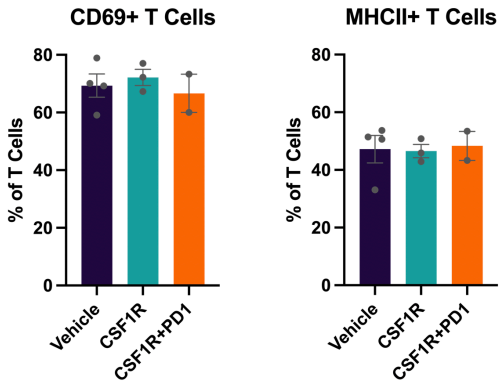
This point was also differently raised by reviewer 1. We had not included these findings in our manuscript as only two populations were significantly affected when we used CSF1R inhibition alone. We are including now in our revised manuscript the results from CyTOF investigations in

the three arms. As shown below, CSF1R treatment decreased the levels of immature (also reduced with the combination) and MHCII^{low}Ly6^{low} macrophages.



11. Are the CD3 cells in the combination treatment active?

We concur with the reviewer that this is an important question. Levels of activation markers (CD69 and MHC class II expression) were not notably different among the various experimental groups and were present in numerous T cells (please refer to the data below). This crude evaluation of T cell activation indicates that these cells within the tumor site are predominantly activated T cells and that the combination treatment does not alter their activation profile significantly but primarily results in an increased recruitment of T cells. Additional studies are needed to further validate these findings and characterize in more depth T cell activation. We have added a statement to this effect in our revised manuscript and hope the reviewer agrees they are beyond the scope of the current study.



12. In the mice that survived, analysis of the lymph nodes would be very informative

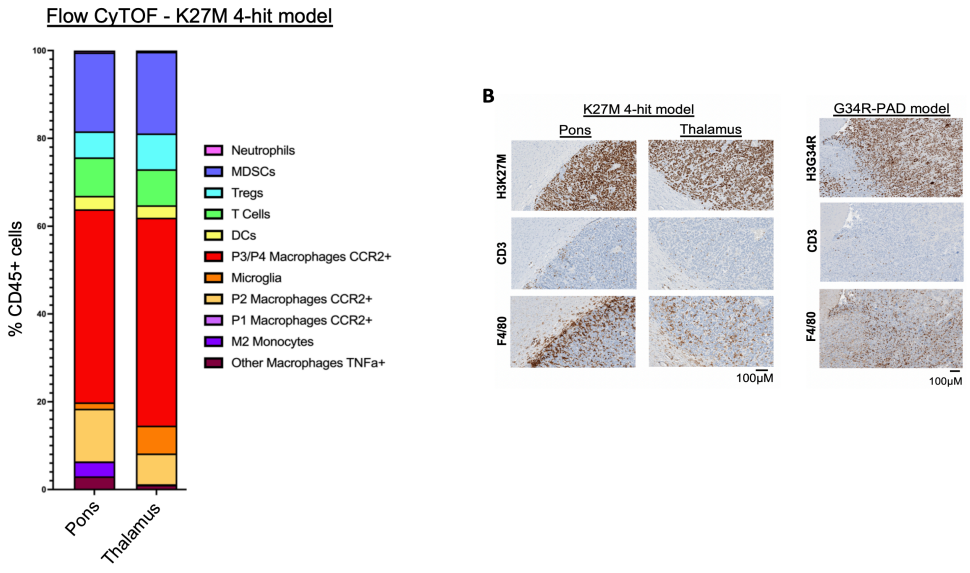
As stated above for draining lymph nodes, we unfortunately have not harvested lymph nodes for the treated mice. This will be done in future studies.

13. What is the difference in the G34R-PAD model with respect to ICI and anti-CSF1R blockade

Due to the limited availability of H3.3G34R human tumors and the need for additional mouse models featuring other PDGFRA mutations, we made the decision to concentrate our investigations on the H3.3K27M models. Investigating G34R tumors and models is important and will be the focus of future studies, where we also plan to delve into the response to ICI and anti-CSF1R blockade. We hope the reviewer agrees that, given the comprehensive data provided in this study as they and Reviewer 1 acknowledge, this aspect can be addressed adequately in future research.

14. Is the TME dependent on the location of the tumor or the mutation it harbors?
This is an important question that we also brought in our response to the first comment to Reviewer 1. IHC results showed a large myeloid and limited lymphoid infiltrate, regardless of tumor location or the H3.3 mutation (cortex for G34R model, and pons or thalamus for K27M models).

Regardless, as this approach mainly provides global overview, we performed flow CyTOF to compare the TME from the two main H3.3K27M targeted brain midline locations, using the K27M 4-hit tumors to engraft them in the pons or the thalamus of C57BL6 mice. We profiled them by flow CyTOF and IHC. Now provided in Fig 6F-G of our revised manuscript. Both tumor locations showed overall similar immune subtypes and proportions and abundant myeloid infiltration. However, we observed increased infiltration of CD45+ cells and microglia in the thalamus, and M2 monocytes in the pons. We did not compare to the cortex as H3.3K27M is exceptionally encountered in the brain hemispheres in human disease, and the tumors we generated in that location were with another oncohistone, H3.3G34R and PDGFRA mutations, which may mitigate results. These results allude to differences in the TME based on the brain location that require more in-depth assessment and have been added to the revised manuscript. Further studies are warranted to investigate more precisely these differences and their impact in tumorigenesis, and extend the comparison to cortical tumors.



15. Not sure what is the purpose of the humanized mouse model since it is not used in any functional or mechanistic study.

We have used the humanized mouse model to validate that when using patient-derived cells we also obtain a rich myeloid infiltrate, as seen in the murine tumor models. This model corroborates that human cells are able to recruit similar immune subtypes as those identified in our syngeneic murine models. We hope the reviewers agrees that while this model was not used in functional studies it provides added confirmation if needed on the predominance of myeloid infiltration in these H3-mutant gliomas.

Reviewer #3:The study by Andrade et al describes the characterization of the immune microenvironment of pediatric Histone H3-mutant gliomas. The authors begin by examination of the immune landscape of H3.3K27M and G34R/V-mutant gliomas using single cell technologies. Utilizing data derived from both human patient tumors as well as glioma mouse models, they infer that the predominant immune cell population in these tumors are of myeloid origin. They show that these cells promote immunosuppression and that while blockade of CSF1R or PD-1 individually did not alter tumor burden or survival, combined blockade of these proteins improved survival in glioma bearing mice which correlated with increased T cell infiltration. Overall, while the manuscript represents a large immune landscape study for pediatric Histone H3-mutant gliomas, the lack of any novel findings that emerge from the analyses dampens the enthusiasm of the reviewer. Furthermore, despite the use of various advanced technologies (CyTOF, scRNA-seq etc.) the data provided does not seem to advance apriori knowledge (e.g., myeloid cell abundance in pediatric gliomas, immunosuppression and targeting CSF1R/PD1, all of which are widely established paradigms). Lastly, the data analyses and interpretation, especially of the scRNA-seq should be improved to obtain meaningful results. Specific comments are shown below.

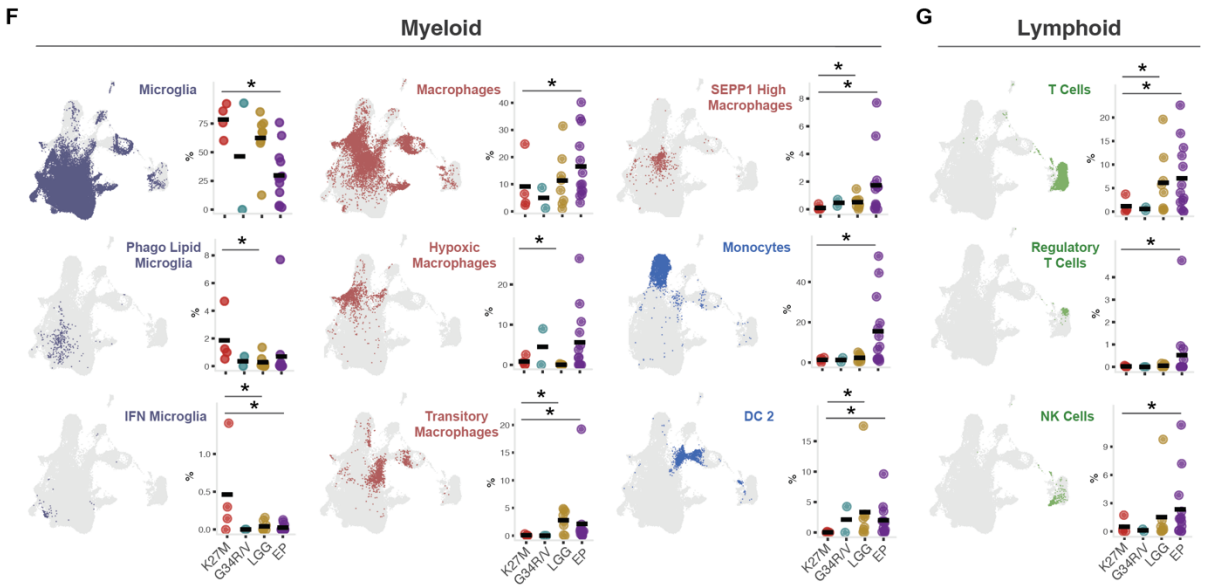
1) There is no information provided on single cell dissociation of resected tissues for the human gliomas. How were the samples processed? If enzymatic digestion was used to separate the tissues, more details are needed. One concern is the estimate of the different cell populations as presented may not be accurate. Different methods can significantly affect the yield of immune and non-immune cells as discussed in Sarcocic et al (PMID 36339814). The authors must provide a detailed methodology for the sample preparation and acknowledge the effects of the method on cell yield.
As we had referred to our previously published papers, we had not elaborated in our initial submission on the methodology of single-cell dissociation to try and respect word count. To address the reviewer's request, we have now updated the methodology section, adding the information regarding sample preparation and processing for single cell.

We agree with the reviewer that single cell dissociation of resected tissues may variably impact specific cell proportions including immune cells as shown by several groups including Sarcocic et al., cited by the reviewer. We performed enzymatic papain dissociation a commonly used dissociation technique in brain tissues in several studies and acknowledge that this may have altered the immune infiltrate, as also suggested by the study cited by the Reviewer. However, we would like to respectfully stress that all samples were treated similarly including those that showed higher lymphoid infiltration. Also, we did not solely rely on single cell transcriptomics to characterize the immune infiltrate and performed spatial proteomics in the form of Imaging Mass Cytometry, which independently validated these findings. Moreover, when doing deconvolution analyses of transcriptomic signatures, we also obtain limited signatures of lymphoid cells.

Nevertheless, to address the reviewer's concern, we now acknowledge the effects of sample preparation on immune composition in our revised manuscript.

2) The sample numbers, although at first read appears adequate for statistical comparisons, is not sufficient to make meaningful inferences. For example, only two tumors were analyzed for G34R group. Broad inferences cannot be made with n=2.

We respectfully point out that, as the sample size for the G34R group is indeed small, we had refrained from making inferences on this group, and only made claims when we had statistical power. The reason to include G34 samples is that they are a valuable resource to the community, given that single-cell resolution data is scarce for these rare tumors.
In any case, to prevent misinterpretations, we have a) included a new statistical test to compare cell proportions with bootstrapping (Miller et al., Cancer Res 2021, https://doi.org/10.1158/0008-5472.CAN-20-3562), and b) moved the panel quantifying cell proportions across samples (former Figure 1D) to supplementary materials (now Figure S1B), and changed the representation of quantifications in Figure 1 to clearly display the number of samples as well as the associated p-values for the relevant comparisons (New Fig 1E-G, new Fig S2B).



3) The lack of batch effect correction on scRNA-seq is worrisome. There are many methods for batch correction, and these are evolving. One example is the study by Yu et al (PMID 36810607) where different methods are compared. The authors must apply robust batch correction measures and show evidence of lower rejection rates before and after the correction.

Since we agree with the reviewer that batch effect is an important factor and we are aware of the many methods available to correct for it (which we use when warranted, e.g. Jessa et al, Nat Genet 2022, https://doi.org/10.1038/s41588-022-01205-w; McNicholas et al., Cancer Discov 2023, https://doi.org/10.1158/2159-8290.CD-23-0004), we had purposely chosen analytical strategies that are not sensitive to batch effects in our analyses.

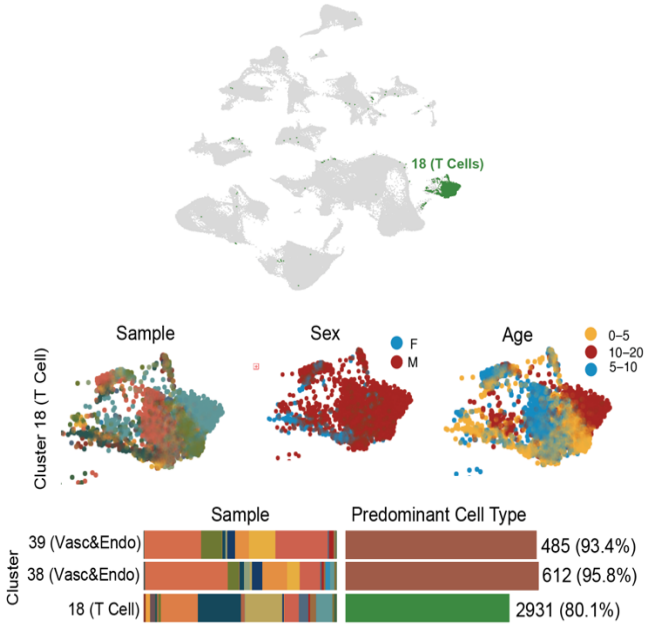
Indeed, in our first submission, we avoided explicit batch correction methods as these are black-box data transformations that we only apply when required. Our main claims here are supported by analyses not affected by batch:

- To identify immune cell types, we annotate cells using a reference mapping approach **performed at the individual cell level**. This approach is not sensitive to batch problems. First, it is not based on clustering (known to be affected by batch), but instead produces labels for each individual cell. The study suggested by the reviewer (Yu et al) evaluates batch correction methods for cluster-based approaches, and is not applicable here. Indeed, we overcome the problems associated with clustering by omitting clustering steps altogether. Second, our annotation approach uses a consensus of 9 machine learning classifiers. While each individual classifier could be potentially sensitive to technical variations such as batch differences, taking the consensus of multiple tools has been shown to overcome this problem and produce results robust to this variation. Finally, each classifier uses a different normalization method from the raw counts; a transformed matrix (the output of batch removal methods) would be incompatible in this case.

- To investigate the diversity of immune types, we quantify proportions **in a per-sample basis**, and compare across tumor types. Once again, batch removal methods would not alter this result.

- To investigate gene expression patterns, we use mainly ssGSEA (single-sample gene set enrichment analysis). This method, as it is ranked-based (instead of relying on absolute expression patterns) and scores lists of genes (instead of individual genes), is also relatively robust to batch variation.

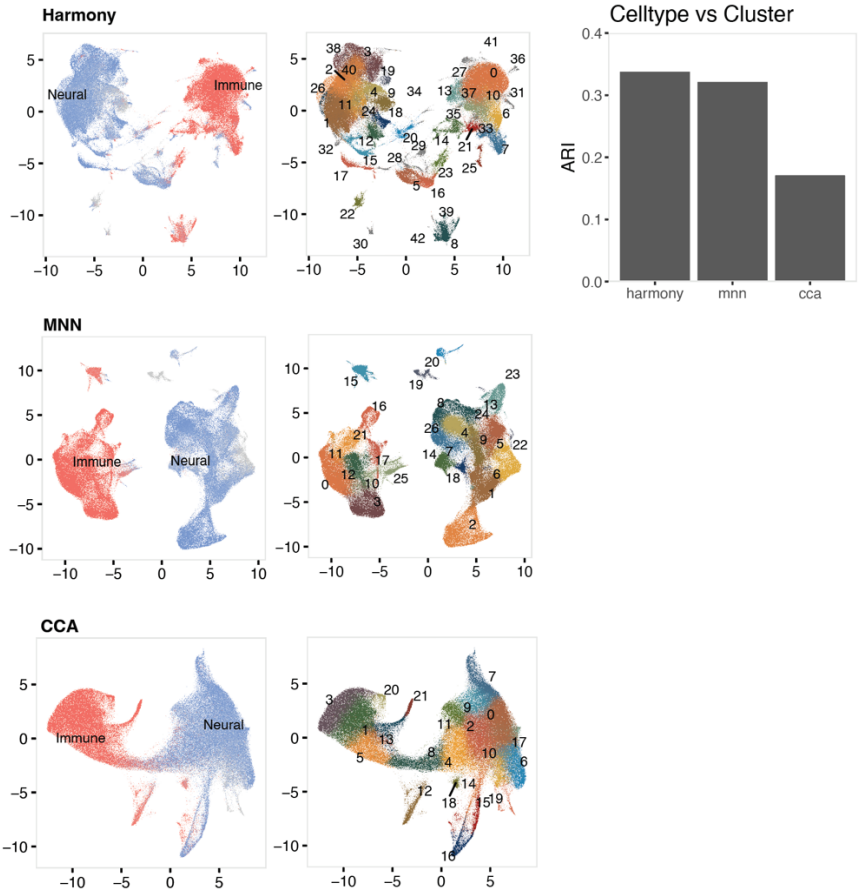
Furthermore, several immune cells (such as T-cells) and other normal cell types from all patients mixed well without requiring integration, indicating minimal batch effects to start with:



***Top**: UMAP of snRNAseq without batch correction applied with T-cells indicated in green.*
***Bottom**: T-cells and normal vascular/endothelial cells clustered together in the unintegrated space independently of sample, indicating minimal batch effects.*

Nonetheless, to address the reviewer's concern, we have now benchmarked and applied 3 batch correction methods: Canonical Correlation Analysis (Butler et al., Nat Biotechnol 2018, https://doi.org/10.1038/nbt.4096), Mutual Nearest Neighbours (Haghverdi et al., Nat Biotechnol 2018, https://doi.org/10.1038/nbt.4091) and Harmony (Korsunsky et al., Nat Methods 2019, https://doi.org/10.1038/s41592-019-0619-0).

To select the method that best integrated samples in this cohort, we performed clustering in the integrated space and computed the agreement between the clustering and the cell type labels using adjusted rand index (ARI):

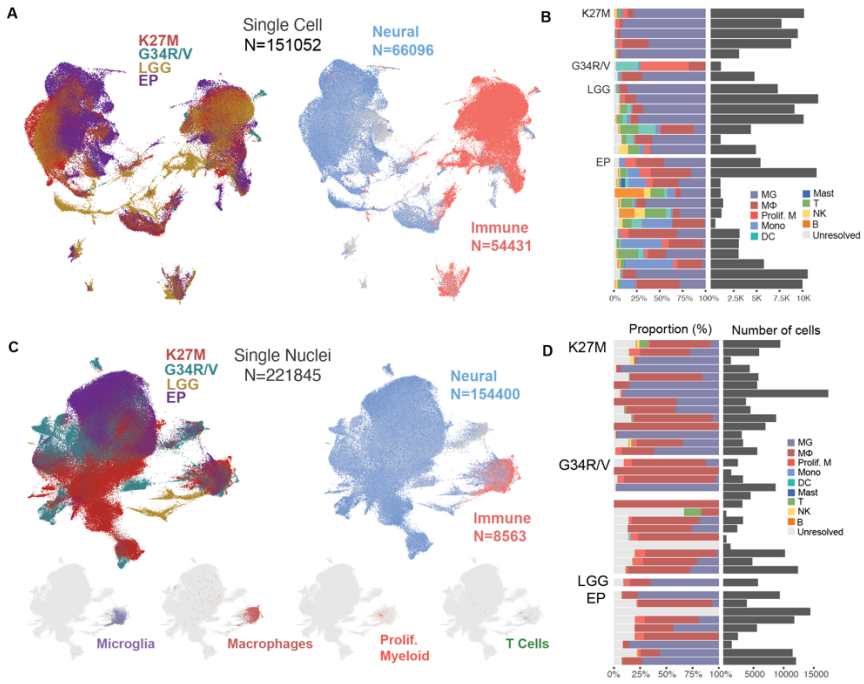


As Harmony outperformed the other methods, we now include visualizations in this batch-corrected, integrated space with Harmony in main figures, and provide the non-transformed results in supplementary figure S1E-F.

4) The study exemplifies a missed opportunity on deeply analyzing single cell RNA-seq data and especially the myeloid cells in pHGG. There is hardly any information on the number of cells

analyzed, immune versus non-immune, sequencing depth etc. How many cells were isolated from each sample?

We would like to respectfully point to the reviewer that these numbers were included in the original submission, in main figures. As they were missed by the reviewer, we have revised the presentation to make this information easier to find. We now included Supplementary Table 1B with detailed information per sample (library protocol, total number of cells, fraction of immune cells, QC metrics.). Relevant numbers of cells are also clearly indicated in Supplementary figure 1A-D:



The authors can further strengthen the cell type analyses by sub-clustering of myeloid populations from the scRNA-seq data since this is the strength of the manuscript. More than 70% of the immune populations are myeloid cells, but beyond classifying them as microglia or macrophages, there is not much inference on the cell states. Gene ontology or publicly available resources such as M-VERSE (https://macroverse.gustaveroussy.fr/2021_M-VERSE) can further strengthen the study with respect the myeloid cell types. This is even more crucial given that the authors claim that myeloid cells are immunosuppressive, but if the identification of CSF1R as a main targetable pathway is the conclusion of the manuscript, there was no need for single cell sequencing, since CSF1R is already shown to play an immunosuppressive role in pediatric and adult gliomas.

We thank the reviewer for the comment and have now revised the analysis of myeloid cell populations accordingly. For this, we have

a) improved on the cell type identification method (reference-based mapping) to produce more granular labels: we have expanded it to include 9 machine learning classifiers and refined the method to integrate these classifications to reach a final cell type label, which now uses a Consensus Accuracy Weighted Score (Large et al., Data Min Knowl Disc2019, https://doi.org/10.1007/s10618-019-00638-y);
b) included a large reference atlas profiling glioblastoma immune cell populations (Pombo Antunes et al, Nat Neuroscience 2021, https://doi.org/10.1038/s41593-020-00789-y) to

train these classifiers. This reference is enriched in myeloid cells and has very granular annotations, allowing the identification of more than 8 distinct myeloid types. We hope the reviewer will agree that this is a more appropriate reference than the M-Verse, which is a murine atlas, mainly focused on Alzheimer disease and aging;

c) included a new bootstrapped permutation test to assess statistically significant changes of these granular cell populations (Fig 1E-F, Fig S2B);
d) characterized gene expression profile changes across tumor types for each myeloid population, including investigation of Hallmark signatures using GSEA analysis.
e) defined gene markers of K27M myeloid cells that are specifically upregulated in this tumor type.

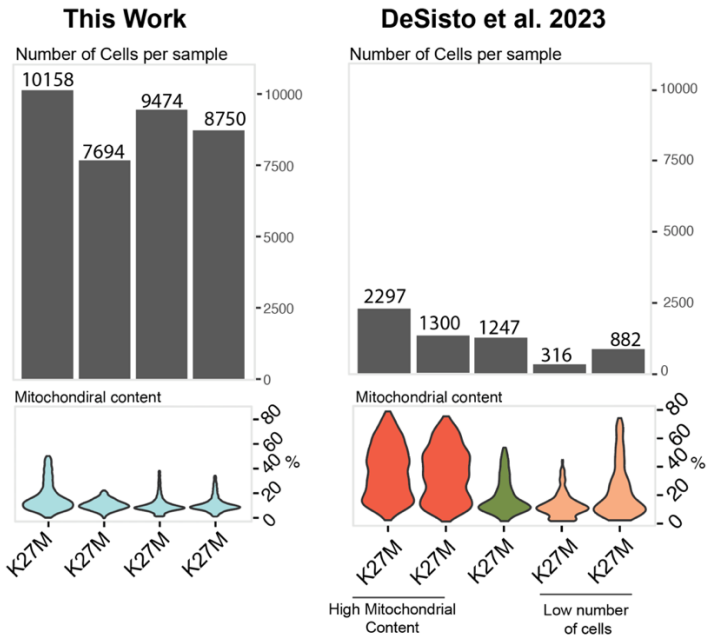
These analyses allowed us to identify myeloid activation states specific to different tumor types, including hypoxic and transitory macrophage populations, IFN-activated and phago-lipid microglia as well as regulatory T cells and NK cells.

Once again, we thank the reviewer for this comment.

5) Many references of previous literature that have shed light on the immune cell landscape on pHGG have not been cited. The authors must (in the discussion section) compare the results from the current study to Plant et al., J Neuroonc, 2021, Griesinger et al, J Immunol, 2013 and the more recent DeSisto et al, Neuro-onc, 2023. How does this study present any advance from these previous reports.

We would like to respectfully remind the reviewer that we did acknowledge previous work published on immune infiltrates in pediatric high-grade gliomas and that most showed predominance of myeloid infiltration albeit on smaller sample size, more limited markers and no spatial information, and have tried to cite the most relevant studies to this effect. We did not cite the two first studies highlighted by the reviewer since they were done on cohorts that consisted mainly of pilocytic astrocytoma, ependymoma, and medulloblastoma and, ***respectively, included only 5 and 3 high-grade glioma samples, with no indication on the molecular driver, patient age and tumor location***. As the H3 status is unknown and the sample size more limited, we could not reliably compare them to H3-mutant high-grade gliomas investigated in this study. Indeed, Griesinger et al. (2013) included only 5 glioblastoma samples labelled as pediatric glioblastoma with no molecular, age or brain location information, compared to other brain tumor types. For the other study, did the reviewer mean Plant et al; Journal of Neuro-oncology 2018 as we have not been able to find Plant et al., J neuroonc 2021? This study only included 3 high-grade gliomas and here as well did not provide molecular data on their samples. As the H3 status is unknow, a direct comparison would be inaccurate.

The third study from DeSisto et al (Neuro-onc, 2023) was published after our manuscript was submitted. To compare their finding with ours, we obtained this dataset and analyzed their single-cell datasets. As the reviewer will see, the quality of this dataset (in terms of numbers of cells, as well as various QC metrics) is not sufficient to properly incorporate it in the current manuscript. Indeed, from the 5 K27M tumors reported, two have very low numbers of cells (preventing a proper profiling of immune diversity), and two have very high mitochondrial content (~80%), indicative of low-quality samples:



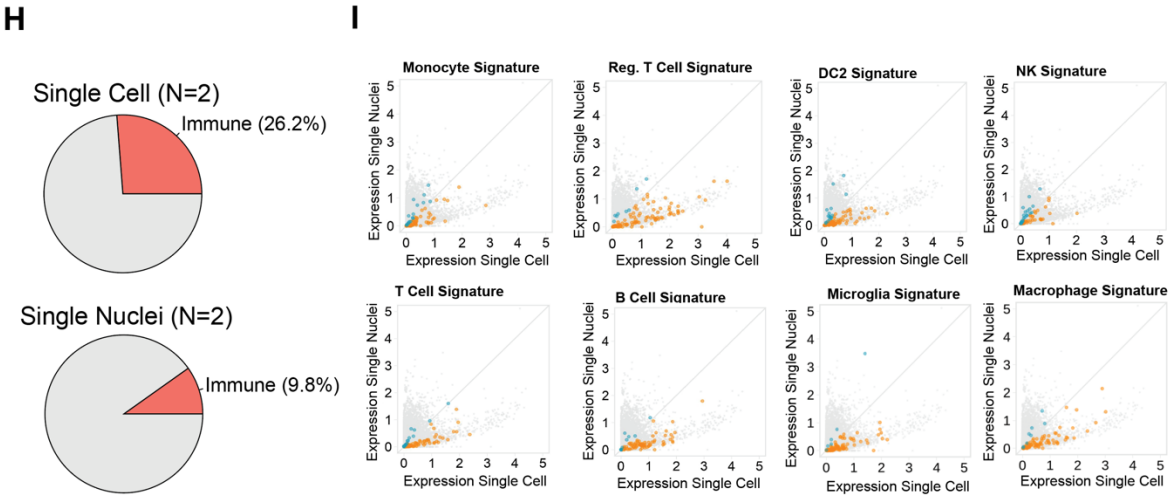
Nonetheless, we now cite this study in our discussion section, and have added:
"… a recent study which profiled 5 H3.3K27M HGG using single-cell transcriptomics also describes a heterogeneous immune infiltrate, including tumor-associated macrophage populations, T, NK and monocytes."

6) Fig. 1B describes the actual number of cells processed for scRNA-seq (n=140743). What is the split for cancer and immune cell contributions?
We have now clearly included this information in the revised version (see also point 4 above). In the case of scRNA-seq, the percentage of immune cells was 36 % (n= 54,431 out of 140,743 total cells).

7) Why is the data only from K27M, but not G34 R/V utilized for comparisons in Fig. 1F and 1G?
As pointed out by this reviewer in point 2 above, the G34R/V group is too small for meaningful comparisons. As noted in our response to point 2, we have refrained from making comparisons where we lack statistical power and included the G34R/V samples as a valuable resource to the research community, to be leveraged in future studies. We hope the new layout of Figure 1 is helpful to avoid misinterpretations of our data.

8) The data shown in Supp Fig. 1D shows that the myeloid cell populations in pediatric gliomas are mainly macrophage, but not microglia. This contrasts with data shown in scRNA-seq related Fig. 1D, where microglia is the major population. How do the authors explain these differences?
As a first step after processing and QC, we performed a careful comparison of the results obtained by single-cell and single-nuclei RNAseq (former figures S1C-D, S1F-G), including an experiment where we profiled the same samples with the two technologies (former Fig S1G). We found that snRNA-seq systematically underestimated the proportion and diversity of immune cells compared to samples processed using scRNA-seq in both human and mouse datasets. Indeed, in the samples

profiled with the two technologies, we see (1) a decrease in proportion of immune cells, (2) less diverse cell types captured, and (3) lower detection of genes from different immune signatures:



Similar results (as stated in our first submission) were reported for human microglia (Slyper et al., Nat Med 2020, https://doi.org/10.1038/s41591-020-0844-1); we now see them for all immune populations, not only for microglia. We believe this effect is observed since most of the gene expression regulation for immune related genes is achieved through post-transcriptional regulation, and thus is missed when extracting only nuclear RNAs.

For these reasons, we do not expect concordance from the two data types, and be base our claims on the single-cell data when there are discrepancies. As for the G34R/V samples, we include the snRNA-seq data as a resource for the community; we believe the analysis we provide demonstrating the limitations of this technology is important for future studies. We have now revised figures Suppl Fig1 to make this point clearer.

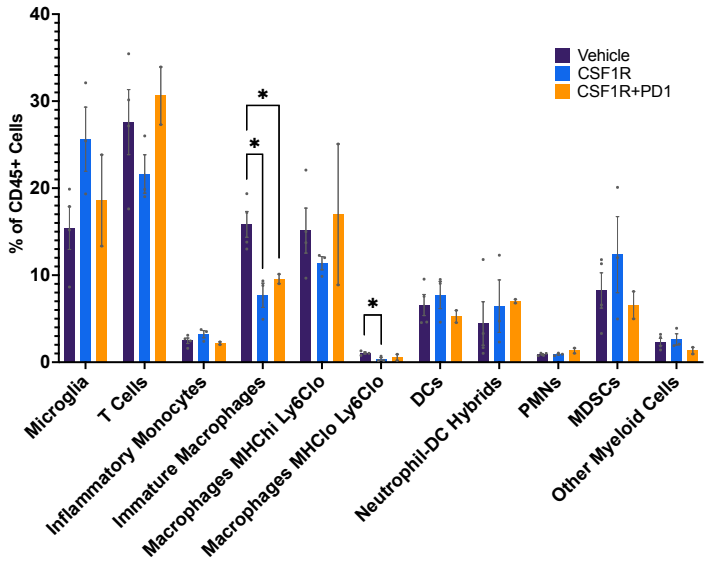
9) Fig. 2 generally validates the scRNA-seq findings that myeloid cells are abundant in gliomas. The data presented in Fig. 2G conveys that microglia and classical monocytes are differential between H3.3K27M- and G34R tumors. This again does not align with the findings from scRNA-seq or scRNA-seq.
We used IMC to expand scRNA-seq findings since it represents a better approach to quantify, at protein level, the cell subtypes present in a certain tissue. We have avoided directly comparing the two methodologies since they represent different approaches (RNA and protein) that are not always consistent. Besides, we have obtained scRNA-seq data for only two H3.3G34R samples, making our claims and comparison very limited. Finally, we point that snRNA-seq samples should not be considered for further analysis since it shows remarkable differences in immune cell populations. With this, we believe we cannot directly compare the number of specific cell subtypes found between IMC and scRNA-seq.

10) Fig. 3 is descriptive and no spatial statistics is provided.
Thank you for your comments and for highlighting the concerns regarding the descriptive nature of Figure 3 (now Figure 4) and the perceived lack of spatial statistics. In our study, we employed a permutation-test-based analysis to rigorously assess spatial interactions and avoidance among

single cells within the tumor microenvironment. For each pairwise comparison, we conducted 50,000 permutations, a methodological choice intended to balance computational feasibility with robust statistical power, given our small sample size. Cells within six pixels of one another were classified as interacting, and the significance of these interactions, or their avoidance, was determined by comparing the observed distributions to those generated through permutations. We set a threshold of 25,000 for our interaction/avoidance scores, which exceeds random chance, ensuring that our findings highlight meaningful biological interactions rather than incidental contact. We acknowledge that while our sample size may appear limited, it was sufficient to detect patterns in cell interactions that are likely to occur more than random chance. The permutation approach provides a robust framework to infer the non-randomness of spatial patterns from the observed data, despite the sample size constraints.

11) The rest of the manuscript does not provide any new findings beyond previously reported efficacy or lack thereof of CSF1R and PD1 treatments. The combination therapy however is promising, but it is not clear what is the mechanism of such a response.
We respectfully disagree with the reviewer, as do Reviewer 1 and 2. The extensive and systematic characterization of the immune landscape in the TME at the single cell transcriptome and proteome level in H3-mutant patient samples is a significant advancement. Our study not only validate these findings across multiple mouse models, from PDOX to syngeneic mouse models, but also includes CyTOF analyses rather than solely relying on IHC, which is the norm in most studies. Furthermore, the potential therapeutic benefit through modulation in a pre-clinical model is meaningful and novel data. Indeed, we bring several new findings, especially for the H3-mutant tumors context. We show the recapitulation of human findings across various murine H3-mutant models, indicating that these observations are not exclusive to specific models but rather inherent traits of H3-mutant gliomas, including the lack of response of PD-1 inhibitors. In addition, we showed that more aggressive and faster growing tumors, in serial engraftments, are associated with an expansion of myeloid cells at the expense of other immune populations, which best mirrored the TME of human H3.3K27M HGGs. And of utmost significance, we report the therapeutic implications, our study explores the therapeutic potential of dual inhibition targeting myeloid cells and immune checkpoints. Our substantial results observed in preclinical syngeneic models pave the way for novel targeted therapies in H3-mutant gliomas. The precise mechanisms that lead to tumor inhibition should be addressed in future studies. The significance and depth of the work we provide have been recognized by the other reviewers and we hope this reviewer will agree based on the clarifications and additional data we are providing herein.

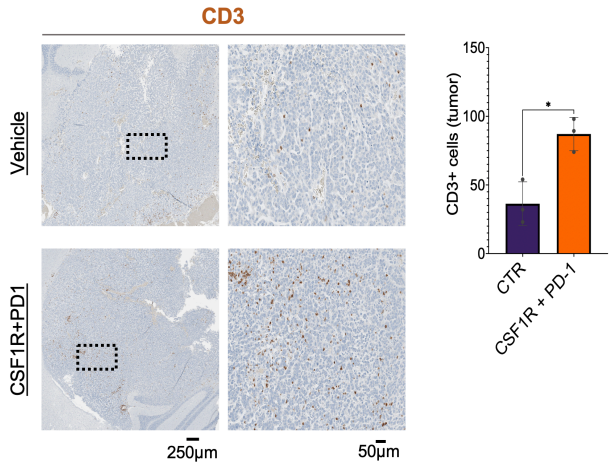
The authors show a general increase in T cell infiltration in CSF1R + PD1 inhibition group vs vehicle (Fig. 5G and 5H), but the data on individual inhibitors is not shown.
Since we have not observed a survival benefit or relevant differences when using CSF1R alone, we had shown CyTOF data only for the vehicle and the combination of inhibitors. To address the reviewer's comments, we have now added the CyTOF results from tumors harvested after use of the CSF1R inhibitor alone in Fig 6H (also please find below the image).



Furthermore, while 5G shows increased CD3+ T cells in the picture, this is not corroborated with any significant increases in 5H.

Out of the seven mice, tumors were observed in only three (with four showing no disease symptoms or tumors). Unfortunately, one of these mice was found dead, resulting in poor tissue quality, which limited further studies. Therefore, we were only able to utilize samples from two mice for IHC and CyTOF analysis.

To validate T cell infiltration and increase the number of mice analyzed, we conducted IHC using tumors from the experiment depicted in Fig. 6E (former Fig5E), where mice were treated with CSF1R for one week and PD1 for four weeks. Despite the shorter duration of anti-CSFR1 treatment, we observed a significant increase in $CD3^{+}$ infiltration in tumors with this therapeutic combination. We have included these findings in Suppl Fig. 6E of our revised manuscript and hope this will address the reviewer's concern.

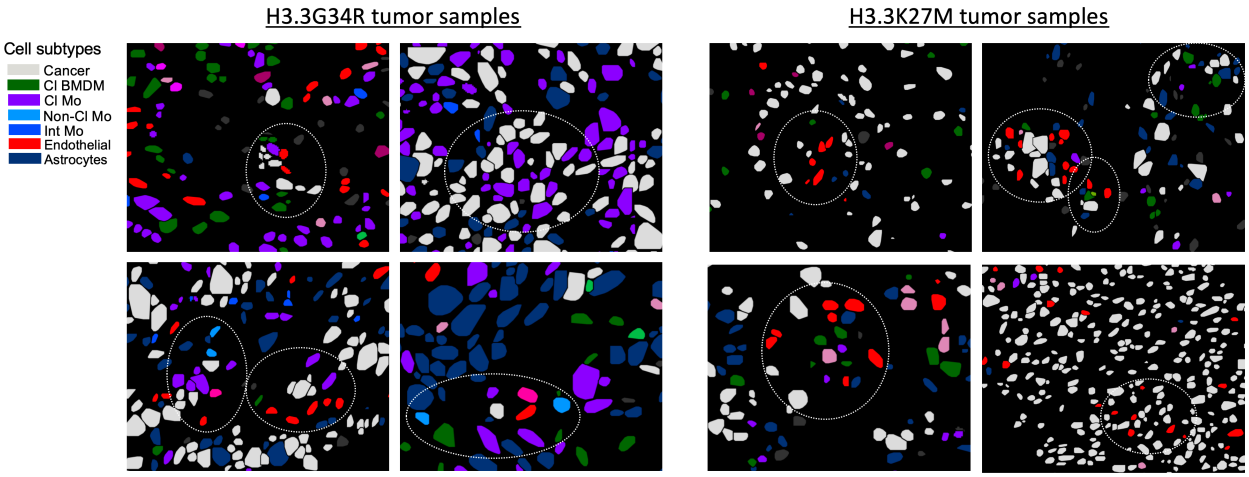


Minor points:

12) What does this mean "Classical BMDMs showed strong interactions with both H3.3K27M-

and G34R-mutant cells 230 while monocyte subsets tended to interact mainly with H3.3G34R cells"

To characterize the patterns of communication between individual cells, we interrogated the positional architecture of tumors using permutation tests to quantify cell–cell co-localization and identify interaction or avoidance behaviors between cell pairs. We have observed that classical BMDMs exhibited a strong likelihood of interacting with H3.3K27M- and G34R-mutant cells. Similarly, monocytes tended to interact with H3.3G34R-mutant cells. Please find some high magnification images that illustrate our findings. Our segmentation pipeline and strategy are robust, and we believe that the accompanying images should provide satisfactory support for our claims.



13) There are typos in the manuscript and will benefit from language editing. One example being "which may atively" in line 381.

We apologize for these typos. The text has been now revised and corrected for typos and edited for language where needed.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors provided a satisfactory revision and addressed the concerns elegantly. They delivered a thorough review of their manuscript which certainly merits publication.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily answered my queries. I commend them on the beautiful work carried out.

Reviewer #3 (Remarks to the Author):

The authors have addressed the critiques raised during the initial submission to my satisfaction.