nature portfolio

Peer Review File

A comparative transcriptomic analysis of mouse demyelination models and Multiple Sclerosis lesions

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
General critique:

This study by Aboelnour et al. combines novel and previously published single-nucleus and single-cell RNA-seq datasets from demyelination models and human MS samples to investigate shared glial reactivity patterns. Given the increasing application of focal demyelination models such as LPC and cuprizone in MS research, this work provides potentially valuable insights for model selection and understanding white matter lesion pathophysiology. A key observation is the partial overlap of glial responses across models, which appears to be recapitulated in human MS tissue. While the data integration is generally well explained, concerns remain regarding dataset quality, integration strategies, and consistency across data sources.

The design of the LPC surgical model appears inadequate. The manuscript describes only a single baseline LPC group, seemingly comprising contralateral, saline-injected mice at 7 dpi. This is problematic, especially for the corpus callosum, where contralateral effects due to interhemispheric projections cannot be excluded. Moreover, the 7 dpi baseline may suffice for comparisons within the demyelination phase but is not suitable for evaluating remyelination at 28 dpi. Separate baseline controls for each phase would have strengthened the experimental design. Notably, microglia proportions were unexpectedly lower in the saline-injected baseline group compared to the cuprizone baseline, complicating inter-model comparisons. These challenges are also evident in the poor integration of the Pandey et al. dataset relative to the other data sources (see Suppl. Fig. 3a).

In summary, while the conceptual framework is strong and the study addresses important questions in the field, the experimental design is insufficient, data integration is suboptimal, and statistical comparisons across groups raise concerns. As a result, many of the study's novel conclusions may be unreliable or confounded by technical limitations.

Specific points:

Figure 1:
- The LPC baseline group shows a higher proportion of microglia compared to the cuprizone baseline, and astrocytes are detected only in the latter. This suggests inconsistencies in sample composition or processing.
- The presence of 'mixed cells' likely represents an artifact of clustering rather than a coherent biological population.

Figure 2:
- Integration of the Pandey et al. dataset, particularly the mature oligodendrocyte lineage (MOLs), appears poor. The leftward UMAP shift suggests batch effects rather than biological divergence, despite both LPC and cuprizone models being present.
- The density plots are difficult to interpret, e.g., Cluster E appears surrounded by Cluster B. Supplementary Fig. 3d offers a clearer view, where MOL_B increases during cuprizone remyelination, a pattern less pronounced in LPC.
- Annotations lack clarity; the metric in the lower left corner is unexplained. Although the Methods state that cuprizone remyelination was excluded from comparison, this group is nonetheless shown. The DEG analysis approach is problematic.
- Despite lower cell numbers, all clusters are present at baseline, raising the question of why comparisons were made to a "proximal" MOL state instead of the same cluster under baseline conditions. UMAP proximity is an unreliable proxy for

transcriptomic similarity and conflates condition-specific and cluster-specific effects, limiting interpretability. This issue also recurs in Figure 3.

Figure 3:
- The UMAP appears over-clustered; for instance, the identity of Cluster D remains unclear. If meaningful, the authors should present defining markers.
- The manuscript claims minimal differences between WM and NAWM in MOL_A-D states. However, Suppl. Fig. 4G indicates increases in MOL_F and MOL_G in NAWM, which contradicts that statement.
- The observation that MOL_A-D are more evenly distributed in human WM than mouse corpus callosum seems to rely on density plots and UMAPs, and thus may be heavily influenced by integration strategy.

Figure 4:
- Figures 4a and Suppl. Fig. 7a show stark differences between cohorts. This raises the question of how meaningful disease-associated shifts can be interpreted in light of baseline discrepancies.
- MG_C, D, and E are grouped for DEG analysis due to shared features. This calls into question the rationale for clustering at such high resolution.
- The claim that LPC induces stronger BAM infiltration and greater microglia proliferation may be biased, given the unusually low microglia proportions in the LPC baseline group.

Figure 6:
- MOL_E is nearly absent in this analysis, and no UMAP is provided showing the integration of the four datasets for oligodendrocytes.
- The reported signatures primarily reflect progenitor states and lack specificity for DAO. Hence, inferred overlap with human DAO populations is weak and should be interpreted cautiously.

Methods:
- Line 1067 indicates different protocols for snRNA-seq and RNAscope validation in the cuprizone model. The rationale for this protocol divergence should be clarified.

Reviewer #2

(Remarks to the Author)
Review Summary
The manuscript from Aboelnour et al., entitled “A comparative transcriptomic analysis of mouse demyelination models and Multiple Sclerosis lesions,” presents a cross-species joint transcriptomic analysis at single-cell and/or single-nucleus resolution to determine which toxin-induced mouse models of multiple sclerosis (MS) most closely resemble glial changes observed in human MS. By integrating three published datasets (Shen et al., 2021; Pandey et al., 2022; and Macnair et al., 2025) with eight newly generated transcriptomic libraries from investigated mouse models, the authors examine both the divergent and convergent properties of glial cells, focusing on oligodendrocyte lineage cells and microglia. The authors have identified an important question in the field of biomedical research. They have demonstrated technical expertise and assembled relevant datasets to tackle this challenge. While the manuscript presents compelling data, we find that the depth of analysis and interpretation, and, as a result, the insights offered in the current version of the manuscript, could be improved.
Technical Details
There are significant concerns regarding the focus of the analysis, the conclusions drawn from the presented data, and the lack of specificity in the descriptions of the manuscript. To help improve future versions, these concerns are outlined point by point below:
Abstract & Introduction:
1. “… it remains unclear whether different demyelination models elicit distinct glial responses, and how comparable these changes are to MS.” While this is a central question raised in the abstract, it is not immediately clear what the authors have discovered in response to it. The abstract and research highlight do not clearly convey the key findings or how the study advances current knowledge relative to existing literature. These sections would benefit greatly from more precise wording and clearer presentation of the quantitative analyses to enhance interpretability and impact.
2. “Interestingly, remyelinating OLs converge on an altered maturation state in both LPC and cuprizone models, potentially due to persistent activation of microglia at remyelination stages.” This is an intriguing hypothesis; however, the manuscript currently lacks the specific analyses needed to support this conclusion. As presented, it appears to be a logical leap, and we recommend that the authors provide clearer evidence or rationale linking microglial activation to altered oligodendrocyte maturation in these models.
3. “A critical gap in the current use of animal models to study remyelination is the lack of understanding of which MS disease mechanisms are replicated. These two toxicity models are often used interchangeably by researchers, with a particular method selected because of experimental design constraints rather than scientific rationale.” Given this important point, it would be valuable if the authors could offer guidance to readers—such as a framework or criteria—for selecting between these models based on their relevance to specific MS-related mechanisms. Do the authors recommend that studies investigating oligodendrocyte protection and remyelination routinely be conducted in both models? Is there evidence that such an approach would enhance translatability? This would enhance the study's translational impact.
4. Page3 citation 16: The citation appeared to be misplaced. Authors should clarify why this comparison excluded mouse EAE data, another commonly used model for MS. Indeed, there is some high-level discussion of EAE toward the end of the paper, which leaves the reader wishing that EAE samples had also been interrogated, even if there is no straightforward way to distinguish demyelination and remyelination in EAE. For example, they could have used methods similar to those

they used for integrating LPC and cuprizone datasets with human MS lesions to compare those same mouse models to EAE.

Results:

5. Fig1 & Sup Data1: Including a timeline in Figure 1 that illustrates the experimental design across studies—clearly labeling the experimental procedures, library numbers, and basic animal demographics—would be helpful in contextualizing the evidence presented.
6. Supplementary Fig1G: Consider integrating some of the information into the main Figure 1 in a more streamlined format. Enhancing annotation of technical replicates, experimental models, dataset sources, distinctions between single-cell (sc) and single-nucleus (sn) approaches, and reporting total sample numbers would greatly aid interpretation.
7. Figure 1D: Recommend presenting a bar graph of group means overlaid with scatter plots of individual replicate values, clearly labeled by condition, study, and cell type. Additionally, performing a proportional statistical test would strengthen the assessment of differences in remyelination composition across conditions, as the current observations are only qualitative.
8. “BAMs and T cells were specifically enriched in the LPC model”: For this and similar claims, it is important to provide supporting statistical analysis to validate the observations.
9. Figure 1G: Given the stated research objectives, separate UMAPs for mouse and human are less informative. Instead, it would be more helpful to begin with a merged mouse and human dataset to allow a harmonized, comparative assessment of cell class composition. Why did the authors choose to integrate only in Figure 6? A deeper evaluation of how differences between snRNA-seq and scRNA-seq mouse datasets relate to the integration with human data would also have been helpful.
10. Figure 2C: Legend title missing (what is the unit). Consider move SupFig3D and 3F to main figure.
11. SupFig3D: Why does cuprizone baseline have more MOL_G? Is it significant?
12. “To quantify these changes, we calculated the differential abundance (DA) across overlapping cell neighborhoods (nhoods) using Milo (Dann et al. 2022)” Informative, method could be applied to other analyses. The citation format is inconsistent.
13. “However, both models enriched the DAO cluster MOL_E at remyelination, suggesting a shared remyelination state.” This suggestion may be premature, as similar enrichment of MOL_E was also observed during the demyelination phase. Additionally, each sample likely represents a mixture of tissue status. It would be helpful to clarify the microdissection strategy used. Including both the collection and injection dates in the experimental design schematic (Fig 1) would further enhance interpretability.
14. “direct connection to MOL_E, suggesting that OLs may undergo an altered maturation state following demyelination (Figure 2A)” Insufficient detail to make this conclusion. It might be helpful to try to construct a connectivity map individually across conditions and compare.
15. “We next asked how the different DAO states compare to each other transcriptionally. We tested for differentially expressed genes (DEGs) using a pseudobulk approach (see Methods)” Method: “For each treatment condition, we made pairwise comparisons of DAO clusters to their closest baseline MOL state (MOL_E vs. MOL_B, MOL_F vs. MOL_D, and MOL_G vs. MOL_A” What is the rationale for comparing each DAO to a different reference group? Would it not be more consistent to compare them to a common subcluster, or to the same subcluster split between control and disease conditions?
16. Figure 2E: According to the Methods, it appears that each group was compared to a different control. This should be clearly labeled in the figure to avoid confusion.
17. “coagulation-related genes were enriched in LPC-treated DAOs, likely due to blood-brain barrier disruption and BAM infiltration following stereotaxic injection” Interesting observation; however, there appears to be a logical gap between this observation and the reported elevation of coagulation-related genes in oligodendrocytes. Did the controls of LPC model injected with PBS shows the same? Please discuss and clarify further.
18. “Socs3, Cdkn1a, Nupr1” are interesting findings and should probably be mentioned in the abstract. Additionally, the presentation of evidence and comparison of these candidates across conditions, cell types, and FISH results should be made more consistent to enhance clarity and interpretation, such as a version of Sup4D plots for all of them. Additional FISH quantification on Nupr1 would strengthen the impact.
19. “Supplementary Figure 4E, F” The contrast between oligodendrocytes and OPCs is intriguing and warrants inclusion in a main figure, especially with supporting FISH data. The findings suggest that the LPC-induced Cdkn1a-high OPC may not progress to mature oligodendrocytes (MOL), or that Cdkn1a expression is downregulated before that transition. Given that LPC leads to more extensive remyelination than cuprizone (Fig1D, higher MOL percentage in recovery phase), this highlights Cdkn1a as a potential therapeutic target—one that may need to be suppressed to promote oligodendrocyte survival and successful remyelination?
20. “Our results support the use of LPC and cuprizone models to study distinct aspects of OL stress and repair in CNS demyelination, and to identify strategies for enhancing OL protection, survival and remyelination” The statements here—and throughout the manuscript—are overly general. A more quantitative perspective is needed, particularly in relation to the specific genes mentioned above. Additionally, the discussion would benefit from being framed within the context of relevant prior literature to strengthen the interpretation and significance of the findings.
21. Figure 3: A very similar naming scheme to human MOL_A–G is used, but it remains unclear whether these labels reflect comparable transcriptomic features as annotated in the mouse data. To clarify this, it would be more informative to initiate the analysis with a combined species clustering. This would allow for direct comparison of cluster identities in the global (combined) space versus the local (species-specific) space. Incorporating a riverplot to visualize these relationships would significantly enhance the clarity and interpretability of cross-species comparisons.
22. “validated the DAO state using a gene signature from a previous MS patient study, confirming that known DAO genes are enriched in MOL_E-F and OPC cluster…” The method authors employed is not an alternative method, so “validation” is too generous a term; phrasing it as "we annotated based on..." would be more appropriate and accurate.
23. “Interestingly, MOL_E- a comparatively small cluster- was highly enriched in two WM samples (44% of cells), both from

85-year-old individuals" Should be based on data, add the plot in the supplementary figure.
24. "suggesting that normal brain aging may induce a stressed state in MOLs" The analysis is insufficient to support this claim. Perform an age-dependent distribution analysis of MOL clusters.
25. "we observed that MOL_A-D populations are more evenly distributed in human WM compared to the mouse corpus callosum, suggesting greater heterogeneity in adult human MOLs" It is unclear, where is the data, and why the conclusion?
26. "Quantification using Milo supported our observations, showing minimal changes in homeostatic MOL_A-D in NAWM, but variable alterations in MS lesions, with the fewest changes observed in RLs (Figure 3D)…" Unclear, please perform stats.
27. "Overall, all MS lesion types were strongly enriched for DAO states MOL_F and G" Is this referring to DAO in a general sense, or the specifically defined DAO population characterized in the mouse dataset introduced in the study?
28. "we performed differential gene expression analysis focused on AL and RL samples, as they are most comparable to our mouse time course data (Supplementary Data 4)" On what basis is their similarity determined? Given the capabilities of the bioinformatics tool employed, shouldn't the comparison be conducted in a more unbiased and comprehensive manner without predefined hypotheses or conclusions about which toxin-induced model most closely resembles specific MS-related pathological changes? In particular, the analysis of chronic active lesions, which are really better characterized as "mixed active-inactive lesions" and have inactive and active areas, should be substantially deeper to enhance relevance to MS.
29. "indicating that DAO cluster MOL_G shifts toward a more homeostatic-like state during remyelination" The conclusion based solely on the number of DEGs is not sufficient or fair, as additional new changes occur beyond the demyelination phase. Moreover, these samples are from different patients, each with diverse genetic backgrounds and demographic profiles. The structure of the sentence is also misleading, as it implies that MOL_G is uniquely affected potentially more so than other MOL subtypes. Have you performed a similar DEG overlap analysis within the mouse MOL_G dataset? Why focus specifically on MOL_G?
30. "Importantly, many of these pathways were previously observed in the original analysis of this human dataset, including TNFα and IFN upregulation, supporting the robustness of our analysis pipeline." Once again, what's novel about your current analysis—specifically in comparison to and building upon your prior mouse data? A joint cross-species analysis from the start would likely be more informative and directly relevant to your research questions.
31. "Finally, we explored the expression pattern of genes previously validated by RNAscope in our mouse models (see Figure 2). CDKN1A and SOCS3 were highly enriched in the MS DAO cluster MOL_G, especially in RLs, indicating persistent activation of pathological pathways in MS lesions (Supplementary Figure 6E)" A new section should begin with this point, moving the earlier preliminary work to the supplementary section. SupFig6E should include the full expression profiles of NUPR1, SOCS3, and CDKN1A, and this panel should be moved to a main figure, especially as it pertains to multiple lesion types. Also, what is the functional role of CDKN1A in this context? A clearer explanation is needed to justify its emphasis.
32. "Together, these findings indicate that both mouse demyelination models exhibit some disease-associated characteristics of MS pathology in Ols" The current comparison is too vague. You should quantify the similarity between mouse MOL_E, F, and G and the human-defined clusters using a similarity score or metric (e.g., correlation, Jaccard index, or another appropriate approach). Additionally, consider assigning informative names to each cluster based on dominant signaling pathways or other defining ensemble features, rather than relying solely on alphabetical labels. This would make cross-species comparisons and functional interpretation much clearer.
33. "We previously observed that microglia undergo a dramatic state shift following injury," Missing citation to data.
34. Fig4-5: Similar to the comments on the oligodendrocyte analysis: the language throughout should be more precise and quantitative. Apply appropriate statistical tests to support your claims, clearly state what is novel in your findings, and interpret results within the context of existing literature. Finally, although it's sometimes difficult to do so cleanly, whenever possible it would help the reader if you use informative naming conventions for clusters based on defining molecular or functional features, rather than abstract labels, to aid interpretability and cross-study comparison.
35. "gene expression changes by comparing the homeostatic state (Mg_A) with DAMs, or BAMs from LPC-induced demyelination" It may be worth performing a direct, head-to-head comparison across the two systems—for example, comparing the same MOL subclusters between LPC- and cuprizone-induced demyelination models (instead of to the homeostatic cluster of the same system or control). Identifying shared or distinct DEGs within corresponding subclusters could provide clearer insights into conserved versus model-specific responses — especially since there seem to be gene expression differences in the control populations despite application of harmonization approaches, raising the possibility that at least some of the model-related differences reported in the paper are technical in origin. Moreover, in the current version the similarity of clustering is relative and should be interpreted in context. In some cases, gene expression levels may change significantly within the same subcluster annotation across conditions, but not to a degree that warrants defining a new, distinct cluster.
36. Fig4G: The method and rationale are unclear. Even if the conclusion is reached that LPC-induced microglial changes are more pronounced, this finding alone is not particularly informative without further context or mechanistic insights.
37. "In contrast, cuprizone-induced demyelination elicits a more gradual response over weeks, leading to weaker DAM enrichment and a near-complete return to homeostasis by remyelination." Doesn't this finding dissociate the role of microglia in oligodendroglia-mediated remyelination? As shown in Fig1D, the cuprizone model indicates no recovery of MOL or OPC percentages. Could this suggest that no further myelin debris is being produced, such that there is no further stimulant for microglial reactivation in the cuprizone model compared to the LPC model?
38. "However, our results highlight LPC as a model for studying robust microglial activation, including the recruitment of BAMs and stronger transcriptional shifts." This is too vague; it needs to be more specific, particularly in the context of MS stages and the involvement of BAMs. How do secretory or diffusive factors contribute to these observations?
39. "Mg_E expresses similar gene expression to Mg_D, but also expresses NUPR1, a gene that is more abundant in white than grey matter, and is critical for repressing iron-dependent cell death" What is the expression pattern of NUPR1 across mouse models? Using FISH to present this evidence would strengthen the conclusion and provide more direct spatial resolution of its expression.

40. “Using the sysVI method to correct for species-specific effects” The code should be shared, and the methods to address differences between single-nucleus (sn) and single-cell (sc) approaches should be clarified. Ensure that cross-species gene homologs are properly addressed. Additionally, how can these findings help explain differences in the inherited diversity in microglial response, particularly with a skew toward cell-type-specific genes? It’s important to clarify the methodology used to get relevant interpretation.
41. Supplementary Figure 11: Should be figure 1. Since UMAP and clustering are sensitive to cell count, it would be prudent to subsample both the mouse and human datasets to an equal number of cells for downstream comparison. This will help ensure that the conclusions drawn are not biased by differences in cell numbers and gene load between the datasets.
42. “We found that the BAM-specific expression of F13A1 is integrated within the microglia cluster that expresses CSF1R, suggesting we lost the specification of macrophages and microglia that we observed in the mouse and human datasets.” How is this phenomenon influenced by the use of the sysVI method? Could biased cross-species correction—particularly favoring certain cell class-specific genes—be contributing to the observed results? This possibility should be examined and clarified.
43. “This observation further supports our hypothesis that OLs have higher conservation across species than microglia. Thus, we focused on the OL cross-species comparison.” A similar analysis presented in Fig 6A should be performed for microglia and other cell classes with improved annotation and naming.
44. Fig 6G: It’s not clear why these specific genes were selected for closer examination in this context. The rationale should be explicitly stated—whether based on prior literature, relevance to MS pathology, differential expression patterns, or pathway involvement—to justify their focus and interpretive value.
45. “We conclude that cuprizone treatment induces a state change in MOLs that more closely resembles active MS lesions than LPC.” However, compared to RL, the number of MOLs in the Cupr_remyel condition does not appear to recover (SupFig11E). What about in the EAE model? Including this comparison would provide a more comprehensive view of MOL dynamics across different demyelination and remyelination contexts.

Discussion
46. “Numerous preclinical therapies have been identified using these models, with 25 out of 88 reported to have entered clinical trials and/or received approval (ref51).” It would be valuable to consult clinicaltrials.gov for updated reports and to mention how many trials are based on LPC-induced versus cuprizone models, though this might be difficult to achieve in practice. Additionally, highlighting any similarities in the mechanisms of action (MoA) of these two models could add depth to the discussion and help justify the choice of model in the context of studying MS pathophysiology and therapeutic relevance. Finally, please avoid the use of the the term “treatment” in describing the toxin used in the models, as this can be confused with salutary therapies.
47. “The only shared enrichment observed was in the TNFα signaling pathway.” This should be mentioned in the abstract and more thoroughly developed throughout the manuscript. The analyses should be clearly framed in the context of multiple sclerosis, with further exploration of subtype diversity, signaling pathways, trajectory dynamics, and their relevance to MS pathology and progression. Importantly, trials with TNF blockers have shown that they can exacerbate MS and precipitate relapse.
48. “yet whether drug targets have been more successful when aligned with the phenotypes we identified—such as P53 signaling, oxidative phosphorylation, or the unfolded protein response—remains an open question and a compelling avenue for future research” Building on point 48, this is a critical issue that should be explicitly addressed in the study to ensure it provides meaningful knowledge advancement. The manuscript should offer some level of guidance for readers—under what conditions or research questions should one choose the LPC model versus the cuprizone model (or others)? Or should both models always be carried out and compared? Clarifying this would not only strengthen the translational value of the work but also support more informed model selection in future MS studies.
49. “Although EAE is induced through immune modulation and is often considered more analogous to MS, we observed upregulation of multiple immune-related genes in MOLs within the toxicity models as well. For example, genes such as Klk8, S100a10, H2-D1, Serpina3n, B2m and other MHC-I genes were particularly upregulated in LPC treatment, where IFN signaling was also strongly induced. These findings indicate that OPC and OL immune responses are not exclusive to EAE-mediated demyelination.” This section feels out of place and emphasizes the important question of why the current study did not include mouse EAE data to enable a more comprehensive cross-model comparison. Incorporating EAE would strengthen the conclusions and better contextualize findings across widely used MS models. Alternatively, the authors should clearly frame the study within the context of remyelination capacity and structure the analyses accordingly. A focused investigation into remyelination dynamics—across models, cell types, and stages—would provide a more coherent narrative and strengthen the study's contribution to understanding MS pathology and repair mechanisms.
50. “Cuprizone and AL DAOs exhibit shared upregulation of stress response genes, including CDKN1A, NUPR1, and FOS.” This appears to be the major finding of the current version and should be expanded upon and followed up with more detailed analyses and discussion to fully convey its significance and implications.
51. “For example, it is not known if these OLs produce functional myelin that matures over time.” The study could also be strengthened by extending the quantification of these MOL subtypes across models using FISH and immunostaining, which would provide valuable spatial-temporal context and enhance the interpretation of their distribution and dynamics in relation to disease progression and remyelination.
52. Supplementary Figure 12: Should not only be introduced in the discussion section.
53. “Additionally, the blood-brain barrier plays a crucial role in damage signaling, and our analysis identified strong enrichment of innate immunity genes in endothelial cells and pericytes.” Citation to the data?

Reviewer #3

(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.

Reviewer #4

(Remarks to the Author)
In this study, Aboelnour et al. investigated how distinct demyelination models trigger different glial responses by integrating newly generated and publicly available single-cell transcriptomic data from the subcortical white matter. They compared glial alterations in the lysophosphatidylcholine and cuprizone models to those observed in multiple sclerosis (MS), and further assessed the relevance of the mouse data by comparing it to human MS samples.
Overall, this comparative analysis highlights key biological and pathological differences in gene expression between mouse demyelination models and human MS lesions, providing a valuable foundation for the effective use of animal models in advancing remyelination therapies. The manuscript, though interesting in topic and well-executed, several aspects require clarification or improvement, as outlined below.

Major concerns:

1，In Figure 2C, distinct UMAP distributions among OLs populations were evident in both the CPZ and LPC models. It is important to consider the localized nature of the LPC injection site, as transcriptomic changes in OLs are likely to exhibit spatial heterogeneity, with only cells near the lesion being significantly affected. In contrast, cuprizone induces more diffuse and widespread OL loss across multiple brain regions through dietary exposure, resulting in more uniform transcriptomic alterations across the sampled tissue. Moreover, the remyelination timelines between LPC and cuprizone models are not fully aligned, potentially confounding interpretation of convergence or divergence in oligodendrocyte recovery states. The authors are encouraged to incorporate spatial transcriptomics, proteomics, or functional imaging to more precisely contextualize molecular changes within the anatomical framework, thereby enhancing the robustness of their conclusions. For example, the authors identified a population of Cdkn1a^{+} OLs as a unique marker of cuprizone-induced OL stress. Notably, CDKN1A was also highly enriched in the MS DAO cluster MOL_G, particularly within remyelinated lesions. To further clarify the functional role of CDKN1A^{+} OLs, it would be beneficial to assess their association with markers of intact versus degraded myelin, which could help determine whether these cells contribute to remyelination or represent a dysfunctional or stalled repair state.

2, Although the white matter is the primary site of demyelination in both mouse models and human MS, the manuscript would be strengthened by discussing whether similar transcriptomic alterations occur in the grey matter in response to CPZ and LPC treatments. Previous single-nucleus RNA-seq studies of both the cortex and corpus callosum (PMID: 40131948; PMID: 36952346) have comprehensively characterized these regions and identified comparable stress-associated oligodendrocyte states during demyelination. A parallel analysis of grey matter lesions in human MS would also provide valuable insights and further support the relevance of these findings across brain regions. Additionally, the study does not explore potential sex- or age-related differences, which may significantly influence demyelination and remyelination dynamics and affect the translational applicability of the findings.

3, MS is characterized by a pronounced peripheral immune component, with significant contributions from T and B lymphocytes. However, these immune cell populations are largely absent or minimally represented in the toxicity-induced mouse models utilized in this study, limiting the capacity of these models to fully recapitulate the immunopathological complexity of MS. The authors are encouraged to explore the integration of immune-mediated models, such as EAE, or to adopt hybrid approaches that combine toxicity paradigms with peripheral immune cell infiltration strategies. At a minimum, these limitations should be explicitly acknowledged and discussed as potential confounding factors in the interpretation of results.

4, In Figure 4, the authors demonstrate that microglial responses to demyelination are highly conserved across both mouse models, with sustained microglial activation from the demyelination phase through to remyelination. It would be of interest to further explore the overlapping and model-specific microglial DEGs between demyelination and remyelination phases. Including Venn diagrams to visualize shared and distinct DEGs across these conditions would provide valuable insight into the temporal dynamics of microglial activation. In addition, the UMAP for the WM condition should be included in Figure S9F to allow for direct comparison with lesion types. Similarly, including the WM UMAP in Figure S12 would enhance the interpretation of astrocyte heterogeneity across different conditions.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
General critique:
The manuscript by Aboelnour et al. has substantially improved by, for example, adding new schematics illustrating dataset

generation, which enhances transparency and help understand the underlying data structure. Major concerns raised in the initial review have been addressed. In particular, the differential expression analysis strategy has been revised, and interspecies comparisons have been removed. These changes, together with the inclusion of the EAE comparison, have considerably strengthened the interpretability and overall impact of the study.

Specific comments:
1. We appreciate the addition of modality and dataset annotations in figures such as Supplementary Fig. 4a. Nevertheless, we still encourage the authors to present the newly generated datasets as separate UMAPs for LPC and CPZ. This would allow readers to more clearly evaluate whether the observed MOL shifts and other patterns arise from genuine biological differences or from dataset-specific characteristics.
2. In their response, the authors note: “Therefore, we see no problems in making inter-model comparisons, as they are controlled by their own datasets’ controls, which should account for technical and experimental-specific artifacts.” Since the Pandey et al. dataset does not include its own controls, this limitation persists. While we recognize that this cannot be fully resolved within the scope of the current study, we recommend explicitly acknowledging this point in the manuscript.

Reviewer #2

(Remarks to the Author)
Review Summary
The manuscript by Aboelnour et al., entitled “A comparative transcriptomic analysis of mouse demyelination models and Multiple Sclerosis lesions,” presents a cross-species analysis comparing glial diversity across disease states in two toxin-induced mouse demyelination models and in multiple sclerosis (MS) lesions. The revised version improves analytical depth, discussion, and readability, and the central message is now clearer. Some remaining point-by-point comments are listed below:
1. separate statistical test comparing the distribution of nhoods…
Clarify what “nhoods” represent to general readers. Also clarify “vs” in Fig. 2d if comparisons are within-condition only.
2. CPZ remyelination samples exhibited high heterogeneity…
State which statistical test was used, whether any DEGs remained significant, and provide the original DEG table.
3. gene set enrichment analysis (GSEA) to determine what signaling pathways…
Clarify whether only upregulated genes or both directions were included in GSEA.
4. supported by shared upregulation of genes Gadd45a/b…
This should be an internal consistency check rather than independent support.
5. performed RNAscope
Clarify the ROI definition and quantification pipeline in the method and also in the caption, how many animals, sections, images, and cells were quantified.
6. B2m encodes the beta-2 microglobulin…
For Fig. 3c, clarify whether nuclear-proximal puncta or diffuse expression is expected, how the background is determined and subsequently corrected?
7. the percentages gradually increased from CPZ demyelination (5 wks) to remyelination (5+3 wks)…
Include statistical testing between CPZ_5wks and CPZ_5+3wks, and explain the absence or presence of significance at baseline → 5wks.
8. altered maturation state following demyelination
Clarify what analysis specifically supports this. If PAGA is used, address whether transitions between DAO states must pass through intermediate MOLs. Have you compared the PAGA results with and without E_DAO, F_DAO, or G_DAO? When these states are analyzed together, there appears to be direct connectivity from E to G; does this imply that the developmental trajectory could transition directly between these DAO states rather than passing through B and C?
9. but dropped significantly at 5 weeks removal from CPZ…
Indicate whether LPC eventually reaches similar levels to CPZ_5+5wks, and what the baseline (EdU-only) population looks like.
10. Human MOL_A–D description
It will be helpful to apply the same concise style used for mouse MOLs for clarity and symmetry.
11. additional connectivity between the DAO states
Clarify whether cluster G connects to E in Supplementary Fig. 6d and whether MFOL link to B is direct or routed through E_DAO in Fig. 2a.
12. Figure 4e
Consider visually marking triple-positive DEGs (MS+LPC+CPZ) directly on the bar graph.
13. Supplementary Figure 7c
This is a compelling finding and might merit inclusion in the main figure. human_MOL_F appears distinct: it aligns most closely with OPCs and MFOL across species, shows similarity to human_MOL_G, yet lacks the pattern seen in younger OL clusters. It is also the predominant population in MS remyelinated lesions (Fig. 4c). What differentiates MOL_F from MOL_G beyond NEDD4L and FGFR1? What drives their cross-species similarity? Aside from GPC5, are there additional genes uniquely marking MOL_G relative to MOL_F?
14. Supplementary Fig. 7e
Which MOLs were combined in each condition?
This figure is confusing; consider separating upregulated and downregulated genes into two plots. The LPC_remyel downregulated profile is highly correlated with the LPC_demyel upregulated profile, which may reduce clarity.
15. while MOL_F and MOL_G are marked by CDKN1A…
This is incorrect, these genes mark MOL_F only.

16. Figure 4f
Rather than using a feature plot, it would be more informative to present the differential loading of these signatures across human MOL subclusters in a dot plot or heatmap. This would better illustrate their similarity to human DAOs rather than homeostatic MOLs. For clarity, plot human subclusters on the x-axis and mouse subclusters on the y-axis, similar to Supplementary Fig. 7c.
17. gene signatures (page11)
Clarify how these signatures were constructed (DEGs? modules? curated lists?).
18. Supplementary Figure 7f
Clarify which pairwise comparisons produced the significant differences.
19. Figure 5f
Add 7dpl and CPZ_5 to the ApoE group, and 42dpl/CPZ_5+5 to the Cd206 group for completeness.
20. Figure 5b
Include subcluster-enriched genes (as in Fig. 6b) to highlight differential loading in Mg_A, Mg_D, and PVMs.
21. Together, these findings suggest that MS induces greater cellular heterogeneity…
Hard to conclude from the current text alone. A matrix plot similar to Supplementary Fig. 7c would help. Consider plotting genes defining human microglia clusters onto the corresponding mouse clusters to better visualize this comparison.
22. Supplementary Figure 12d
Same recommendation as for the OL portion.
23. Supplementary Figure 12c
Consider moving this panel to the main figure; it conveys more information than Fig. 6f.
24. overall DEG overlap and gene signature patterns support…
Consider inverting the analysis to show how human signatures load onto mouse clusters. Can you apply PAGA to examine the relationships between human_F_DAM, D_DAM, and E_DAM, to identify potential transition states between these clusters?
25. were most strongly enriched in the human Mg_F DAM…
If human Mg_F represents “repairing microglia,” could this explain why remyelination is more robust in mice, with Mg_F-like microglia driving the process? In this context, could we argue that mouse microglia exhibit greater diversity rather than less, given that the same human Mg_F maps onto three subclusters in mouse?
26. CPZ DAO state exhibits greater DEG overlap with DAOs in EAE…
Extend this comparison to microglia: compare CPZ and LPC microglia with EAE DAM states.
27. As OPCs and DAMs in the mouse toxicity models also share similar signaling pathways…
Add citations to the specific datasets referenced.
28. but both OPCs and DAMs exhibit wide heterogeneity
Provide references for this statement.
29. However, our data suggests that there is a diversity of OPC states…
Point to the specific figure(s) that demonstrate this.

Reviewer #3

(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.

Aboelnour et al. Response to Reviewers:

We thank the reviewers for their positive and thoughtful comments to our paper. The revised version of the manuscript is significantly strengthened by the new data and analysis generated in response to these critiques. Our study now provides a comprehensive and statistically robust characterization of the response of oligodendrocyte lineage cells and microglia to demyelination. Overall, our study provides the first direct comparison of lysolecithin (LPC) and cuprizone (CPZ) mouse toxicity models of demyelination to each other, as well as to different MS lesions types.

In response to the reviewers' comments, we have significantly revised our original manuscript through additional experimental validation, revised gene expression analysis, increased statistical testing, and more precise and specific writing. Importantly, the revised manuscript now includes the following new analysis:

- *In vivo* validation of *Nupr1* and *B2m* expression in oligodendrocyte lineage cells following demyelination. We have expanded our *in vivo* analysis of mouse disease-associated oligodendrocytes (DAOs) at both demyelination and remyelination timepoints.

- *Socs3* expression in EdU-labeled newly generated oligodendrocytes. This data now provides direct evidence that *Socs3* is expressed in newly generated oligodendrocytes, thereby representing an altered maturation pathway following demyelination.

- Comparison of LPC, CPZ, and EAE oligodendrocyte gene expression changes. Using a published EAE scRNAseq dataset, we now include comparison of DAO genes across the three most commonly used mouse models of demyelination.

- An *in vivo* analysis of the microglia/macrophage response across LPC and CPZ demyelination models. To strengthen our analysis of microglial dynamics, we now provide evidence that perivascular macrophages are enriched solely in LPC demyelination and disease-associated microglia (DAMs) remain activated for longer in the LPC model.

- Analysis of oligodendrocyte and microglia cell state and gene expression changes across all MS lesion types. We have expanded our original analysis to include all MS lesion types, providing additional molecular insights into the human disease pathology.

As extensive changes have been made throughout the manuscript, we have provided a summarized list of figure changes, followed by a point-by-point response to individual reviewers' comments.

New figures:

1. Figure 3, characterization of the DAO state at remyelination with new *B2m* RNAscope and EdU analysis.
2. Supplemental figure 2, statistical comparison across human metadata.
3. Supplemental figure 5, comparison of DAO genes between the EAE, LPC, and CPZ models.
4. Supplemental figure 7, human oligodendrocyte DEG analysis across all MS lesion types.
5. Supplemental figure 9, analysis of OPC gene expression changes across MS lesion types and a comparison with mouse OPCs.
6. Reviewer figure 1, comparison of integration strategies.
7. Reviewer figure 2, bar graph of cell type proportions across different mouse datasets.

Modified figures:

1. Figure 1 – new panels (a and e) have been added to provide an experimental overview of the sc/snRNAseq datasets. New panels (c and g) have been added to show cell counts for each dataset. Stacked bar charts have been moved into supplemental figures 1f (mouse) and 3d (human).
2. Figure 2 – density plots have been replaced with Milo differential abundance of neighborhoods (panel c). To statistically compare increased and decreased neighborhoods, we generated stacked bar charts and performed pairwise comparisons (panel d). Upset plot has been replaced with Venn diagram of upregulated DEGs (panel f). Heatmaps of DEGs within GSEA pathways have been added (panel g). Socs3 RNAscope has been moved to new Figure 3. New panels (j and k) have been added for Nupr1 RNAscope.
3. Figure 4 (previously Figure 3) – density plots have been replaced with Milo differential abundance of neighborhoods and bar charts (panel c). Venn diagrams have been replaced with bar charts showing shared up- and downregulated genes across MS lesion type and LPC/CPZ (new panel e). Upset plots have moved into supplemental figure 7b.
4. Figure 5 (previously Figure 4) – density plots have been replaced with Milo differential abundance of neighborhoods (panel c) and bar charts (panel d). New panels have been added for perivascular macrophage analysis (e) and DAM analysis (f). Upset plots have been replaced with Venn diagrams in panel h. Scatter plots have been removed and replaced with heatmap of DEGs within GSEA pathways (panel i).
5. Figure 6 (previously Figure 5) – density plots have been replaced with Milo differential abundance of neighborhoods and bar charts (panel c). Upset plots have been moved to supplemental figure 12b. New bar charts showing shared up- and downregulated genes in microglia across MS lesion type and LPC/CPZ have been added to panel e. UMAPs showing distribution of individual gene expression have been moved to supplemental figure 12h.
6. Supplemental Figure 1 – scCODA analysis was added in new panel g. Table of cell numbers and distribution of cell type across batches were removed and replaced with cell counts bar charts in Figure 1.
7. Supplemental Figure 3 (previously Supp Fig 2) – scCODA analysis results were added as a bar chart in panel d. UMAP plots separated by human disease status were added to panel e. Table of cell numbers was removed and replaced with cell counts bar chart in Figure 1.
8. Supplemental Figure 4 (previously Supp Fig 3) – Stacked bar chart of proportions of cell types per condition and table of cell counts were removed. New panel showing number of DEGs in OL lineage across mouse model added to panel d. New panel of Venn diagram of downregulated DEGs in OLs added to panel e.
9. Supplemental Figure 6 (previously Supp Fig 5) – Stacked bar chart of proportions of cell types per condition and table of cell counts were removed due to redundancy. New panel showing counts of human MOL_E across lesion types added in e.
10. Supplemental Figure 8 (previously Supp Fig 4) – Bar chart and Venn diagrams of mouse MOL DEGs in panels a and b were moved to other figures. RNAscope images for *Cdkn1a* and *Socs3* expression were added in panels c and f. Quantification of RNAscope for DAO gene expression in mouse OPCs were added in panels d,e,g,h. UMAPs of *Gadd45g* and *Col120a1* expression were removed.
11. Supplemental Figure 10 (previously Supp Fig 7) – Stacked bar chart of proportions of cell types per condition and table of cell counts were removed due to redundancy. MILO density plots were moved to Figure 5c. Bar chart of numbers of mouse microglia DEGs was added in panel d. GSEA analysis of PVMs and upset plots were moved from original main figure 4 to panels e and f.
12. Supplemental Figure 11 (previously Supp Fig 9) – Stacked bar chart of proportions of cell types per lesion type and table of cell counts were removed due to redundancy. MILO density plots were moved to Figure 6c.

13. Supplemental Figure 12 (previously Supp Fig 10) – Bar chart of human microglia DEGs per lesion type was added in panel a. Upset plots of microglia DEG overlap across MS lesions was added in panel b. MetaNeighbor heatmap of mouse and human microglia was added to panel c. Bar chart of shared DEGs between LPC remyelination and MS microglia was added in panel d. Overlap index/Spearman analysis added in panel e. Heatmap of DEGs within GSEA pathways added in panel f.

Removed figures:

1. Original Figure 6, cross-species integration.
2. Original Supplemental figure 11, QC for cross-species integration.
3. Original Supplemental figure 12, human astrocyte lineage subclustering analysis.

We have included below a point-by-point response (in blue) for each reviewer's comments:

Reviewer #1 (Remarks to the Author):

General critique:
This study by Aboelnour et al. combines novel and previously published single-nucleus and single-cell RNA-seq datasets from demyelination models and human MS samples to investigate shared glial reactivity patterns. Given the increasing application of focal demyelination models such as LPC and cuprizone in MS research, this work provides potentially valuable insights for model selection and understanding white matter lesion pathophysiology. A key observation is the partial overlap of glial responses across models, which appears to be recapitulated in human MS tissue. While the data integration is generally well explained, concerns remain regarding dataset quality, integration strategies, and consistency across data sources.

We thank the reviewer for recognizing the value of our analysis for the glial biology and MS research fields.

The design of the LPC surgical model appears inadequate. The manuscript describes only a single baseline LPC group, seemingly comprising contralateral, saline-injected mice at 7 dpi. This is problematic, especially for the corpus callosum, where contralateral effects due to interhemispheric projections cannot be excluded. Moreover, the 7 dpi baseline may suffice for comparisons within the demyelination phase but is not suitable for evaluating remyelination at 28 dpi. Separate baseline controls for each phase would have strengthened the experimental design.

Thank you for bringing up this important point. Unfortunately, the Pandey et al. LPC scRNAseq dataset did not include saline-injected controls, but we have now performed comparisons with contralateral, saline-injected mice at the equivalent timepoint (7dpl, 28dpl, and 42dpl) for all of our validation experiments *in vivo.* We do not see any statistical differences between our different saline controls. Interestingly, for some of our remyelination timepoints, we did see slight increases in DAO gene expression (*Socs3* and *B2m*) within the saline control, compared to uninjected control animals (baseline for CPZ). This may reflect a cellular response to the injection itself or interhemisphere effects.

Notably, microglia proportions were unexpectedly lower in the saline-injected baseline group compared to the cuprizone baseline, complicating inter-model comparisons.

These types of differences likely arise because of upstream sample preparation choices. The Shen data set, which was focused on a microglia knockout phenotype, may have been optimized to maximize microglia enrichment, or may have required a longer experimental prep time, which could have led to the induction of the larger proportion of Mg_B state (Supplementary Figure 7E). Microglia proportions, as counted in MILO, are specifically made against the baseline of only the corresponding controls to help alleviate these types of

differences. Therefore, we see no problems in making inter-model comparisons, as they are controlled by their own datasets' controls, which should account for technical and experimental-specific artifacts.

These challenges are also evident in the poor integration of the Pandey et al. dataset relative to the other data sources (see Suppl. Fig. 3a).

We disagree and do not think this interpretation of the integration for the Pandey data is fair. The Pandey et al. dataset consists of only LPC de-and remyelination samples, the Shen et al. dataset consists of only CPZ de- and remyelination samples, and our own new dataset consists of both. To help clarify we have added labels to these publication-specific UMAPs with the specific demyelination model, in addition to the type of library (single cell or single nuclei) to make it more clear. We address this concern in more detail below.

In summary, while the conceptual framework is strong and the study addresses important questions in the field, the experimental design is insufficient, data integration is suboptimal, and statistical comparisons across groups raise concerns. As a result, many of the study's novel conclusions may be unreliable or confounded by technical limitations.

Again, we thank the reviewer for their recognition of the importance of our work. We have provided additional details of the experimental design, provided rationale below for our data integration strategy, and increased our statistical comparisons.

Specific points:

Figure 1:
- The LPC baseline group shows a higher proportion of microglia compared to the cuprizone baseline, and astrocytes are detected only in the latter. This suggests inconsistencies in sample composition or processing.

Yes, there were differences in the sample processing across the different sc/snRNAseq datasets, as mentioned above. Cell proportions are not quantitative, but qualitative, so while there may be discrepancies between datasets, these are controlled by using the controls within each dataset and then comparing the effects (i.e. cell type abundance or differentially expressed genes) across treatments.

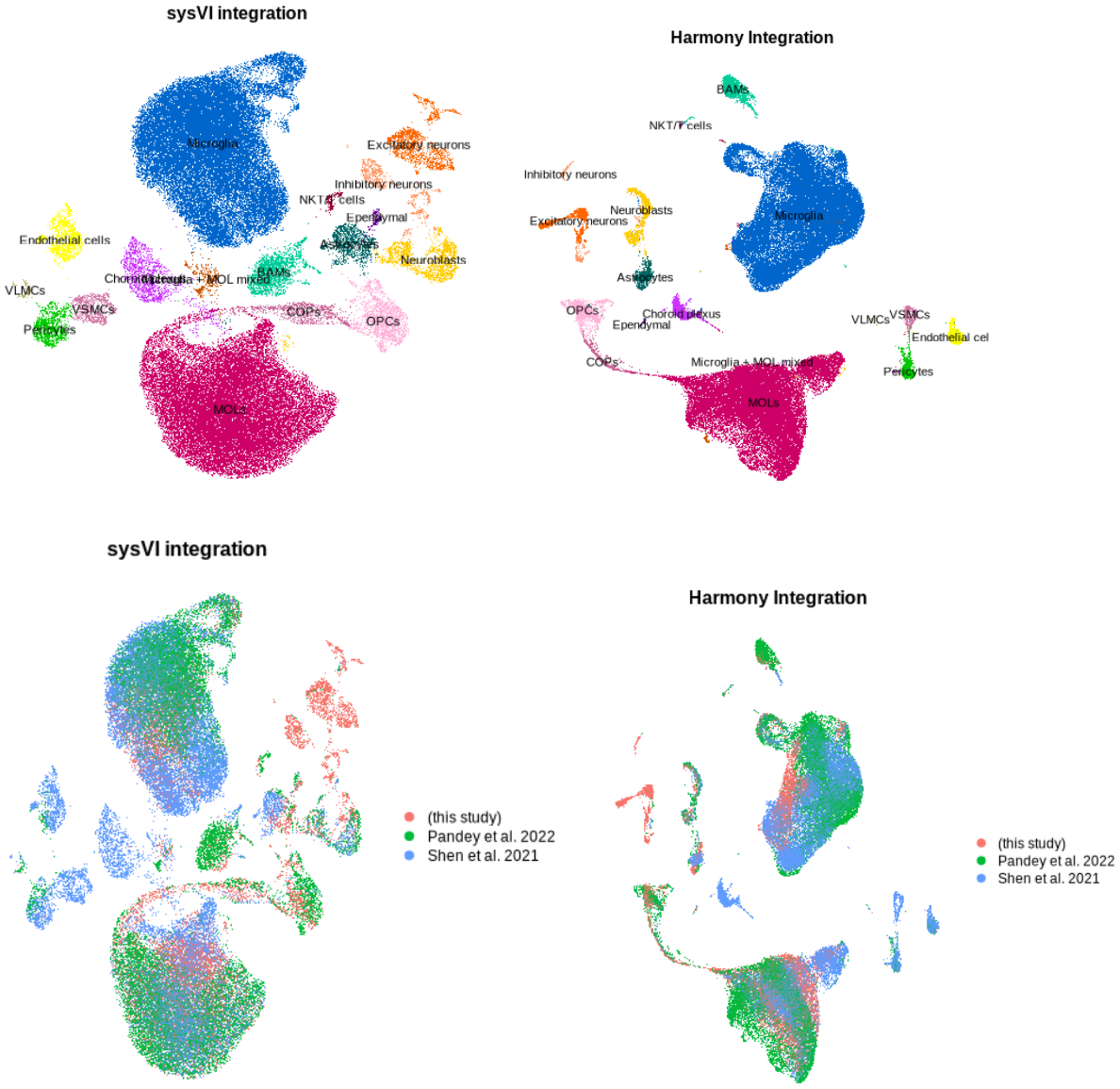
- The presence of 'mixed cells' likely represents an artifact of clustering rather than a coherent biological population.

Overall, the mixed cells represent a low proportion of the total cells (for example, the mixed cells represent only 0.5% of the total cells in the CPZ dataset). We followed current standard best practices for sc/snRNAseq data analysis, including ambient RNA removal, stringent doublet removal criteria, and removal of cells with high gene counts or UMIs. It is possible that these are a real population of microglia potentially engulfing myelin debris or a result of ambient myelin RNA contamination. They have been described in other studies (Olah et al. Nat Comm 2020). Similarly, the human dataset mixed cells show a mix of oligodendrocyte and astrocyte genes, which could be a real population that is predominantly enriched in the active lesion condition. We do not make any biological conclusions about these cells, verify their existence in tissue, nor use them for downstream analysis. We could simply drop them from the data, but since we are happy with our QC, we kept them as they appear to be real, and there is no computationally valid reason to remove them.

Figure 2:

- Integration of the Pandey et al. dataset, particularly the mature oligodendrocyte lineage (MOLs), appears poor. The leftward UMAP shift suggests batch effects rather than biological divergence, despite both LPC and cuprizone models being present.

As we mention above, the reviewer's interpretation is incorrect, as the Pandey et al. dataset has only LPC samples. We have added this annotation in several figures to make this more clear. Additionally, we provide a reviewer figure here demonstrating a second integration approach using Harmony. The Harmony integration shows dataset overlap, but we feel that it does not appear as good as the sysVI integration.



Reviewer Figure 1: UMAP plots colored by cell type (top) and publication of origin (bottom) comparing the sysVI integration (left) and Harmony integration (right). We observe similar separation between cell types, but better resolution of publication specific effects in the sysVI model. We observe a similar spatial separation of the cuprizone-specific oligodendrocyte cluster.

- The density plots are difficult to interpret, e.g., Cluster E appears surrounded by Cluster B. Supplementary Fig. 3d offers a clearer view, where MOL_B increases during cuprizone remyelination, a pattern less pronounced in LPC.

We agree and have therefore removed the density plots and focused instead on the MILO plots, which are quantified in the adjacent graph in Figure 2d.

- Annotations lack clarity; the metric in the lower left corner is unexplained. Although the Methods state that cuprizone remyelination was excluded from comparison, this group is nonetheless shown. The DEG analysis approach is problematic.

We have checked every figure to make sure all legends and scale bars are properly labeled, and removed the cuprizone remyelination group as the data is not robust and is not used in the DEG plots. We have revised our DEG analysis approach, detailed in the comment below.

- Despite lower cell numbers, all clusters are present at baseline, raising the question of why comparisons were made to a "proximal" MOL state instead of the same cluster under baseline conditions. UMAP proximity is an unreliable proxy for transcriptomic similarity and conflates condition-specific and cluster-specific effects, limiting interpretability. This issue also recurs in Figure 3.

Concern over our cluster-specific DEG analysis was also raised by reviewer 2. While we originally wanted to explore the genes that were uniquely changing across the different DAO and DAM clusters to uncover the biology of these specific cell states, we acknowledge both reviewer 1 and 2's concerns. We therefore have changed our DEG analysis to a pseudobulk approach where we compare all mature oligodendrocytes (MOLs) within the de/remyelination samples to their respective baseline control (Figure 2). We use this new approach for the human MOLs in Figure 4, mouse microglia in Figure 5, and human microglia in Figure 6. We find that the overall enriched pathways and DEGs do not significantly change from our original approach, but some of the cluster-specific DEGs are less prominent due to a mixture of cell states.

Figure 3:
- The UMAP appears over-clustered; for instance, the identity of Cluster D remains unclear. If meaningful, the authors should present defining markers.

Human MOL_D expresses elevated levels of *FTL* (iron sequestration, oxidative stress resistance), *PEBP1* (ERK/MAPK pathway, anti-apoptotic and protective against oxidative stress), and *CSRP1* (cytoskeleton-associated protein) compared to the other MOL clusters, as we show in Figure 4b, suggesting upregulation of stress-related responses. Interestingly, MOL_D cells are proportionally most abundant in NAWM, where they comprise ~21% of all oligodendrocyte-lineage cells. In contrast, they represent ~14% of OL lineage cells in non-lesional WM, and <10% within all MS lesion types (AL, CIL, RL, and CAL). This indicates that the MOL_D state is present in all samples, but with greater relative enrichment in NAWM compared to MS lesions. Comparatively, the homeostatic cluster MOL_C is proportionally more uniformly distributed (26% AL and WM each, 23% CIL, 22% RL, 20% NAWM and 19% CAL).

- The manuscript claims minimal differences between WM and NAWM in MOL_A-D states. However, Suppl. Fig. 4G indicates increases in MOL_F and MOL_G in NAWM, which contradicts that statement.

We apologize for the confusion. Human MOL_F and MOL_G are disease-associated OL clusters (or DAOs), whereas human MOL_A-D are homeostatic OL clusters. We do not see significant changes in the proportion of the homeostatic MOLs (A-D) between NAWM and control WM, as shown in Figure 4c. We do see a significant increase in DAO clusters MOL_F and G. We have labeled MOL_F and MOL_G as DAO clusters in the UMAP in Figure 3a to help clarify this.

- The observation that MOL_A-D are more evenly distributed in human WM than mouse corpus callosum seems to rely on density plots and UMAPs, and thus may be heavily influenced by integration strategy.

We agree and have decided to omit this statement as it was a minor observation in our previous version.

Figure 4:
- Figures 4a and Suppl. Fig. 7a show stark differences between cohorts. This raises the question of how meaningful disease-associated shifts can be interpreted in light of baseline discrepancies.

We have included labeling for the models and the type of libraries (now Supp Figure 10a) to help clarify that the general cell types that we see are specific to the model (ie. PVMs, previously called BAMs are specific to the LPC models). The biggest difference we see is that the Shen et al dataset has more coverage of microglia, suggesting that their collection favored conditions to obtain microglia. However, we find differences are meaningful and that the proper comparison to baseline in our DEG analysis allows us to make robust conclusions.

- MG_C, D, and E are grouped for DEG analysis due to shared features. This calls into question the rationale for clustering at such high resolution.

We have revised our DEG analysis approach to a bulk approach, which is not impacted by our clustering. There are slight changes in expression level of various genes across these different microglia clusters, as shown in the dot plot in Figure 5a, which may reflect transitions across different cell states. We did not find many unique cluster markers, which we are transparent about in Supplemental Figure 10c. Our clustering of the mouse microglia may be lower compared to other published studies, possibly as a limitation of the difference in library types and the sysVI model, though we and others observe these microglial states as a continuum in the single cell space (see Sankowski and Prinz, Nature Neuro. 2025. https://doi.org/10.1038/s41593-025-01978-3).

- The claim that LPC induces stronger BAM infiltration and greater microglia proliferation may be biased, given the unusually low microglia proportions in the LPC baseline group.

We now provide *in vivo* analysis of the number of BAMs (renamed perivascular macrophages or PVMs) in both LPC and CPZ demyelination. We find that LPC demyelinated lesions do contain significantly more PVMs (Figure 5e).

Figure 6:
- MOL_E is nearly absent in this analysis, and no UMAP is provided showing the integration of the four datasets for oligodendrocytes.

After careful consideration, we have decided to remove the cross-species analysis (previous Figure 6) due to the high difficulty in the integration of the samples, which achieves low resolution, and loss of gene level information for non-homologous genes.

- The reported signatures primarily reflect progenitor states and lack specificity for DAO. Hence, inferred overlap with human DAO populations is weak and should be interpreted cautiously.

We have moved our DAO gene signature analysis to Figure 4 and have added new gene signatures for DAMs in Figure 6. For the DAO signature, we used genes that are differentially expressed in the MOL_G state for

CPZ demyelination and MOL_E for the remyelination signature. The expression within the progenitors on the UMAPs reflects that many DAO genes are also upregulated in OPCs, which we mention in the results.

Methods:
- Line 1067 indicates different protocols for snRNA-seq and RNAscope validation in the cuprizone model. The rationale for this protocol divergence should be clarified.

The snRNAseq experiments were performed at Dr. Adams' postdoctoral institution, whereas the RNAscope validation experiments were performed in Dr. Adams' independent lab at the University of Notre Dame. We found that the 0.2% cuprizone pellets were not inducing as robust of a demyelination phenotype after this transition, so we decided to switch to 0.3% cuprizone pellets, which have been previously reported to be more effective when using pre-packaged pellets instead of mixing the cuprizone into chow on site (Sullivan et al. *Acta Neuro. Comm*. 2020).

Reviewer #2 (Remarks to the Author):

Review Summary
The manuscript from Aboelnour et al., entitled "A comparative transcriptomic analysis of mouse demyelination models and Multiple Sclerosis lesions," presents a cross-species joint transcriptomic analysis at single-cell and/or single-nucleus resolution to determine which toxin-induced mouse models of multiple sclerosis (MS) most closely resemble glial changes observed in human MS. By integrating three published datasets (Shen et al., 2021; Pandey et al., 2022; and Macnair et al., 2025) with eight newly generated transcriptomic libraries from investigated mouse models, the authors examine both the divergent and convergent properties of glial cells, focusing on oligodendrocyte lineage cells and microglia. The authors have identified an important question in the field of biomedical research. They have demonstrated technical expertise and assembled relevant datasets to tackle this challenge. While the manuscript presents compelling data, we find that the depth of analysis and interpretation, and, as a result, the insights offered in the current version of the manuscript, could be improved.

We thank the reviewer for recognizing the importance of our research question and our technical expertise.

Technical Details
There are significant concerns regarding the focus of the analysis, the conclusions drawn from the presented data, and the lack of specificity in the descriptions of the manuscript. To help improve future versions, these concerns are outlined point by point below:

Abstract & Introduction:
1. "… it remains unclear whether different demyelination models elicit distinct glial responses, and how comparable these changes are to MS." While this is a central question raised in the abstract, it is not immediately clear what the authors have discovered in response to it. The abstract and research highlight do not clearly convey the key findings or how the study advances current knowledge relative to existing literature. These sections would benefit greatly from more precise wording and clearer presentation of the quantitative analyses to enhance interpretability and impact.

We thank the reviewer for their constructive criticism and have revised the abstract to clearly state the key findings of our study.

2. “Interestingly, remyelinating OLs converge on an altered maturation state in both LPC and cuprizone models, potentially due to persistent activation of microglia at remyelination stages.” This is an intriguing hypothesis; however, the manuscript currently lacks the specific analyses needed to support this conclusion. As presented, it appears to be a logical leap, and we recommend that the authors provide clearer evidence or rationale linking microglial activation to altered oligodendrocyte maturation in these models.

We agree that additional experimental evidence is needed to make this statement. While we would love to dissect the relationship between microglia activation and oligodendrocyte remyelination, we feel this is beyond the scope of the current study. However, we do now provide experimental evidence of an altered oligodendrocyte maturation state at remyelination timepoints in our new Figure 3, as well as analysis of microglia activation at late stages of remyelination in Figure 5. We postulate about this potential relationship in the discussion section in the context of other published studies, but do not mention it in the abstract or introduction.

3. “A critical gap in the current use of animal models to study remyelination is the lack of understanding of which MS disease mechanisms are replicated. These two toxicity models are often used interchangeably by researchers, with a particular method selected because of experimental design constraints rather than scientific rationale.” Given this important point, it would be valuable if the authors could offer guidance to readers—such as a framework or criteria—for selecting between these models based on their relevance to specific MS-related mechanisms. Do the authors recommend that studies investigating oligodendrocyte protection and remyelination routinely be conducted in both models? Is there evidence that such an approach would enhance translatability? This would enhance the study's translational impact.

We acknowledge the reviewer’s point and have now included a paragraph in the discussion section that describes our views on selecting the appropriate demyelination model based on our results. However, we feel that this is not a simple task as there are many different factors to consider, and our study is not all encompassing. Therefore, we do not feel comfortable providing a sweeping guidance that states, “X model should be used over Y model”. This requires greater depth of discussion and would be better served as a short commentary or review article.

4. Page3 citation 16: The citation appeared to be misplaced. Authors should clarify why this comparison excluded mouse EAE data, another commonly used model for MS. Indeed, there is some high-level discussion of EAE toward the end of the paper, which leaves the reader wishing that EAE samples had also been interrogated, even if there is no straightforward way to distinguish demyelination and remyelination in EAE. For example, they could have used methods similar to those they used for integrating LPC and cuprizone datasets with human MS lesions to compare those same mouse models to EAE.

Reviewers 2/3 and 4 both requested a comparison with the EAE model, so we now provide a new supplemental figure 5, where we compare DEGs from a published scRNAseq dataset of oligodendrocyte lineage cells isolated from the spinal cords of EAE mice. We chose to not integrate this dataset with our LPC and CPZ datasets for several reasons: (1) our study is focused on demyelination of the subcortical white matter, whereas the EAE dataset is from the spinal cord. There is evidence that glial cells display differences across different CNS regions, especially between the brain and spinal cord (Floriddia et al. Nature Communications 2020). To our knowledge, an equivalent sc/snRNAseq dataset of all cell types within the corpus callosum of EAE mice has not been performed. (2) The experimental setup and single cell technology that was used in the EAE study (Smartseq2) was different from the other datasets (10x Genomics), which could cause problems with integration, and we did not want to disrupt our analysis of the LPC and CPZ models.

Results:
5. Fig1 & Sup Data1: Including a timeline in Figure 1 that illustrates the experimental design across studies—clearly labeling the experimental procedures, library numbers, and basic animal demographics—would be helpful in contextualizing the evidence presented.

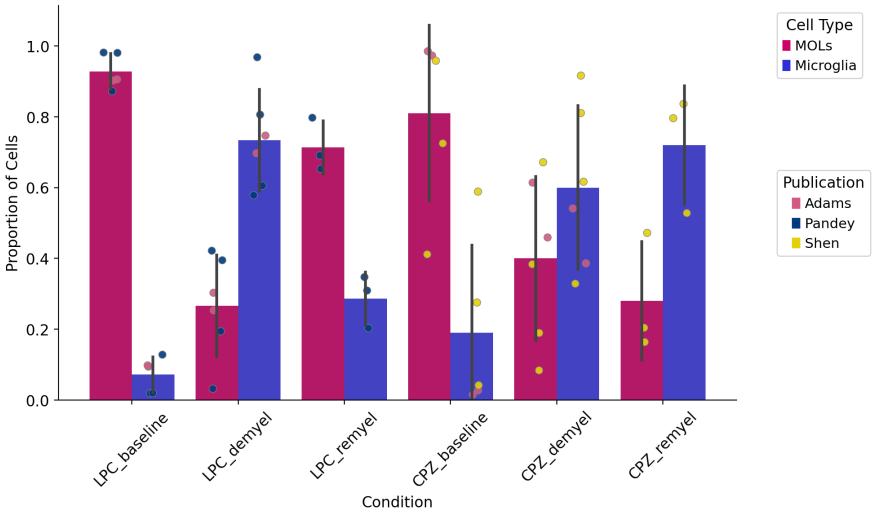
We thank the reviewer for this suggestion and have added a schematic in Figure 1a and 1e to show the data and replicates that were collected and used in the paper.

6. Supplementary Fig1G: Consider integrating some of the information into the main Figure 1 in a more streamlined format. Enhancing annotation of technical replicates, experimental models, dataset sources, distinctions between single-cell (sc) and single-nucleus (sn) approaches, and reporting total sample numbers would greatly aid interpretation.

The schematic diagram in Figure 1a, and the inset map in 1b should help to create a map for readers about what the data is. We have included supplementary table 1 with metadata related to the mouse samples. The table shows the individual sample IDs, treatment, timepoint, and the library type, as well as a second tab showing the cell counts for all replicates. Readers can use this table to look at replicate information and cell counts.

7. Figure 1D: Recommend presenting a bar graph of group means overlaid with scatter plots of individual replicate values, clearly labeled by condition, study, and cell type. Additionally, performing a proportional statistical test would strengthen the assessment of differences in remyelination composition across conditions, as the current observations are only qualitative.

We now provide bar chars of the counts for each cell type for mouse and human datasets (Figure 1c,g). We also provide a reviewer figure below with this data, showing a bar chart with the replicates. To add proportional statistical testing, we have now quantified compositional changes in the total mouse and human UMAPs using scCODA (Supplemental Figures 1e and 3d). For the subcluster analyses, we performed statistical analysis on our MILO outputs. To compare the distribution of nhoods across conditions, we aggregated counts of upregulated, downregulated, and non-significant nhoods by cell type and condition. For each cell type and significance category, we constructed contingency tables of observed counts and totals, and applied either Fisher's Exact Test (for tables with <100 total counts) or Pearson's Chi-squared Test (for larger tables). Resulting p-values were corrected for multiple testing using the Benjamini–Hochberg procedure. For visualization, we generated stacked bar plots showing the proportions of upregulated, downregulated, and non-significant neighborhoods per cell type in each condition. Significant differences between conditions were denoted with asterisks on the bar with higher proportions of significant nhoods that corresponded to adjusted p-value thresholds (Figures 2d, 4c, 5d, and 6c).



Reviewer Figure 2: Bar chart showing the major glial cell types analysed herein, labeled with individual replicates that are colored by the publication of origin.

8. "BAMs and T cells were specifically enriched in the LPC model": For this and similar claims, it is important to provide supporting statistical analysis to validate the observations.

We have now performed scCODA analysis on the LPC and CPZ models (Supplemental Figure 1g). We did find significant upregulation of T cells in the LPC remyelination samples and BAMs (renamed perivascular macrophages, or PVMs) in the LPC demyelination samples. We also now include *in vivo* quantification of PVMs using IHC for CD206, a BAM marker (Figure 5e).

9. Figure 1G: Given the stated research objectives, separate UMAPs for mouse and human are less informative. Instead, it would be more helpful to begin with a merged mouse and human dataset to allow a harmonized, comparative assessment of cell class composition. Why did the authors choose to integrate only in Figure 6? A deeper evaluation of how differences between snRNA-seq and scRNA-seq mouse datasets relate to the integration with human data would also have been helpful.

We have edited the manuscript to emphasize the point that we want to first compare the mouse models that are most commonly used to study remyelination of the CNS, and second, to compare the phenotypes we describe in mice with human MS patient samples. While we acknowledge the advantages of creating a shared embedding space, the cross-species analysis is limited to homologues, which affects our interpretation of the mouse models. In addition, the cross-species integration is a challenging computational task, with large technical and biological variations. There are more challenges in performing compositional analysis and differential expression testing in mixed species models, as well as in mixed single cell and single nuclei experiments. We originally used this cross-species approach to complement our more detailed analysis comparing single species, but during our revisions we found that it does not add any new information to the story. For this reason, we decided to remove the cross-species analysis and focused on optimizing computational approaches to most robustly compare mouse models, and secondarily the mouse vs human data.

10. Figure 2C: Legend title missing (what is the unit). Consider move SupFig3D and 3F to main figure.

We have removed the density plots and replaced them with the MILO plots for all main figures to reinforce the quantification of differential abundance in neighborhoods. We have also removed the stacked barchart due to redundancy with the MILO analysis, which is more quantitative.

11. SupFig3D: Why does cuprizone baseline have more MOL_G? Is it significant?

We hypothesize that the small percentage of MOL_G in the CPZ baseline samples (5% of the total OL lineage cells, and only comes from the Shen et al. data) could be an artifact of their tissue collection process, possibly due to the stress of dissociation and FACS sorting, or contamination from the cuprizone samples. Importantly, we do not observe markers for MOL_G in our IHC/RNAscope validation in Figure 2, supporting that this DAO state is not present in healthy white matter.

12. "To quantify these changes, we calculated the differential abundance (DA) across overlapping cell neighborhoods (nhoods) using Milo (Dann et al. 2022)" Informative, method could be applied to other analyses. The citation format is inconsistent.

We have fixed the citation, thank you.

13. "However, both models enriched the DAO cluster MOL_E at remyelination, suggesting a shared remyelination state." This suggestion may be premature, as similar enrichment of MOL_E was also observed during the demyelination phase. Additionally, each sample likely represents a mixture of tissue status. It would be helpful to clarify the microdissection strategy used. Including both the collection and injection dates in the experimental design schematic (Fig 1) would further enhance interpretability.

We agree with the reviewer that each sample likely represents a mixture of tissue status. We now provide additional RNAscope analysis of this MOL_E state at remyelination timepoints in a new Figure 3. Our methods section contains details on our own microdissection strategy, and we have provided citations for the other datasets. We have added the specific timepoints represented within the mouse datasets to our new schematic in Figure 1a.

14. "direct connection to MOL_E, suggesting that OLs may undergo an altered maturation state following demyelination (Figure 2A)" Insufficient detail to make this conclusion. It might be helpful to try to construct a connectivity map individually across conditions and compare.

We have performed additional experiments to address this point. We injected mice with EdU at demyelination stages to label proliferating progenitors and then collected tissue at remyelination stages. We find that EdU+ cells are primarily mature OLs (Olig2+ Pdgfra-) in both models, and importantly, that these newly-generated OLs express *Socs3* - a gene upregulated in the MOL_E cluster. This data is shown in our new Figure 3 (panels e-i).

15. "We next asked how the different DAO states compare to each other transcriptionally. We tested for differentially expressed genes (DEGs) using a pseudobulk approach (see Methods)" Method: "For each treatment condition, we made pairwise comparisons of DAO clusters to their closest baseline MOL state (MOL_E vs. MOL_B, MOL_F vs. MOL_D, and MOL_G vs. MOL_A" What is the rationale for comparing each DAO to a different reference group? Would it not be more consistent to compare them to a common subcluster, or to the same subcluster split between control and disease conditions?

Our original reasoning behind this approach was to identify the most uniquely altered genes within these DAO states, rather than general changes across the population. The subclusters are not present in sufficient numbers across all samples to compare between baseline and de-/remyelination groups (i.e. the DAO clusters are not present in the baseline samples). However, based on comments from both reviewer 1 and 2, we decided to change our DEG analysis approach to a pseudobulk approach, which is not impacted by our

clustering. We still see similar GSEA enriched pathways and DEGs with this new analysis, showing that the transcriptional changes are being driven by the DAO groups.

16. Figure 2E: According to the Methods, it appears that each group was compared to a different control. This should be clearly labeled in the figure to avoid confusion.

As mentioned in our above comment, we have changed our DEG approach. We explicitly describe in our methods section (pages 22-23) how we performed the DEG analysis.

17. "coagulation-related genes were enriched in LPC-treated DAOs, likely due to blood-brain barrier disruption and BAM infiltration following stereotaxic injection" Interesting observation; however, there appears to be a logical gap between this observation and the reported elevation of coagulation-related genes in oligodendrocytes. Did the controls of LPC model injected with PBS shows the same? Please discuss and clarify further.

We agree that we do not have direct experimental evidence to support a role for BAM/PVM infiltration and coagulation-related genes in the LPC-injected samples. However, we do now show the PVMs are increased solely in LPC demyelination (Figure 5e). This was a minor point in our original version so we have removed it from the revised manuscript.

18. "Socs3, Cdkn1a, Nupr1" are interesting findings and should probably be mentioned in the abstract. Additionally, the presentation of evidence and comparison of these candidates across conditions, cell types, and FISH results should be made more consistent to enhance clarity and interpretation, such as a version of Sup4D plots for all of them. Additional FISH quantification on Nupr1 would strengthen the impact.

We have revised our abstract to include these results. We have performed additional RNAscope validation of these genes within oligodendrocytes and OPCs, and our figures now show a uniform presentation of this data. We have not included UMAP plots for these genes due to space limitations and redundancy with the RNAscope results.

19. "Supplementary Figure 4E, F" The contrast between oligodendrocytes and OPCs is intriguing and warrants inclusion in a main figure, especially with supporting FISH data. The findings suggest that the LPC-induced Cdkn1a-high OPC may not progress to mature oligodendrocytes (MOL), or that Cdkn1a expression is downregulated before that transition. Given that LPC leads to more extensive remyelination than cuprizone (Fig1D, higher MOL percentage in recovery phase), this highlights Cdkn1a as a potential therapeutic target—one that may need to be suppressed to promote oligodendrocyte survival and successful remyelination?

We now include expanded RNAscope analysis of the mouse OPCs, analyzing their expression of Cdkn1a, Nupr1, Socs3, and B2m (Supplementary figure 8). We also provide new analysis of the human OPCs (Supplementary figure 9). We switched our quantification method to calculate the percentage of OPCs that are Cdkn1a+, etc. instead of the total number. This gives a better idea of how broadly these DAO genes are expressed within the OPCs. We find that ~10% of OPCs express Cdkn1a in LPC demyelination, so it is not a very high proportion. This may indicate that OPCs downregulate Cdkn1a before differentiation, or it may indicate that they are progressively lost due to reduced survival. Distinguishing between these possibilities would require fate labeling of Cdkn1a+ OPCs, which we hope to do in future studies.

20. "Our results support the use of LPC and cuprizone models to study distinct aspects of OL stress and repair in CNS demyelination, and to identify strategies for enhancing OL protection, survival and remyelination" The statements here—and throughout the manuscript—are overly general. A more quantitative perspective is

needed, particularly in relation to the specific genes mentioned above. Additionally, the discussion would benefit from being framed within the context of relevant prior literature to strengthen the interpretation and significance of the findings.

We have revised our writing throughout the manuscript to emphasize a more quantitative approach, citing specific numbers within the text, as well as increasing our statistical analysis. We have also significantly revised our discussion to include relevant prior literature.

21. Figure 3: A very similar naming scheme to human MOL_A–G is used, but it remains unclear whether these labels reflect comparable transcriptomic features as annotated in the mouse data. To clarify this, it would be more informative to initiate the analysis with a combined species clustering. This would allow for direct comparison of cluster identities in the global (combined) space versus the local (species-specific) space. Incorporating a riverplot to visualize these relationships would significantly enhance the clarity and interpretability of cross-species comparisons.

We appreciate the reviewer's point about the combined species clustering and have responded to that suggestion in a previous comment (#9). The annotations of the mouse and human oligodendrocyte lineages (and microglia) are performed independently of each other, which we have clearly stated in our results section. The dot plots show marker genes for each cluster, and the reader can see that different genes were used to define the mouse vs. human states. We have also used the meta-neighborhood analysis in Supplementary Figures 7 and 12 to show similarities across cell types between species. We feel that this analysis shows nice intermingling of both mouse and human cells within a given subcluster.

22. "validated the DAO state using a gene signature from a previous MS patient study, confirming that known DAO genes are enriched in MOL_E-F and OPC cluster…" The method authors employed is not an alternative method, so "validation" is too generous a term; phrasing it as "we annotated based on..." would be more appropriate and accurate.

We have rewritten this statement.

23. "Interestingly, MOL_E- a comparatively small cluster- was highly enriched in two WM samples (44% of cells), both from 85-year-old individuals" Should be based on data, add the plot in the supplementary figure.

We have added the appropriate plot to Supplementary Figure 6e.

24. "suggesting that normal brain aging may induce a stressed state in MOLs" The analysis is insufficient to support this claim. Perform an age-dependent distribution analysis of MOL clusters.

We have added the appropriate plot to Supplementary Figure 6e.

25. "we observed that MOL_A-D populations are more evenly distributed in human WM compared to the mouse corpus callosum, suggesting greater heterogeneity in adult human MOLs" It is unclear, where is the data, and why the conclusion?

Reviewer 1 also commented on this statement. We have removed it from our revised version.

26. "Quantification using Milo supported our observations, showing minimal changes in homeostatic MOL_A-D in NAWM, but variable alterations in MS lesions, with the fewest changes observed in RLs (Figure 3D)…" Unclear, please perform stats.

To compare the distribution of nhoods across conditions, we aggregated counts of upregulated, downregulated, and non-significant nhoods by cell type and condition. For each cell type and significance category, we constructed contingency tables of observed counts and totals, and applied either Fisher's Exact Test (for tables with <100 total counts) or Pearson's Chi-squared Test (for larger tables). Resulting p-values were corrected for multiple testing using the Benjamini–Hochberg procedure. For visualization, we generated stacked bar plots showing the proportions of upregulated, downregulated, and non-significant neighborhoods per cell type in each condition. Significant differences between conditions were denoted with asterisks on the bar with higher proportions of significant nhoods that corresponded to adjusted p-value thresholds (Figures 2d, 4c, 5d, and 6c).

27. "Overall, all MS lesion types were strongly enriched for DAO states MOL_F and G" Is this referring to DAO in a general sense, or the specifically defined DAO population characterized in the mouse dataset introduced in the study?

It is referring to the human DAO states that we annotated in Figure 4a,b.

28. *"we performed differential gene expression analysis focused on AL and RL samples, as they are most comparable to our mouse time course data (Supplementary Data 4)" On what basis is their similarity determined? Given the capabilities of the bioinformatics tool employed, shouldn't the comparison be conducted in a more unbiased and comprehensive manner without predefined hypotheses or conclusions about which toxin-induced model most closely resembles specific MS-related pathological changes? In particular, the analysis of chronic active lesions, which are really better characterized as "mixed active-inactive lesions" and have inactive and active areas, should be substantially deeper to enhance relevance to MS.*

We agree with the reviewer and appreciate the suggestion. We have now performed the analyses across all MS lesion types.

29. "indicating that DAO cluster MOL_G shifts toward a more homeostatic-like state during remyelination" The conclusion based solely on the number of DEGs is not sufficient or fair, as additional new changes occur beyond the demyelination phase. Moreover, these samples are from different patients, each with diverse genetic backgrounds and demographic profiles. The structure of the sentence is also misleading, as it implies that MOL_G is uniquely affected potentially more so than other MOL subtypes. Have you performed a similar DEG overlap analysis within the mouse MOL_G dataset? Why focus specifically on MOL_G?

Our new pseudobulk DEG analysis incorporates all MOLs within the different MS lesion types, so we no longer focus specifically on MOL_G.

30. "Importantly, many of these pathways were previously observed in the original analysis of this human dataset, including TNFα and IFN upregulation, supporting the robustness of our analysis pipeline." Once again, what's novel about your current analysis—specifically in comparison to and building upon your prior mouse data? A joint cross-species analysis from the start would likely be more informative and directly relevant to your research questions.

As explained in other responses (comment #9), we chose not to focus foremost on the cross-species comparison. Our revised analysis of the human dataset provides additional details of the cellular heterogeneity across different MS lesions, which was not the primary focus of the original Macnair et al. study. In addition, our dataset includes only the white matter samples, whereas Macnair et al. included both gray and white matter in their analyses. The greatest increase in new knowledge comes from our comparison of the mouse

models with the human data, which we feel that our approach is able to do without the joint cross-species analysis.

31. "Finally, we explored the expression pattern of genes previously validated by RNAscope in our mouse models (see Figure 2). CDKN1A and SOCS3 were highly enriched in the MS DAO cluster MOL_G, especially in RLs, indicating persistent activation of pathological pathways in MS lesions (Supplementary Figure 6E)" A new section should begin with this point, moving the earlier preliminary work to the supplementary section. SupFig6E should include the full expression profiles of NUPR1, SOCS3, and CDKN1A, and this panel should be moved to a main figure, especially as it pertains to multiple lesion types. Also, what is the functional role of CDKN1A in this context? A clearer explanation is needed to justify its emphasis.

Our revised human MOL analysis now includes heatmaps showing example DEGs that are significant across some/all of the MS lesion types (Supplementary Figure 7f). *SOCS3* and *CDKN1A* are both significantly increased, and NUPR1 almost reaches our significance threshold. We state this within the results section. We also provide more discussion into the potential functional role of CDKN1A in the discussion section.

32. "Together, these findings indicate that both mouse demyelination models exhibit some disease-associated characteristics of MS pathology in OIs" The current comparison is too vague. You should quantify the similarity between mouse MOL_E, F, and G and the human-defined clusters using a similarity score or metric (e.g., correlation, Jaccard index, or another appropriate approach). Additionally, consider assigning informative names to each cluster based on dominant signaling pathways or other defining ensemble features, rather than relying solely on alphabetical labels. This would make cross-species comparisons and functional interpretation much clearer.

We have now calculated the overlap coefficient and Spearman correlations of mouse and human up- and downregulated DEGs for both MOLs and microglia (Supplementary figures 7e and 12e). For each dataset pair, we separately assessed overlap among significantly upregulated and downregulated genes. The degree of overlap was measured using the overlap coefficient, defined as the size of the intersection divided by the size of the smaller set. This metric is more robust to differences in list size than union-based measures such as the Jaccard index, and captures the degree to which the smaller gene set is contained within the larger. The overlap coefficient was calculated independently for upregulated and downregulated DEGs.

To evaluate concordance in the magnitude and direction of differential expression, we computed the Spearman rank correlation of the logFC for the shared DEGs between each dataset pair. Only genes detected (though not necessarily significantly differentially expressed by our thresholds) in both datasets were used for correlation analysis. Statistical significance of correlations was determined using two-tailed tests, with $p < 0.05$ considered significant.

We have considered alternative naming strategies for the different clusters but ultimately decided against it for several reasons. First, we have not performed any functional analyses so we can not state definitively what the likely function or biological role of each subcluster is. Second, many of the enriched signaling pathways or DEGs are shared across multiple subclusters, which makes it difficult to assign these labels. While we appreciate the reviewer's point, we have revised our writing to hopefully make it easier to understand.

33. "We previously observed that microglia undergo a dramatic state shift following injury," Missing citation to data.

Now cited as Figure 1d.

34. Fig4-5: Similar to the comments on the oligodendrocyte analysis: the language throughout should be more precise and quantitative. Apply appropriate statistical tests to support your claims, clearly state what is novel in your findings, and interpret results within the context of existing literature. Finally, although it's sometimes difficult to do so cleanly, whenever possible it would help the reader if you use informative naming conventions for clusters based on defining molecular or functional features, rather than abstract labels, to aid interpretability and cross-study comparison.

We have now performed additional statistical analyses and *in vivo* validation experiments for the microglia analyses, mirroring the changes that we made to the oligodendrocyte figures. We have also increased discussion of the microglia in the context of existing literature. We acknowledge that it may be easier for readers if we provide functional names for the subclusters, however we are very hesitant to attach biological functions to these different cell states. In the results section, we discuss some possibilities of what these different DAM states might be based on some marker genes and how they've been previously referred to in other studies, but we purposefully do not include this within the figures. As more scRNAseq and functional studies are performed on different disease tissue samples, these biological labels may change. There is already discussion in the field as to how best to approach this issue (Paolicelli et al. Neuron 2022). For this reason, we focus on the GSEA enriched pathways and specific DEGs.

35. "gene expression changes by comparing the homeostatic state (Mg_A) with DAMs, or BAMs from LPC-induced demyelination" It may be worth performing a direct, head-to-head comparison across the two systems—for example, comparing the same MOL subclusters between LPC- and cuprizone-induced demyelination models (instead of to the homeostatic cluster of the same system or control). Identifying shared or distinct DEGs within corresponding subclusters could provide clearer insights into conserved versus model-specific responses — especially since there seem to be gene expression differences in the control populations despite application of harmonization approaches, raising the possibility that at least some of the model-related differences reported in the paper are technical in origin. Moreover, in the current version the similarity of clustering is relative and should be interpreted in context. In some cases, gene expression levels may change significantly within the same subcluster annotation across conditions, but not to a degree that warrants defining a new, distinct cluster.

We agree that directly comparing matched subclusters between the LPC and cuprizone models is an informative approach, and we did explore this analysis during the development of the study. However, we found that these direct comparisons were at least partially influenced by differences between each model which persisted even after batch correcting methods. Because we saw baseline differences contributing to the resulting DEG lists, the direct cross-model comparisons obscured the biological signals and ultimately we decided to stick with the comparison to baseline, which we felt provided clearer biological resolution and more reproducible detection of DEGs.

36. Fig4G: The method and rationale are unclear. Even if the conclusion is reached that LPC-induced microglial changes are more pronounced, this finding alone is not particularly informative without further context or mechanistic insights.

We agree with the reviewer and have removed these plots.

37. "In contrast, cuprizone-induced demyelination elicits a more gradual response over weeks, leading to weaker DAM enrichment and a near-complete return to homeostasis by remyelination." Doesn't this finding dissociate the role of microglia in oligodendroglia-mediated remyelination? As shown in Fig1D, the cuprizone model indicates no recovery of MOL or OPC percentages. Could this suggest that no further myelin debris is

being produced, such that there is no further stimulant for microglial reactivation in the cuprizone model compared to the LPC model?

We have now quantified the percentage of activated microglia at late remyelination timepoints, using Apoe as our DAM marker since it was strongly upregulated in both LPC and CPZ microglia. We do find Apoe+ microglia at late LPC remyelination stages (42dpl), but not in CPZ remyelination (5+ 5 weeks). In our oligodendrocyte MILO analysis, we find that CPZ has not completely recovered homeostatic MOLs. This does suggest that microglia activation is important for remyelination, which has been shown in other studies (Baaklini et al. Cell Reports 2023, Boutou et al. Cell Reports 2024). Since myelin debris is presumably not being generated in either model at these late stages, it suggests a potential alternative reason for why microglia remain activated in the LPC model.

38. "However, our results highlight LPC as a model for studying robust microglial activation, including the recruitment of BAMs and stronger transcriptional shifts." This is too vague; it needs to be more specific, particularly in the context of MS stages and the involvement of BAMs. How do secretory or diffusive factors contribute to these observations?

We have revised our writing throughout the manuscript to be more specific. Interestingly, we do not observe any changes in the proportion of BAMs (renamed as PVMs in current version) across the different MS lesion types. To perform a direct comparison of gene expression changes specifically within microglia between the mouse models and MS lesions, we did not include PVMs within our pseudobulked human microglia DEG analysis.

39. "Mg_E expresses similar gene expression to Mg_D, but also expresses NUPR1, a gene that is more abundant in white than grey matter, and is critical for repressing iron-dependent cell death" What is the expression pattern of NUPR1 across mouse models? Using FISH to present this evidence would strengthen the conclusion and provide more direct spatial resolution of its expression.

We now present RNAscope data for Nupr1 expression in both the LPC and CPZ models for OLs. We did not perform RNAscope for Nupr1 expression in microglia, we see that it is broadly but lowly expressed in the RNA-seq data.

40. "Using the sysVI method to correct for species-specific effects" The code should be shared, and the methods to address differences between single-nucleus (sn) and single-cell (sc) approaches should be clarified. Ensure that cross-species gene homologs are properly addressed. Additionally, how can these findings help explain differences in the inherited diversity in microglial response, particularly with a skew toward cell-type-specific genes? It's important to clarify the methodology used to get relevant interpretation.

All of our code is shared on our lab's github page, and this repository is currently being updated to reflect the revisions in the manuscript and includes scripts for preprocessing, ortholog mapping, model training, and downstream analyses. For the cross-species integration, human–mouse gene orthologs were mapped using Ensembl gene IDs corresponding to GRCh38.p14 (human) and GRCm39 (mouse). Only one-to-one ortholog pairs were retained to ensure direct comparability. We followed sysVI example code presented by the authors and directly contacted them by email to ensure our approach with their model was suitable. However, we have now removed this cross-species embedding and analysis in favor of the species specific integration and comparisons.

41. Supplementary Figure 11: Should be figure 1. Since UMAP and clustering are sensitive to cell count, it would be prudent to subsample both the mouse and human datasets to an equal number of cells for

downstream comparison. This will help ensure that the conclusions drawn are not biased by differences in cell numbers and gene load between the datasets.

As described above in our response to comment #9, we have decided to remove the cross-species analysis (original Figure 6 and supplementary figure 11).

42. "We found that the BAM-specific expression of F13A1 is integrated within the microglia cluster that expresses CSF1R, suggesting we lost the specification of macrophages and microglia that we observed in the mouse and human datasets." How is this phenomenon influenced by the use of the sysVI method? Could biased cross-species correction—particularly favoring certain cell class-specific genes—be contributing to the observed results? This possibility should be examined and clarified.

No longer relevant as this section has been removed from the manuscript.

43. "This observation further supports our hypothesis that OLs have higher conservation across species than microglia. Thus, we focused on the OL cross-species comparison." A similar analysis presented in Fig 6A should be performed for microglia and other cell classes with improved annotation and naming.

We have now performed meta-neighborhood analysis for the microglia, which shows nice mixing of the human and mouse microglial states. This is shown in supplementary figure 12c. Because of our decision to remove the combined-species analysis, this section of the manuscript has been removed.

44. Fig 6G: It's not clear why these specific genes were selected for closer examination in this context. The rationale should be explicitly stated—whether based on prior literature, relevance to MS pathology, differential expression patterns, or pathway involvement—to justify their focus and interpretive value.

These UMAPs have been removed as they are no longer relevant to the figures.

45. "We conclude that cuprizone treatment induces a state change in MOLs that more closely resembles active MS lesions than LPC." However, compared to RL, the number of MOLs in the Cupr_remyel condition does not appear to recover (SupFig11E). What about in the EAE model? Including this comparison would provide a more comprehensive view of MOL dynamics across different demyelination and remyelination contexts.

As described above in comment #4, we now provide a new supplemental figure 5 comparing DEGs from EAE oligodendrocytes with LPC and CPZ. It is difficult to assess demyelination vs remyelination timepoints within the EAE model, so this aspect is not included in our analysis.

Discussion

46. "Numerous preclinical therapies have been identified using these models, with 25 out of 88 reported to have entered clinical trials and/or received approval (ref51)." It would be valuable to consult clinicaltrials.gov for updated reports and to mention how many trials are based on LPC-induced versus cuprizone models, though this might be difficult to achieve in practice. Additionally, highlighting any similarities in the mechanisms of action (MoA) of these two models could add depth to the discussion and help justify the choice of model in the context of studying MS pathophysiology and therapeutic relevance. Finally, please avoid the use of the the term "treatment" in describing the toxin used in the models, as this can be confused with salutary therapies.

We appreciate the reviewer's suggestions and agree that this information would be interesting. However, obtaining information on how many clinical trials are based on LPC versus CPZ is not straightforward, and we feel this would be better suited as a meta-analysis in a separate follow-up commentary or review article. We

include a paragraph in our discussion section that describes some parameters for justifying the choice of demyelination model, but again we feel this would be better served in a separate article. We have removed the term “treatment” when talking of LPC or CPZ.

47. “The only shared enrichment observed was in the TNFα signaling pathway.” This should be mentioned in the abstract and more thoroughly developed throughout the manuscript. The analyses should be clearly framed in the context of multiple sclerosis, with further exploration of subtype diversity, signaling pathways, trajectory dynamics, and their relevance to MS pathology and progression. Importantly, trials with TNF blockers have shown that they can exacerbate MS and precipitate relapse.

We have revised our discussion of the shared pathways between LPC and CPZ DAOs, based on our new DEG analysis and GSEA dot plots. TNFa signaling remains enriched across all conditions, and we have added additional details to the discussion that discuss the role of TNFa signaling in MS. We appreciate the reviewer’s opinion that all analyses should be framed in the context of Multiple Sclerosis, and we do our best to discuss this, given space constraints. Our primary goal of the manuscript is to describe and compare the LPC and CPZ demyelination models to each other, which has implications for a wide range of CNS pathologies and diseases where demyelination occurs.

48. “yet whether drug targets have been more successful when aligned with the phenotypes we identified—such as P53 signaling, oxidative phosphorylation, or the unfolded protein response—remains an open question and a compelling avenue for future research” Building on point 48, this is a critical issue that should be explicitly addressed in the study to ensure it provides meaningful knowledge advancement. The manuscript should offer some level of guidance for readers—under what conditions or research questions should one choose the LPC model versus the cuprizone model (or others)? Or should both models always be carried out and compared? Clarifying this would not only strengthen the translational value of the work but also support more informed model selection in future MS studies.

We address this request in our response to comment #3. We have included a paragraph in the discussion section that describes our views on selecting the appropriate demyelination model based on our results.

49. “Although EAE is induced through immune modulation and is often considered more analogous to MS, we observed upregulation of multiple immune-related genes in MOLs within the toxicity models as well. For example, genes such as Klk8, S100a10, H2-D1, Serpina3n, B2m and other MHC-I genes were particularly upregulated in LPC treatment, where IFN signaling was also strongly induced. These findings indicate that OPC and OL immune responses are not exclusive to EAE-mediated demyelination.” This section feels out of place and emphasizes the important question of why the current study did not include mouse EAE data to enable a more comprehensive cross-model comparison. Incorporating EAE would strengthen the conclusions and better contextualize findings across widely used MS models. Alternatively, the authors should clearly frame the study within the context of remyelination capacity and structure the analyses accordingly. A focused investigation into remyelination dynamics—across models, cell types, and stages—would provide a more coherent narrative and strengthen the study's contribution to understanding MS pathology and repair mechanisms.

As mentioned in previous comments, we now include a comparison with the EAE model. In addition, we do highlight the similar and different dynamics of remyelination between the two models. However, as the different MS lesion types do not necessarily follow a temporal progression, we avoided analyzing the MS data within this context.

50. "Cuprizone and AL DAOs exhibit shared upregulation of stress response genes, including CDKN1A, NUPR1, and FOS." This appears to be the major finding of the current version and should be expanded upon and followed up with more detailed analyses and discussion to fully convey its significance and implications.

We have expanded our analysis and discussion of Cdkn1a and Nupr1.

51. "For example, it is not known if these OLs produce functional myelin that matures over time." The study could also be strengthened by extending the quantification of these MOL subtypes across models using FISH and immunostaining, which would provide valuable spatial-temporal context and enhance the interpretation of their distribution and dynamics in relation to disease progression and remyelination.

We have performed RNAscope for *Cdkn1a* and *Nupr1* in both mouse models at demyelination and remyelination timepoints. We only see elevation of these genes within oligodendrocytes in CPZ demyelination samples. For the human dataset, we see significant upregulation of *CDKN1a* in oligodendrocytes in CILs and *NUPR1* is elevated in CALs. Future studies examining the fate of these cells using reporter mice will hopefully shed light on their dynamics.

52. Supplementary Figure 12: Should not only be introduced in the discussion section.

We have removed this characterization of the human astrocyte subclusters. By itself it does not add significant new information into astrocyte pathology in MS over what has been shown in other studies, and due to the lack of sufficient astrocyte numbers in our mouse dataset, we cannot compare astrocytes across species.

53. "Additionally, the blood-brain barrier plays a crucial role in damage signaling, and our analysis identified strong enrichment of innate immunity genes in endothelial cells and pericytes." Citation to the data?

This statement is no longer included in the revised version.

Reviewer #3 (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.

Reviewer #4 (Remarks to the Author):

In this study, Aboelnour et al. investigated how distinct demyelination models trigger different glial responses by integrating newly generated and publicly available single-cell transcriptomic data from the subcortical white matter. They compared glial alterations in the lysophosphatidylcholine and cuprizone models to those observed in multiple sclerosis (MS), and further assessed the relevance of the mouse data by comparing it to human MS samples.

Overall, this comparative analysis highlights key biological and pathological differences in gene expression between mouse demyelination models and human MS lesions, providing a valuable foundation for the effective use of animal models in advancing remyelination therapies. The manuscript, though interesting in topic and well-executed, several aspects require clarification or improvement, as outlined below.

We thank the reviewer for their favorable comments on our manuscript.

Major concerns:

1, In Figure 2C, distinct UMAP distributions among OLs populations were evident in both the CPZ and LPC models. It is important to consider the localized nature of the LPC injection site, as transcriptomic changes in OLs are likely to exhibit spatial heterogeneity, with only cells near the lesion being significantly affected. In contrast, cuprizone induces more diffuse and widespread OL loss across multiple brain regions through dietary exposure, resulting in more uniform transcriptomic alterations across the sampled tissue. Moreover, the remyelination timelines between LPC and cuprizone models are not fully aligned, potentially confounding interpretation of convergence or divergence in oligodendrocyte recovery states. The authors are encouraged to incorporate spatial transcriptomics, proteomics, or functional imaging to more precisely contextualize molecular changes within the anatomical framework, thereby enhancing the robustness of their conclusions.

We appreciate the author's concerns regarding the inherent differences in LPC- and CPZ-induced demyelination. We have removed the density plots that the reviewer is referring to in original Figure 2c, and replaced them with the MILO plots, which are more robust. We agree that the remyelination timelines between the two models are not perfectly aligned, but as they show similar patterns of cell state changes for both oligodendrocytes and microglia, we feel confident that we can make comparisons between the two. We have added additional timepoints for our *in vivo* validation experiments (2 days post LPC, 42 days post LPC, and 5 weeks after cuprizone removal), which gives us the ability to make statements more definitively. We acknowledge that other analyses, such as spatial sequencing, proteomics, and functional imaging, would provide additional levels of information, but as these are very expensive and laborious experiments, we feel that they are beyond the scope of the current study.

For example, the authors identified a population of Cdkn1a$^+$ OLs as a unique marker of cuprizone-induced OL stress. Notably, CDKN1A was also highly enriched in the MS DAO cluster MOL_G, particularly within remyelinated lesions. To further clarify the functional role of CDKN1A$^+$ OLs, it would be beneficial to assess their association with markers of intact versus degraded myelin, which could help determine whether these cells contribute to remyelination or represent a dysfunctional or stalled repair state.

We find that *CDKN1A* is significantly upregulated in oligodendrocytes within chronic inactive lesions (CILs). As we mention in our above responses to comments, the functional role of CDKN1A+ oligodendrocytes is an outstanding question, and we discuss some possibilities within the discussion section. Based on their presence solely within the CPZ demyelination samples, we hypothesize that they represent a dysfunctional state. However, without a Cdkn1a-reporter mouse strain, we cannot assess if they contribute to remyelination.

2, Although the white matter is the primary site of demyelination in both mouse models and human MS, the manuscript would be strengthened by discussing whether similar transcriptomic alterations occur in the grey matter in response to CPZ and LPC treatments. Previous single-nucleus RNA-seq studies of both the cortex and corpus callosum (PMID: 40131948; PMID: 36952346) have comprehensively characterized these regions and identified comparable stress-associated oligodendrocyte states during demyelination. A parallel analysis of grey matter lesions in human MS would also provide valuable insights and further support the relevance of these findings across brain regions. Additionally, the study does not explore potential sex- or age-related differences, which may significantly influence demyelination and remyelination dynamics and affect the translational applicability of the findings.

We agree with the reviewer that a comparison between white and gray matter in response to CPZ and LPC demyelination would be interesting. However, to our knowledge there are no scRNAseq datasets of LPC-induced demyelination of the cortex. In the CPZ studies that the reviewer lists above, the authors combined gray and white matter for their nuclei isolation and snRNAseq, so these different brain regions can not be separated within this dataset. We purposefully chose not to use this dataset as we wanted to perform a direct comparison between the two toxicity models, focused on the same brain region. This is why we also took the extra step of separating out only the white matter samples from the published human MS dataset.

We have now included some plots (Supplementary Figure 2) that show the age distribution of the human dataset, and include a statistical test to show there is not a significant difference between control and MS patients. We were not seeking to make translational statements about MS in sex and age related differences, which is why we tested for whether we need to control for these variables in analyses such as gene expression. The original paper using this data (Macnair et al.) focused on the clinical features of the dataset, and found that patients stratify into a few subgroups of glial-gene expression changes, suggesting particular treatments may be best suited to some patients but not others.

3, MS is characterized by a pronounced peripheral immune component, with significant contributions from T and B lymphocytes. However, these immune cell populations are largely absent or minimally represented in the toxicity-induced mouse models utilized in this study, limiting the capacity of these models to fully recapitulate the immunopathological complexity of MS. The authors are encouraged to explore the integration of immune-mediated models, such as EAE, or to adopt hybrid approaches that combine toxicity paradigms with peripheral immune cell infiltration strategies. At a minimum, these limitations should be explicitly acknowledged and discussed as potential confounding factors in the interpretation of results.

We have now provided a new supplemental figure that compares gene expression changes in oligodendrocytes in the EAE model with those we see in the LPC and CPZ models. Interestingly, we see many DEGs are shared across the three models, indicating that some of the changes within the EAE model may be due to demyelination-induced inflammation, rather than peripheral immune cell infiltration. We have added discussion of this to the discussion section and also mention the limitations of these toxicity models in modeling MS.

4, In Figure 4, the authors demonstrate that microglial responses to demyelination are highly conserved across both mouse models, with sustained microglial activation from the demyelination phase through to remyelination. It would be of interest to further explore the overlapping and model-specific microglial DEGs between demyelination and remyelination phases. Including Venn diagrams to visualize shared and distinct DEGs across these conditions would provide valuable insight into the temporal dynamics of microglial activation. In addition, the UMAP for the WM condition should be included in Figure S9F to allow for direct comparison with lesion types. Similarly, including the WM UMAP in Figure S12 would enhance the interpretation of astrocyte heterogeneity across different conditions.

We have provided new Venn diagrams that show the overlap in DEGs between LPC and CPZ microglia. We highlight some of the genes that are shared and model-specific (Figure 5h), and also provide supplemental lists of the DEGs as there are too many to discuss. Our new analysis of the microglial response to demyelination highlights interesting differences between LPC demyelination and remyelination stages – for example, microglia in LPC remyelination are enriched for genes related to complement, hypoxia/glycolysis, and inflammation.

We appreciate the difficulty in remembering cell type specific UMAPs and have tried to include these analyses within the same figure as much as possible.

Aboelnour et al. — Response to Reviewers, 2nd resubmission

We sincerely thank the reviewers for their careful evaluation and constructive feedback. Their comments have substantially strengthened the manuscript. We have incorporated additional analyses and revised the text throughout for clarity and precision. The revised manuscript now provides a more comprehensive and rigorously validated comparison of oligodendrocyte lineage cells and microglia across lysolecithin (LPC) and cuprizone (CPZ) models of demyelination, as well as across distinct multiple sclerosis (MS) lesion types.

Newly added analyses:

- Cross-comparison of CPZ- and LPC-induced microglial states with EAE microglia
- Visualization of the spatial distribution of disease-associated oligodendrocytes (DAOs)
- Expanded quality control for individual dataset integration for single nuclei/cell cohorts
- Quantification of demyelination time-point ApoE$^+$ microglia

New figures:

- Supplementary Figure 5 – Spatial distribution of LPC- and CPZ-induced DAOs
- Supplementary Figure 12 – Quality control of dataset integration
- Supplementary Figure 13 – Differential expression analysis of CPZ and LPC microglia/PVMs with comparison to EAE

Revised figures

- Figure 4 – Expanded cross-species gene examples and updated gene scores
- Figure 5 – Added quantification of ApoE$^+$ microglia at 7dpl LPC and 5 weeks CPZ
- Figure 6 – Expanded cross-species gene examples and updated gene scores
- Supplementary Figure 7 – added MOL_F and MOL_G counts per sample
- Supplementary Figure 8 – Added gene score heatmap, removed Spearman correlation
- Supplementary Figure 11 – Moved microglia gene expression analyses
- Supplementary Figure 15 – Added gene score heatmap, removed Spearman correlation

Below, we provide a detailed, point-by-point response (blue text) to each reviewer comment.

Reviewer #1 (Remarks to the Author)

General critique:
The manuscript by Aboelnour et al. has substantially improved by, for example, adding new schematics illustrating dataset generation, which enhances transparency and help understand the underlying data structure. Major concerns raised in the initial review have been addressed. In particular, the differential expression analysis strategy has been revised, and interspecies comparisons have been removed. These changes, together with the inclusion of the EAE comparison, have considerably strengthened the interpretability and overall impact of the study.

We thank the reviewer for their positive assessment of the revised manuscript and for acknowledging the improvements made, including the addition of new schematics, revision of the differential expression strategy, removal of interspecies comparisons, and inclusion of the EAE comparison. We appreciate the constructive feedback that has helped strengthen the study, enabling us to compare the commonly used toxicity models of demyelination with each other, and with MS patient samples at the transcriptional level in white matter.

Specific comments:
1. We appreciate the addition of modality and dataset annotations in figures such as Supplementary Fig. 4a. Nevertheless, we still encourage the authors to present the newly generated datasets as separate UMAPs for LPC and CPZ. This would allow readers to more clearly evaluate whether the observed MOL shifts and other patterns arise from genuine biological differences or from dataset-specific characteristics.

We thank the reviewer for this helpful suggestion. In response, we have now included an additional supplementary figure presenting both the single-nuclei and single-cell datasets integrated independently, for completeness. In Supplementary Figure 12 we provide UMAPs for each full dataset, as well as OL and microglia-specific UMAPs, alongside proportions of cell types across the timepoints included in each dataset. These plots confirm the expected patterns, including expansion of DAO populations (MOL_E–G) during demyelination and remyelination, microglial proliferation under demyelination, and perivascular macrophage infiltration specifically in LPC. We find that the dataset-specific analysis supports the conclusions drawn from our integrated analysis. Presenting the datasets independently increases transparency and allows readers to better distinguish genuine biological differences from potential dataset-specific effects.

2. In their response, the authors note: “Therefore, we see no problems in making inter-model comparisons, as they are controlled by their own datasets’ controls, which should account for technical and experimental-specific artifacts.” Since the Pandey et al. dataset does not include its own controls, this limitation persists. While we recognize that this cannot be fully resolved within the scope of the current study, we recommend explicitly acknowledging this point in the manuscript.

We appreciate the reviewer raising this important point. While the Pandey dataset does not include saline-injected controls, it does contain baseline (uninjected) control animals (GSE182846), which serve as internal controls within that dataset and help mitigate technical or experimental artifacts.

To ensure clarity and transparency, we have now explicitly acknowledged this point in the manuscript. In the opening paragraph of the section "Compiling single cell/nuclei transcriptomic datasets of mouse demyelination models and MS patient samples," we added the following clarification:

> *"Notably, the LPC baseline controls in the snRNA-seq datasets were saline injected, whereas in the scRNA-seq dataset they were untreated."*

We hope this addition sufficiently clarifies the distinction between control types and addresses the reviewer's concern.

Reviewer #2 (Remarks to the Author)

Review Summary
The manuscript by Aboelnour et al., entitled "A comparative transcriptomic analysis of mouse demyelination models and Multiple Sclerosis lesions," presents a cross-species analysis comparing glial diversity across disease states in two toxin-induced mouse demyelination models and in multiple sclerosis (MS) lesions. The revised version improves analytical depth, discussion, and readability, and the central message is now clearer. Some remaining point-by-point comments are listed below:

We thank the reviewer for their positive assessment of the revised manuscript and for acknowledging the improvements in analytical depth, discussion, and clarity. We address the remaining point-by-point comments below.

1. separate statistical test comparing the distribution of nhoods…
Clarify what "nhoods" represent to general readers. Also clarify "vs" in Fig. 2d if comparisons are within-condition only.

We appreciate this suggestion and have clarified the terminology in the second section "Cuprizone demyelination induces a distinct DAO state not present in LPC demyelination." We now state:

> *"In Milo, cellular states are modeled by assigning cells to partially overlapping groups of cells ('nhoods') defined on a k-nearest neighbor (KNN) graph."*

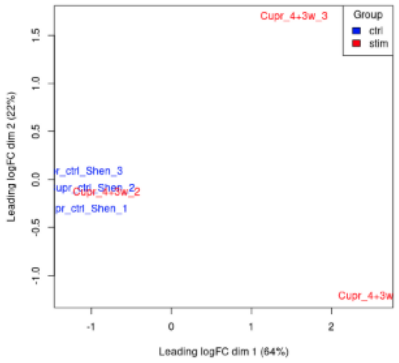
To further clarify the statistical comparisons shown in Figure 2d, we have added the following description:

"To determine whether there were differences in cellular composition between CPZ and LPC, we performed a separate statistical test comparing the distribution of nhoods by aggregating the counts of upregulated, downregulated, and non-significant nhoods by cell type and condition. Statistical testing was performed between demyelination timepoints and remyelination timepoints separately."

This clarifies that comparisons were performed within matched phases (demyelination or remyelination), rather than across conditions indiscriminately.

2. CPZ remyelination samples exhibited high heterogeneity…
State which statistical test was used, whether any DEGs remained significant, and provide the original DEG table.

Differential gene expression analysis was performed using voom with quality weights within the limma/edgeR framework on pseudobulk counts derived from the total mature oligodendrocyte population.



The multidimensional scaling (MDS) plot demonstrates that CPZ remyelination samples (shown in red) do not cluster along the primary axis of variation. This marked biological heterogeneity within the CPZ remyelination group violates the assumption of within-group homogeneity required for standard differential expression testing.

Although we proceeded with standard DEG analysis, the results were largely non-significant, as expected under these conditions. Specifically, only two genes met the adjusted significance threshold (padj < 0.05), while all other genes were non-significant. Given the instability introduced by within-group heterogeneity, we interpreted these results with caution.

For full transparency, we have now included the complete DEG output table as Supplementary Table 4.

3. gene set enrichment analysis (GSEA) to determine what signaling pathways…
Clarify whether only upregulated genes or both directions were included in GSEA.

Thank you for this request for clarification. We have now explicitly stated that GSEA was performed using a ranked gene list including all genes tested in the differential expression analysis, not only upregulated genes:

"We then performed gene set enrichment analysis (GSEA) using a ranking metric on all genes tested in the DEG analysis to determine what signaling pathways are enriched in MOLs across demyelination (Figure 2e)."

This clarifies that enrichment reflects coordinated shifts across the full ranked dataset.

4. supported by shared upregulation of genes Gadd45a/b…
This should be an internal consistency check rather than independent support.

We agree with this point and have revised the wording accordingly:

> *"TNFα signaling via NFκB and interferon (IFN) signaling were the primary pathways enriched across all conditions, with shared upregulation of genes Gadd45a/b, Stat1/3, B2m, H2-D1, and Socs3."*

This phrasing reflects these genes as representative components of the enriched pathways rather than independent lines of evidence.

5. performed RNAscope
Clarify the ROI definition and quantification pipeline in the method and also in the caption, how many animals, sections, images, and cells were quantified.

We thank the reviewer for highlighting this need for clarity. We have now added a detailed definition of the ROI and quantification workflow to the Methods section, including the number of animals, sections, and cells analyzed:

> *"Sections were imaged using the Leica Stellaris 8 DIVE confocal microscope and at least 3 sections per animal were analyzed by FIJI for each stain. Positive cells for each probe were determined by the presence of at least two fluorescent puncta within the Olig2+ DAPI+ nucleus, which represented expression above background levels, as determined using baseline controls. Olig2^{+} cells positive for Pdgfra were counted as OPCs, and Olig2^{+} cells negative for Pdgfra were counted as MOLs. Approximately 400-900 cells were counted per animal, with 3-6 animals analyzed per marker combination. The exact number of animals for each experiment is reported in the figure legends."*

6. B2m encodes the beta-2 microglobulin…
For Fig. 3c, clarify whether nuclear-proximal puncta or diffuse expression is expected, how the background is determined and subsequently corrected?

We thank the reviewer for the opportunity to clarify this point. B2m is highly expressed in microglia, and as a result, the diffuse or background signal likely reflects B2m expression in surrounding microglia rather than nonspecific staining. In contrast, Socs3 is expressed at much lower levels in other cell types, which accounts for the comparatively minimal background signal observed for that probe. We did not perform background correction for our RNAscope quantification; instead, we applied stringent cell type-specific counting criteria. In the MOL analysis shown in Figure 3, we restricted quantification to Olig2^{+} Pdgfra^{-} cells and counted only

clearly defined B2m puncta that were localized within Olig2$^+$ DAPI$^+$ nuclei corresponding to these cells. Diffuse staining outside of Olig2$^+$ nuclei was excluded from analysis. Importantly, all images were acquired as confocal z-stacks, allowing us to determine whether B2m puncta were localized within MOL or OPC nuclei versus neighboring DAPI$^+$ cells. This approach enabled us to distinguish true cell-associated signals from surrounding high-expressing microglia. We have clarified these methodological details in the revised Methods section, as described in the above comment.

7. the percentages gradually increased from CPZ demyelination (5 wks) to remyelination (5+3 wks)…

Include statistical testing between CPZ_5wks and CPZ_5+3wks, and explain the absence or presence of significance at baseline → 5wks.

We have now reported on these statistical comparisons in the text, under section “At remyelination stages, LPC and cuprizone converge onto a shared DAO state that upregulates immune-related genes”:

> *While B2m$^+$ OLs did not significantly differ between CPZ_5wks and CPZ_5+3wks ($p = 0.08$), these trends suggest progressive emergence of immune-associated OL programs during recovery. The comparison between baseline and CPZ_5wks approached significance, consistent with partial remyelination initiating during prolonged CPZ exposure.*

8. altered maturation state following demyelination

Clarify what analysis specifically supports this. If PAGA is used, address whether transitions between DAO states must pass through intermediate MOLs. Have you compared the PAGA results with and without E_DAO, F_DAO, or G_DAO? When these states are analyzed together, there appears to be direct connectivity from E to G; does this imply that the developmental trajectory could transition directly between these DAO states rather than passing through B and C?

We appreciate this thoughtful question. PAGA models the single-cell k-nearest neighbor graph as a cluster-level graph, where edge weights reflect the statistical strength of connectivity between clusters and the inferred likelihood of transitions between cell states. In the PAGA analysis we include all cell states, and we do not observe connectivity between E_DAO and G_DAO but we do observe connectivity between E_DAO and F_DAO. We have not repeated the PAGA analysis excluding DAO states, as this would require a new dataset integration.

We used PAGA primarily to demonstrate that the established differentiation trajectory from progenitors to mature oligodendrocytes is preserved in our dataset and to assess relationships among MOL populations. However, without lineage tracing or functional experiments, it remains difficult to determine whether the inferred connectivity represents true dynamic transitions. This hypothesis motivated the experimental validation in Figure 3. We have clarified this interpretation in the revised manuscript with the following:

Because DAO populations are absent under homeostatic conditions, we reasoned that these states arise either from stressed mature OLs or from newly generated OLs adopting a DAO-like transcriptional program. Supporting the latter possibility, MFOLs displayed direct connectivity to MOL_E in PAGA analysis (Figure 2a), suggesting that demyelination alters OL maturation trajectories rather than inducing a terminally damaged fate.

9. but dropped significantly at 5 weeks removal from CPZ…

Indicate whether LPC eventually reaches similar levels to CPZ_5+5wks, and what the baseline (EdU-only) population looks like.

We agree with the reviewer that this is an interesting question. The prolonged persistence of Socs3+ MOLs in the LPC remyelination timepoints may reflect sustained inflammatory signaling in the LPC lesion environment, consistent with the continued presence of $Cd11b^{+}$/$Apoe^{+}$ microglia (Figure 5f), which is less prominent in CPZ at comparable stages. While we agree that it would be interesting to determine if LPC eventually reaches similar levels of Socs3+ MOLs as CPZ, we feel that determining this timeline is beyond the scope of our current study. The main point of Figure 3 is that both LPC and CPZ demyelination induce a similar DAO state at remyelination stages. The majority of published studies that use LPC as a demyelination model only use 21-28 dpl as their latest recovery timepoint. We have gone beyond these studies already by including a 42 dpl timepoint.

The baseline EdU-only population is low for the majority of the conditions and timepoints (approximately 10-20% of EdU+ cells did not express Olig2). We do see a higher percentage of EdU-only cells in the 28dpi LPC samples (~50%), which most likely correspond to proliferating microglia. Interestingly, the 42dpi LPC samples had only ~20% EdU-only cells, suggesting that this population of proliferating microglia was lost due to apoptosis or other mechanisms.

We have expanded the discussion to note that the mechanisms governing DAO resolution, the role of cell–cell crosstalk, whether DAO states contribute functionally to remyelination, and the precise timing of their resolution in LPC remain important open questions and represent key future directions.

10. Human MOL_A–D description
It will be helpful to apply the same concise style used for mouse MOLs for clarity and symmetry.

We agree, and have revised the human MOL state descriptions to match the concise style used for mouse MOLs. The text now reads:

> *MOL states are characterized by cluster-specific markers (MOL_A: $RBFOX1^{high}$ and $SCL5A11^{high}$; MOL_B: expresses MOL_A markers, $CTNND2^{low}$ and $PLXDC2^{low}$; MOL_C: loses RBFOX1 and SLC5A11, expresses $CTNND2^{high}$ and $PLXDC2^{high}$ and gains OPALIN and LAMA2 expression; and MOL_D: similar to MOL_C but increased FTL^{high}, $CSPRP1^{high}$).*

11. additional connectivity between the DAO states

Clarify whether cluster G connects to E in Supplementary Fig. 6d and whether MFOL link to B is direct or routed through E_DAO in Fig. 2a.

Thank you for requesting clarification. In the PAGA analysis for human OLs (now Supplementary Figure 8d), there is no connectivity between E_DAO and G_DAO. We observe connectivity between E_DAO and F_DAO.

Additionally, there is no direct link between MFOL and MOL_B. The inferred connectivity suggests a transition from MFOL → MOL_A → MOL_B, rather than a direct MFOL → MOL_B route. We have clarified these relationships in the revised manuscript to avoid any ambiguity in the interpretation of cluster connectivity.

12. Figure 4e
Consider visually marking triple-positive DEGs (MS+LPC+CPZ) directly on the bar graph.

Thank you for this helpful suggestion. In Figure 4e, we now visually mark DEGs shared between MS lesions and both LPC and CPZ as hashed bars, allowing direct visualization of the triple-overlap within the bar graph. We agree that this improves clarity and strengthens interpretation of cross-model consistency.

13. Supplementary Figure 7c
This is a compelling finding and might merit inclusion in the main figure. human_MOL_F appears distinct: it aligns most closely with OPCs and MFOL across species, shows similarity to human_MOL_G, yet lacks the pattern seen in younger OL clusters. It is also the predominant population in MS remyelinated lesions (Fig. 4c). What differentiates MOL_F from MOL_G beyond NEDD4L and FGFR1? What drives their cross-species similarity? Aside from GPC5, are there additional genes uniquely marking MOL_G relative to MOL_F?

We appreciate the reviewer highlighting this important observation. Human MOL_F_DAO shares transcriptional similarities with progenitor populations, as well as strong similarity with human MOL_G_DAO and modest similarity with mouse DAO MOL_E and MOL_G. Notably, mouse MOL_G_DAO (the CPZ-associated DAO population) also shows similarity to mouse progenitor populations, which may contribute to the observed cross-species alignment.

A focused DEG comparison between human MOL_F and MOL_G reveals additional distinguishing features. Beyond NEDD4L and FGFR1 (enriched in MOL_F) and GPC5 (enriched in MOL_G), MOL_F shows higher expression of genes including CHL1, S100A10, CARMIL1, TNFRSF10B, DLGAP1, and PLCB1. In contrast, MOL_G largely shares DEGs with MOL_F but more prominently upregulates genes such as GBP2, EGR1, ANXA2, and SERPINE1.

Interestingly, several genes enriched in MOL_F are associated with stress or apoptotic signaling, which may relate to its comparatively low abundance in the dataset (mean ~33 cells per sample across 92 samples, compared to ~245 for MOL_G). Although Figure 4c shows strong enrichment of MOL_F neighborhoods in remyelinated lesions, MOL_G remains the predominant DAO-like population numerically across lesion types.

To clarify distribution across samples, we have now added box plots of cell counts per sample in Supplementary Figure 7e–f, providing improved transparency regarding relative abundance and variability.

We agree that these populations warrant further mechanistic investigation, though this is outside the current scope of this study, and note this in the Discussion as an important future direction.

14. Supplementary Fig. 7e
Which MOLs were combined in each condition?
This figure is confusing; consider separating upregulated and downregulated genes into two plots. The LPC_remyel downregulated profile is highly correlated with the LPC_demyel upregulated profile, which may reduce clarity.

We agree that the original plots were potentially confusing and did not substantially strengthen the main conclusions. To improve clarity and focus, we have removed these plots. Instead, we now present heatmaps summarizing gene score comparisons, which more clearly illustrate cross-condition relationships. We believe this revision strengthens interpretability and streamlines the narrative.

15. while MOL_F and MOL_G are marked by CDKN1A…
This is incorrect, these genes mark MOL_F only.

Thank you for catching this, we have corrected the text for accuracy. The revised sentence now reads: *“Specifically, human MOL_E expresses high levels of HSPH1, FOS, and JUN, while MOL_F, and to a lesser extent MOL_G, are marked by CDKN1A, NEDD4L, and GADD45B expression, and MOL_G lowly expresses GPC5 and CD63.”*

16. Figure 4f
Rather than using a feature plot, it would be more informative to present the differential loading of these signatures across human MOL subclusters in a dot plot or heatmap. This would better illustrate their similarity to human DAOs rather than homeostatic MOLs. For clarity, plot human subclusters on the x-axis and mouse subclusters on the y-axis, similar to Supplementary Fig. 7c.

We agree that a matrix-style visualization improves cross-species interpretability. We have now included heatmaps displaying gene signature scores across subclusters, provided in Supplementary Figures 9e and 15e. We believe this revision more clearly illustrates the similarity between human DAO-like MOLs and mouse DAO populations relative to homeostatic MOL states.

17. gene signatures (page11)
Clarify how these signatures were constructed (DEGs? modules? curated lists?).

For MOL signatures, genes were selected from differential expression outputs. Specifically, we retained genes that were upregulated ≥4-fold in the condition of interest, then visually assessed

their specificity and expression level on UMAPs across timepoints/treatments. Genes were retained only if they had a human orthologue that was expressed in our dataset.

For microglial signatures, we began with genes from the Hallmark Hypoxia and Inflammatory Response gene sets and retained only those with both human and mouse orthologues. We then filtered for genes upregulated ≥2-fold in at least one human lesion condition (AL, CAL, CIL, or RL) or in mouse DAM populations.

To improve transparency, we have: (1) added a detailed description of gene selection criteria in the Methods, (2) included an additional tab in the Supplementary DEG tables listing the gene scores (logFC and adjusted p-values) across treatment conditions. We hope this clarifies the construction process.

18. Supplementary Figure 7f
Clarify which pairwise comparisons produced the significant differences.

Each lesion type was compared directly to white matter controls to calculate DEGs. The asterisks reflect adjusted p-values derived from differential expression testing, using a logFC threshold ≥1.5-fold. We have clarified this explicitly in the figure legend.

19. Figure 5f
Add 7dpl and CPZ_5 to the ApoE group, and 42dpl/CPZ_5+5 to the Cd206 group for completeness.

We have added the early timepoints (7 dpl LPC and 5wk CPZ) to the ApoE group for completeness. Importantly, this analysis revealed that a higher percentage of microglia expressed Apoe following LPC demyelination, compared to CPZ demyelination. This supports our overall conclusion that LPC demyelination induces a more robust DAM response than CPZ. The scRNAseq data do not support the presence of perivascular macrophages (PVMs) at the late timepoints; therefore, we do not believe it is necessary to analyze the late recovery timepoints for Cd206+ cells.

20. Figure 5b

Include subcluster-enriched genes (as in Fig. 6b) to highlight differential loading in Mg_A, Mg_D, and PVMs.

We have revised the dot plot to separate shared activated genes, DAM-enriched genes, and representative PVM-enriched genes. We note, however, that relatively few genes are discretely expressed in only one cluster. Instead, differences are largely driven by expression level and the proportion of expressing cells, rather than strictly binary cluster specificity. The revised visualization better reflects this biology.

21. Together, these findings suggest that MS induces greater cellular heterogeneity…

Hard to conclude from the current text alone. A matrix plot similar to Supplementary Fig. 7c would help. Consider plotting genes defining human microglia clusters onto the corresponding mouse clusters to better visualize this comparison.

The requested matrix-style comparison is included as Supplementary Figure 15C (previously Supplementary Figure 12), directly comparing cell types across species. We believe this strengthens the basis for the statement.

22. Supplementary Figure 12d
Same recommendation as for the OL portion.

We have expanded the gene panels to include additional representative genes to improve clarity and symmetry with the OL analysis.

23. Supplementary Figure 12c
Consider moving this panel to the main figure; it conveys more information than Fig. 6f.

While we appreciate this suggestion, we have chosen to retain the MetaNeighbor plots in the Supplementary material, as they serve as supportive validation rather than primary evidence. Instead, we focus on the DEG analysis overlaps and shared signatures in the main figures, which provide specific genes to focus on. We have decided to prioritize gene score UMAPs for the main figures, which we find more intuitive and directly interpretable.

24. overall DEG overlap and gene signature patterns support…

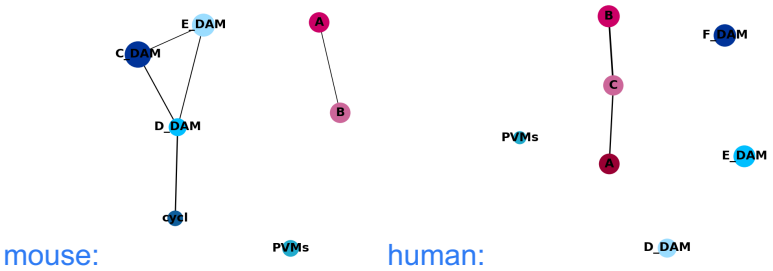
Consider inverting the analysis to show how human signatures load onto mouse clusters. Can you apply PAGA to examine the relationships between human_F_DAM, D_DAM, and E_DAM, to identify potential transition states between these clusters?

We agree that alternative analytical framings may provide complementary perspectives on the cross-species relationships. To address this point, we explored additional approaches, including projection of human gene signatures onto mouse microglial clusters as well as application of PAGA to examine potential connectivity among microglial states.

For the PAGA analysis, we applied the method separately to mouse and human microglia, using a threshold set to the lowest value that avoided artifactual connectivity between microglia and macrophage populations. Under these conditions, we observed limited connectivity primarily between homeostatic and DAM populations. However, we did not detect robust or directional relationships among the human Mg_F, Mg_D, and Mg_E DAM states that would suggest clear transition trajectories (outputs shared below).

Importantly, these alternative analyses were consistent with the transcriptional overlap and gene signature comparisons already presented and did not materially alter the interpretation of the data. Specifically, they support the conclusion that while a conserved DAM core is evident across species, human MS microglia exhibit greater diversification rather than a simple linear

progression between defined DAM states. Therefore, we believe the current presentation captures the principal cross-species relationships in a clear and mechanistically informative manner. We have added clarifying text in the revised manuscript to acknowledge these additional analyses.



25. were most strongly enriched in the human Mg_F DAM…
If human Mg_F represents “repairing microglia,” could this explain why remyelination is more robust in mice, with Mg_F-like microglia driving the process? In this context, could we argue that mouse microglia exhibit greater diversity rather than less, given that the same human Mg_F maps onto three subclusters in mouse?

This is an interesting perspective. Human_Mg_F_DAMs show similarity to PVM-like populations, while human and mouse Mg_E states are more closely aligned. However, we do not observe strong evidence to conclude that the mouse clusters are very diverse. Differences across the mouse models appear more related to timing and large logFC in DEGs than fundamentally greater state diversity. We have therefore avoided making broader claims regarding heterogeneity.

26. CPZ DAO state exhibits greater DEG overlap with DAOs in EAE…
Extend this comparison to microglia: compare CPZ and LPC microglia with EAE DAM states.

We have now included this comparison in a new Supplementary Figure 13, incorporating brain microglia scRNAseq data from an EAE study (*Sinner et al., 2023; DOI: 10.1038/s41467-023-40370-2*). This analysis extends the cross-model comparison to DAM populations and complements the oligodendrocyte findings.

27. As OPCs and DAMs in the mouse toxicity models also share similar signaling pathways…
Add citations to the specific datasets referenced.

We agree that this statement requires clarification. Upon review, we found that the GO term overlap was not sufficiently strong to justify the claim as written. We have therefore revised the text to avoid overstating this overlap.

28. but both OPCs and DAMs exhibit wide heterogeneity
Provide references for this statement.

We now explicitly reference: Supplementary Figure 10b–d for human OPC heterogeneity (distinct pathway enrichment and low DEG overlap), and Figure 6d (distinct pathway enrichment) and Supplementary Figure 14b for DAM heterogeneity (low DEG overlap across lesion types). These figures are now cited directly in the text.

29. However, our data suggests that there is a diversity of OPC states…
Point to the specific figure(s) that demonstrate this.

We have clarified that diversity of human OPC states is supported by Supplementary Figure 10, specifically: (b) distinct pathway enrichment, (c) different DEGs across conditions,(d) low overlap of DEGs. These references are now explicitly included in the manuscript.

Reviewer #1 additional comments on the response to Reviewer #4's major concern 1:

I appreciate the authors' response but do not think this point has been fully addressed. Although the previously shown plot has been removed, the reliance on MILO based analyses (including the PAGA representation) remains inherently biased, as the selection of start and end points effectively imposes a supervised trajectory structure on the data. In this sense, the analysis is not fully unsupervised. Complementary approaches that are less constrained such as LIANA+ for the inference of spatial cell cell interactions would provide a more unbiased perspective.

We thank the reviewer for this thoughtful comment and the opportunity to further clarify our analytical framework. First, we respectfully note that MILO and LIANA address different biological questions and are therefore not interchangeable. MILO is specifically designed to detect differential abundance across neighborhoods in high-dimensional single-cell space, whereas LIANA infers ligand–receptor–based cell–cell communication. While both are valuable, they interrogate distinct aspects of the data.

Regarding the concern about supervision: neither MILO nor PAGA requires the specification of start or end points. MILO performs differential abundance testing across graph-defined neighborhoods constructed in an unsupervised manner. PAGA infers connectivity between clusters based on graph topology and does not impose trajectory directionality unless explicitly combined with pseudotime approaches, which we did not perform here. Thus, the analyses presented remain unsupervised with respect to trajectory specification.

We agree that complementary approaches are valuable. We did explore LIANA; however, the current implementation does not include statistical correction frameworks that account for differences between single-cell and single-nucleus datasets (analogous to edgeR/limma-style modeling in differential expression). Given the known differences between these modalities, we could not confidently interpret LIANA outputs in a statistically robust manner within the current framework.

We also considered scCODA as an alternative compositional approach. However, scCODA requires discrete cluster-level annotation and a stable reference population across conditions.

Because our populations of interest (e.g., MOL/DAO and DAM states) form continuous rather than discrete, invariant clusters, this assumption is not satisfied. For this reason, we determined that scCODA would not provide a less biased or more appropriate framework in this specific context.

I agree that large-scale spatial validation using platforms may be beyond the scope of the current manuscript. However, targeted spatial validation of a broader set of differentially expressed genes within defined spatial niches and across time points would substantially strengthen the conclusions. In particular, demonstrating both overlapping and distinct expression patterns between the LPC and CPZ models would add important spatial context.

We acknowledge the reviewer's point and thank the reviewer for their recognition that spatial transcriptomics is beyond the scope of the current manuscript.

We would like to emphasize that our current validation of *Cdkn1a, Nupr1, Socs3*, and *B2m* already represents a substantial group of DEGs that show overlapping and distinct expression patterns between LPC and CPZ models. Importantly, these markers were validated across multiple time points and in both mouse models, allowing us to assess both temporal dynamics and model-specific expression patterns. We appreciate that there could be interesting spatial components to the demyelination and remyelination responses, but we did not observe any within the subcortical white matter (described in further detail below). As the overall goal of our study was to perform a direct comparison of the LPC and CPZ models, we actively chose to restrict our analysis to the same brain region so that gene expression differences due to regional differences would be minimized.

While large-scale spatial transcriptomic profiling would indeed be informative, we believe such an approach is beyond the scope of the current study. Nevertheless, to strengthen spatial context, we have expanded the presentation of our existing data as detailed below.

Finally, while the newly added Nupr1 staining in Fig. 2j is appreciated, the immunostainings in panels h-i are shown exclusively as high-magnification zoom-ins. This makes it difficult to assess their spatial relationship to lesion versus non-lesion areas (in the LPC model) or to defined anatomical regions such as the corpus callosum (in the CPZ model). I would therefore recommend including lower-magnification overview images to better convey spatial context, ideally combined with additional markers such as a microglial marker (e.g. Iba1) and a myelin marker (e.g. Mbp).

We thank the reviewer for this helpful suggestion. To give some additional context to our data: we present the RNAscope immunostainings as high-magnification images because of the difficulty in visualizing the small RNA puncta in lower-magnification images. Importantly, several of these DEGs are not exclusively expressed by MOLs following demyelination but are also upregulated by microglia and/or astrocytes. Therefore, it is vital that we include MOL markers in these analyses and do not simply show single channel images for *Cdkn1a*, *Nupr1*, etc. as that would misrepresent the data.

To address your request, we have added a new supplementary figure (Supp Figure 5) that includes tiled images of the corpus callosum that have been stained for 1) *Cdkn1a*, Olig2, and *Pdgfra* (to identify mouse MOL_G_DAOs) and 2) *Socs3*, Olig2, *Pdgfra* (to identify mouse MOL_E_DAOs). These low-magnification images were compiled from stitched 40x images across the corpus callosum, which were then colored in FIJI to highlight the distribution of the relevant cell populations. To clearly delineate lesion versus non-lesion regions for LPC samples, and show the extent of demyelination in CPZ, we also performed antibody staining for Iba1 and Mbp to define microglial distribution and myelin integrity, respectively.

Cdkn1a^{+} MOL_G were strongly induced at 5 weeks of CPZ exposure and resolved by 5+3 weeks, localizing predominantly to the corpus callosum with minimal presence in deep grey matter and striatum (Supplementary Fig. 5a). In contrast, *Socs3*$^{+}$ MOL_E were sparse during demyelination but increased during remyelination, distributing broadly throughout the white matter. No significant medial–lateral differences in DAO abundance were detected in CPZ. In the focal LPC model, *Socs3*$^{+}$ MOL_E were confined to the lesion core, appearing at low frequency at 7 dpl and increasing by 28 dpl.

These additions now allow direct visualization of marker expression within defined lesion areas (LPC) and within the corpus callosum (CPZ), and clarify the spatial relationship between gene expression, microglial activation, and myelin status.