nature portfolio

Peer Review File

A Lactate–H4K12la–HDAC1 Epigenetic Axis Governs Microglial State Transitions During Experimental Autoimmune Encephalomyelitis in Female Mice

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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)
The present study is an interesting investigation of potential effects of lactate to reprogram microglia function in MS, a process mediated by histone lactylation. The subject is of great interest. The experiments are logical and in general seem to be well performed, although questions on small animal numbers and reproducibility need to be answered (see below).

Comments
1. The number of mice investigated for a number of important experiments seems to be very small: in Fig.2F/H n=4, in Fig.3E n=3, in Fig.4 (conditional KO mice) n=4. These numbers are too small to be able to draw solid conclusions: it is even questionable that unpaired t-tests should be performed in groups of n=3. The experiments need to be validated in sufficient numbers and repeated to show reproducibility of the results.
2. It is questionable that the effects of intracellular lactate in microglia can be mimicked by exogenous administration of lactate intraperitoneally. Intracellular and extracellular lactate have been shown to have different effects, and lactate has been shown to be immunosuppressive independently of histone lactylation. Does an administration of lactate intraperitoneally increase the lactate concentrations in CSF and brain?
3. Lactate can be also used as a fuel for energy production, could that explain downregulation of glycolysis and oxidative phosphorylation?
4. The authors use overexpression of HDAC1 in a microglial cell line as a model to demonstrate that histone lactylation is responsible for the modulation of expression of neurotrophic factors. However, HDACs are not specific for histone lactylation, they have been mostly described as deacetylases (hence the name). Did the authors check histone acetylation in their model, and how do they know that not acetylation is responsible for these effects?
5. One potential manner to discriminate whether HDAC effects depend on lactylayion or acetylation is to repeat the experiment in the presence of a strong glycolysis inhibitor: if HDAC overexpression still inhibit the neurotrophic factors even in the absence of lactate, this may be due to acetylation.
6. There are many CNS infections and inflammatory processes characterized by high lactate. Can the authors speculate whether similar effects as described here are to be expected in those diseases?

Reviewer #2

(Remarks to the Author)
In the manuscript by Jiahong Li, Su Meng, Jiajian Li et al, the team uses the EAE model of multiple sclerosis to address a potential role of lactate metabolism and histone lactylation in neuroinflammation. Though the topic is of relevance and interest, unfortunately, due to several fundamental flaws in the approach and analyses I cannot recommend publishing the manuscript

The main issues are
1. The grouped together analyses of myeloid cells, that is presented as though the data reflect changes in microglia. The staining of IBA1 (as for example presented in fig 2) and quantification of other proteins/modifications within IBA1 positive

cells in this case does not necessarily reflect a change in microglia. In peak EAE there is huge number of infiltrating cells from the blood, of which many also express IBA1. Therefore all the differences reported most likely reflect a difference in cell composition, rather than a difference in microglia. This is also a problem for all analyses performed on samples of isolated 'microglia' where CD11B beads were used. As is also demonstrated in supplemental figure S3, in the different stages of EAE (and control) different populations will be captured by this approach and the outcomes do not allow to allocate these effects to microglia.

2. The mouse experiments presented in figure 4, related to the conditional knockout of Ldha. These experiments lack the appropriate controls. The control that is used, controls for the presence of floxed Ldha alleles and the treatment with tamoxifen. What should have also been controlled for is 1. The activation of Cre by tamoxifen, since Cre is not 100% specific and it's activation will result in DNA damage in unintended areas of the genome. And 2. The effect of lacking CX3CR1. In this model CreERT2 expression is controlled by the Cx3cr1 promoter, rendering the Cx3cr1 gene damaged, so in this case the homozygous (I expect the animals are homozygous based on the diagram in figure 4a and the genotyping data in the supplement, but as indicated below, many experimental details are not clear) animals are in fact deficient for CX3CR1 expression. Previous work has demonstrated that in EAE there is an effect of deletion of Cx3cr1 on EAE progression corresponding to the effect observed here (eg PMIDs 23817416 and 30617416). To address these issues the experiment needs to be repeated with as a minimum a 3rd group that is homozygous Cx3cr1:CreERT2 animals that were treated with tamoxifen

3. Many experimental details are lacking in the figures, results and in particular the methods sections, which is not providing sufficient detail to interpret or in future repeat the experiments. (For example (but list is not exhausitive). This makes it difficult to assess the quality and validity of the work
- For the IIDD patients (particular MS) whose csf was used to measure lactate levels: please provide in the table their disease activity status. Was there ongoing demyelination and inflammation in the CNS at the time of sampling?
- Where in the spinal cord are the images taken, inside a lesion? Outside? At what level? Thoracic? Lumbar? Is the whole spinal cord used to isolate cells?
- the method used to generate the scRNAseq data are not provided, was it 10x genomics, something else?
- the methods used to analyze the scRNAseq data are very poorly described, were doublets removed, was ambient RNA correction performed? These steps are critical for obtaining a high quality dataset, but they are not indicated
- what were the parameters used for clustering analysis of the scRNAseq data? What do the clustering dendrogram and elbow plots look like that were used to make the decision to only generate 2 clusters?
- for all mice used in the manuscript: provide the clinical EAE score and day after immunization at the time of their termination

4. The analysis and presentation of the single cell RNAseq data does not meet the standards of the field. Any basic overview of the QC of the data should be presented in supplement (eg RNA counts, mito, scrublet score). The fact that the UMAP show many 'strings', the overall lack of clear specificity in expression of cell type markers (see dotplot in figure s2) and eg. the closeness of 'neurons' to the microglia (while the macrophages who should be more similar in expression pattern are far away) suggesting that there are still many doublets in the data, or that the data is not clean and contains high levels of ambient RNA.
If the whole spinal cord was homogenized, where are the neurons and oligodendrocytes? I appreciate that there is an influx of immune cells, but I expected more of the CNS resident cells in the total object.
Where in the UMAP (in which cells) are the relevant proteins for lactate metabolism expressed?
For the subclustering of microglia: were the CAMs/BAMs removed or included in the analysis? Why was a resolution chosen that resulted in only 2 clusters? There is a clear difference between the 2 groups, where the control animals have a group of cells on the right-side of the UMAP that should be further explored. I expect these to represent a separate cluster that warrants further investigation.
It is not helpful to make the distinction between homeostatic and activated microglia as is done. What does activated mean in this context? Are there any functional processes altered in these microglia (again here an increased clustering resolution might be helpful in identifying relevant microglia populations)?

Other points.
- The title refers to several concepts that are not (confidently) addressed in the manuscript, including trained immunity, microglia and importantly also MS. As detailed above, the authors analysed a pool of myeloid cells whose exact cellular composition was different in the different groups analysed. Trained immunity is a concept that partially describes a functional state that can only be uncoverd by a second (immune) stimulus. The experiments the authors did in my opinion do not address this aspect of trained immunity. For MS: in the manuscript the authors have studied EAE (apart from measuring lactate levels as depicted in figure 2). This is not the same as MS (if anything EAE is more of a model for MOGAD, though it is an acceptable model for several aspects of MS) and therefore that should not be in the title
- How do the levels of lactate in the csf correlate to levels in the blood? Both in patients and the animal model. This is also relevant given the confounding factor of infiltration of the peripheral immune system at the peak of EAE
- The quantification of fluorescence intensity as presented in fig 2F is not an appropriate estimation of protein levels. I recommend depicting the data similar as for astrocytes (panel 2H) and, given the caveats associated with this type of analysis, refrain from using intensity as a quantitative measure.
- At the end of the legend of Figure 1 the authors write 'promoting effective neuroprotection'. In the manuscript there is no evidence provided that demonstrates neuroprotection. Increased expression of a few genes does not constitute the same.
- In figure legend of 2B it is written that n=8 animals were used, the figure itself only has 5 datapoints. Where are the missing 3 samples per group?

- The bulk sequencing data is only partially depicted and should be shown as a whole. What was the number of genes detected and differentially expressed? What do the comparisons look like in a volcano plot? (or 4-way plot or something else that is a visual representation of the actual comparisons)
- Many of the western blots depicted are oversaturated, shorter exposures should be provided. As ECL was used to develop the signal, and this was used to quantify the protein levels, this is a serious issue. ECL does not result in a linear signal, so preferable 2 different exposure times are quantified to make sure any differences are presented accurately. Again, how the measurements were done is not clearly indicated in the methods.
- The reference to M1 and M2 like microglia markers should be removed from the manuscript. As the authors are aware (their reference #36) this is an outdated concept that does not contribute to our understanding of microglia function in the CNS.

Minor points:
- The authors fail in the paper to use the appropriate nomenclature that is common for gene names vs protein names from different species. Not all instances where genes are indicated are in italic and protein names should be written in all caps.
- Fig 6a indicates tSNE, while the authors have used a UMAP approach in the actual data analysis and visualization
- In figure 6, panels C/D vs E: it is confusing that the same colors mean different things in these panels. Eg blue is activated microglia in C/D, while it refers to all microglia from control in E
- Line 161 references to fig 3A, this should be 4A
- Figure S2D: the hierarchical tree is cut off on the left side and the legend representing the colors (and connected information on what is depicted: z-scores, expression values??) is missing
- The colors in figure s2c do not contribute as the gene names are already listed on the top of the figure panel

Reviewer #3

(Remarks to the Author)
Li et al. identify histone H4 lysine 12 lactylation (H4K12la) as a key mechanism to re-educate microglia and mediate neuroprotection in neuroinflammation. They performed analyses with samples from patients with multiple sclerosis (MS), functional assays with microglia cell lines, as well as in vivo EAE experiments to link inflammation-associated lactate accumulation with the suppression of pro-inflammatory microglia responses.
This is an interesting and well-written manuscript, particularly notable for its combined use of human and murine samples. The findings are novel and may have therapeutic implications for MS. However, the manuscript requires extensive revision prior to publication.

Major Criticisms:

1. The authors use the term „trained immunity“ to describe the reprogramming of microglia in response to lactate. However, most of the data is derived from the EAE model in which lactate accumulation as a response to inflammation may sustain throughout the observation period. More experiments are required to support the hypothesis that microglia are „trained“ to support neuroprotection by epigenetic reprogramming. This could involve in vitro treatment of microglia cells with L-lactate and monitoring of H4K12la over time after decontinuation of the treatment (potentially in the presence or absence of an HDAC1 inhibitor such as valproid acid). Furthermore, if their hypothesis holds true, local short-term administration of L-lactate to the CNS prior to EAE induction should be sufficient re-educate microglia to mediate neuroprotection once disease is induced.

2. Fig. 2ff: The authors provide representative images of Pan Kla and H4K12la in tissue sections. Staining of histone marks should be confined to the nucleus, but appear to also be present in the cytoplasm. The authors should provide additional data to evaluate subcellular localization (i.e. enlarged images with DAPI counterstain, immunoblot of nuclear vs. cytoplasmic fraction).

3. Fig. 3: Li et al. aimed to enrich microglia to perform proteomic and transcriptomic analyses. However, they used magentic enrichment of CD11b+ cells from the CNS. During acute EAE the CD11b+ fraction contains a large proportion of infiltrating myeloid cells. Therefore, all subsequent analyses may be biased by variations in cellular composition of the sample rather than by disease-induced changes in microglia. They should repeat these experiments using flow-cytometry enriched CD45dim CD11b+ cells.

4. Fig. 5: The authors treat animals with a dose of 500 mg/kg lactate per day. In a previous study Sanmarco et al. (ref. 20) used a dose of 125 mg/kg in the same animal model. The authors should provide data on lactate levels in the CNS after lactate supplementation and compare this to elevated lactate levels during neuroinflammation.

5. Fig. 5, related to previous comment: In this study the authors started lactate treatment during the preclinical phase (day 10 post-immunization). While with this approach effects on T cell priming may be negligible, it can profoundly effect inflammatory monocytes and other myeloid cells. Indeed the authors detect a significant reduction in neuroinflammatory cell infiltration upon lactate-treatment, but they don’t further characterize the infiltrate using cell type-specific antibodies such as CD3 or Mac-3. To assess whether lactate treatment efficiently blocks progression via reprogramming of microglia rather than influx of peripheral myeloid cells, the authors should provide data from EAE animals treated from the peak of disease onwards (approx. day 17 post-immunization).

6. Fig. 7 and 8: The authors claim that histone deacetylases, especially HDAC1, are downregulated in response to lactate treatment in vivo. One the one hand this is not visible in the heatmaps provided in this manuscript. On the other hand the

histological data provided in Fig. 7L and 7P does not allow the reader to assess how HDAC1 is regulated during the course of neuroinflammation without lactate treatment. The authors should quantify both total and microglial HDAC1 expression throughout different disease stages. Treatment of EAE animals with an HDAC1 inhibitor from the acute phase onwards and monitoring of H4K12la would further strengthen the functional link between lactate-H4K12la-HDAC1 and neuroprotection in vivo.

7. Fig. 8: The authors claim that stimulation of HMC3 cells with IFNγ and LPS can mediate H4K12la accumulation in vitro. However, the authors should provide stainings of H4K12la in HMC3 cells to prove that this is the case. They should additionally include L-lactate treatment and a more disease-relevant stimulation regimen such as IFNγ and TFNα rather than LPS stimulation.

8. Fig. 8, related to the previous comment: The authors mention that stimulation of HMC3 cells to induce H4K12la may drive expression of Gdnf and Fgf1, which they corroborate by fact that HDAC1 overexpression lowers Gdnf and Fgf1 levels while also lowering H4K12la accumulation. To support this functional link the authors should provide stainings of GDNF and FGF-1 on HMC3 cells. These experiments should be performed with the stimulation regimens mentioned above, and in the presence of absence of an HDAC1 inhibitor.

9. The statistical analysis must be revisited. The authors mostly rely on unpaired t-tests, yet, most of their data does not seem to follow a normal distribution. Therefore, they should use a Mann-Whitney U test (two experimental groups) or a Kruskal-Wallis test (three or more groups).

Minor Criticisms:

1. Fig. 2C: The line depicting the linear regression analysis in the upper left graph appears to be driven by a single data point in the upper right corner. The authors should consider performing an outlier analysis.

2. Fig. 2D: Quantification of Pan Kla normalized to an appropriate housekeeping protein alongside with the image of the immunoblot should be provided.

3. Fig.2J and I: Label should be „NeuN“ instead of „Neun“.

4. Fig. 3: It is not clear at which time point EAE samples were collected to perform LC/MS. This should be specified in the figure legend.

5. Fig. S3: In the text the authors talk about Ly6C+ activated microglia. Ly6C is a marker for bone marrow-derived myeloid cells and is not expressed on microglia. The authors should therefore revisit their data.

6. Fig. 4: Li et al. aim to limit Ldha knockout to microglia by inducing EAE two weeks after the last tamoxifen injection. This is rather short and therefore peripheral CX3CR1+ cells may still harbour reduced LDH levels. To support their hypothesis that loss of microglial Ldha mediates the observed effect, they should provide data from peripheral myeloid cells two weeks post-tamoxifen injection to show that their LDH levels are not affected.

7. Fig. 4: The authors show that Ldha icKO mice have a marked increase in neuroinflammatory cell infiltration. If their phenotype was solely explained by a loss of neuroprotective properties in microglia in response to LDH loss, infiltration by peripheral immune cells should not be modulated during the acute disease phase. The authors should discuss this finding.

8. Fig. 4D: The authors should provide individual data points rather than a simple bar plot.

9. Fig. S4B: Font sizes in the figures are too small and should be increased to improve readability.

10. Fig. 5: The authors should use a consistent color code for the groups analyzed in Fig. 5B-5L.

11. Fig. 7: When describing the phenotype of microglia in EAE, the authors should refrain from using the terms M1 and M2. Furthermore, they should include additional functional markers to characterize the microglia phenotype in addition to Arg1 and iNOS (i.e. IL-1β, MHC II, CD68, Areg).

12. Panels within each figure are not refered to in the correct order. Please adjust in the text or figures.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors responded to my comments.

Reviewer #2

(Remarks to the Author)
I appreciate the extensive efforts made by the authors to improve their manuscript and clarify the methodology and interpretation of the results.

In particular the analysis and presentation of the scRNAseq data is much improved and now allows for biological interpretation.

My only remaining point relates to the inconsistent use of terminology for CNS myeloid cells. While in the rebuttal renaming to CNS myeloid compartment' is mentioned, for example the figure title of figure 4 still refers to microglia (while IBA1 staining was used, which the authors agree that this is not specific).

Reviewer #3

(Remarks to the Author)
Remarks to the Author:
Overall, the authors took the reviewers' comments seriously and amended the revised version of their manuscript accordingly. The quality of the study substantially improved and the functional link of lactate–H4K12la–HDAC1-mediated reprogramming of microglia in neuroinflammation. However, even after the inclusion of additional experimental data questions about the scientific rigor remain and need to be addressed prior to publication.

Major concerns
1. As requested, the authors provide an immunoblot for a housekeeping protein to quantify Pan Kla expression (Fig. S1C). Yet, they did not provide the requested quantification of the Pan Kla expression in comparison to the housekeeping protein. Also, when having a closer look at the provided immunoblot image of the ß-actin bands, the membrane appears to be cropped right above and below the band. The authors should provide immunoblot images that allow the reader to judge the size of the individual bands and see potential unspecific bands.
2. The authors clarified in the text that for the initial screening experiments CNS myeloid cells rather than pure microglia populations were used (Fig. 2 for proteomics, Fig. 7 for bulk RNA-seq). They argue that their findings are further supported by their in-depth single cell RNA sequencing analysis, which allows them to specifically assess modulations in microglia (Fig. 6). While this is in general a valid approach, their single cell data lacks information about biological replicates and statistical comparisons. They use the same dataset to support their statement about the functional phenotype of microglia in response to lactate (Fig. S6), but also here do not provide any statistics. Some key findings should therefore be validated using microglia from individual bioreplicates.
3. As suggested, the authors performed additional experiments with exogenous lactate supplementation at the peak of disease (day 14 post-immunization) and claim that this treatment regimen significantly improved clinical scores. However, from Fig. 5B it becomes evident that the animals have not been stratified correctly to individual treatment arms since the dpi14 lactate group (blue) starts with a decreased disease severity prior to treatment start. Therefore, all additional readouts (AUC, LFB, etc.) are not reliable. They also argue that the immune cell infiltrate (T cells, monocytes) does not differ, but only provide frequencies rather than absolute cell counts. This part needs to be revisited.

Minor concerns
1. Fig. 2A and 7A: The schematic representation of the experimental workflow still suggests that the author analyzed microglia, when indeed CNS myeloid cells were used. Please adjust. Also, there is a type in „Magnetic labeling/separation“.
2. As suggested, the authors included an HDAC inhibitor (MS-275) to corroborate their findings and refer previous studies that identified HDAC1 as „eraser“ (ll. 227-229). However, they fail to provide a reference for this statement.
3. The authors state that they corrected the type in „NeuN“ in Figure 1H and 1I (formerly 2I and 2J). However, this typo is still present in the provided version.
4. To corroborate their findings in microglia-specific Ldha knockout mice, the authors were asked to provide supporting data to validate the restriction of their knockout to microglia. They argue that after 2 weeks all targeted peripheral cells have been replaced without providing additional data. Based on their suggested publication > 10% of peripheral Ly6C+ monocytes (from initally 40%) remain labeled with a marker fluorochrome after 2 weeks, indicating that the turnover is slower. They furthermore refer to immunhistochemistry data in Iba1+ cells. However, since in naive mice only sparse infiltrates by peripheral myeloid cells are present, this is not informative. Therefore, the authors should provide data of lactate levels in peripheral myeloid cells 2 weeks after tamoxifen injection.
5. The authors adjusted the color code for their lactate treatment EAE (now Fig. 4 and 5). However, it is still misleading that in the schematic representation they use pink and blue to mark the acute and chronic phase of the disease, but also use these colors in B-L to label their treatment groups. Please revise.
6. The authors state they have rearranged figure panels to appear in sequential order. However, in the revised version of the manuscript the supplementary figures are not refered to in a chronological order, which makes it difficult to read (i.e. Fig. S11 and S10 right after S2).
7. The discussion lacks some references for their statements in ll. 546-557.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I would like to thank the authors for their thorough and thoughtful revision of the manuscript. They have invested considerable effort in addressing all of the comments raised during review process, and the resulting revised manuscript has significantly improved. I am satisfied with the revisions and support publication in Nature Communications.

Response to Reviewers

We thank the Reviewers for their positive and thorough review of our manuscript and their insightful comments and suggestions. We have addressed all the issues raised and hope that the manuscript is now ready for publication. We proceed with a point-by-point reply (the comments of the reviewers are printed in italics).

Note on the Revised Manuscript:
To facilitate the review process, we have provided both a clean version and a tracked-changes version of the revised manuscript. In the tracked-changes version, major modifications and newly added sections are highlighted in blue text. Furthermore, we have inserted marginal comments next to these highlights to explicitly indicate which reviewer's specific comment is being addressed (e.g., [Addressed Reviewer #2 (Comment 4)]). Minor formatting and typographical corrections have been incorporated silently to maintain readability.

Reviewer #1 (Remarks to the Author):

The present study is an interesting investigation of potential effects of lactate to reprogram microglia function in MS, a process mediated by histone lactylation. The subject is of great interest. The experiments are logical and in general seem to be well performed, although questions on small animal numbers and reproducibility need to be answered (see below).

Response: We thank the reviewer for the positive and encouraging comments on our work.

Comments

1. The number of mice investigated for a number of important experiments seems to be very small: in Fig.2F/H n=4, in Fig.3E n=3, in Fig.4 (conditional KO mice) n=4. These numbers are too small to be able to draw solid conclusions: it is even questionable that unpaired t-tests should be performed in groups of n=3. The experiments need to be validated in sufficient numbers and repeated to show reproducibility of the results.

Response: We fully agree that the originally reported animal numbers in several experiments (originally Fig. 2F/H, Fig. 3E, and Fig. 4) were limited and that such small group sizes may compromise statistical power. We have repeated these experiments using independent biological replicates and explicitly mapped the updated data to the revised manuscript. Specifically, the western blot and immunofluorescence data (originally Fig. 2F/H) are now presented in Fig. 2F-G and Fig. 2H-K in the revised manuscript; the H&E staining data (originally Fig. 3E) is now in Fig. 3E-F; and the conditional knockout mice experiments (originally Fig. 4) are now detailed in Fig. 3. We increased the sample size to n=5-8 mice per group for all these experiments.

2. It is questionable that the effects of intracellular lactate in microglia can be mimicked by exogenous administration of lactate intraperitoneally. Intracellular and extracellular lactate have been shown to have different effects, and lactate has been shown to be immunosuppressive independently of histone lactylation. Does an administration of lactate intraperitoneally increase the lactate concentrations in CSF and brain?

Response: We thank the reviewer for this insightful and critical question. We fully agree that it is essential to clarify whether systemically administered lactate can effectively reach the CNS, and to distinguish its intracellular epigenetic roles from its potential extracellular immunosuppressive effects.

To directly address whether intraperitoneal (i.p.) administration effectively increases lactate concentrations within the CNS, we quantified lactate levels in the spinal cord tissues. As demonstrated in our Supplementary Figure S10A, i.p. administration of lactate (500 mg/kg) significantly elevated the overall lactate concentrations in the spinal cords of EAE mice compared to vehicle-treated EAE controls. This result provides direct evidence that exogenously supplemented lactate can successfully cross the blood-brain/spinal cord barrier and accumulate in the inflamed CNS parenchyma.

Regarding the mechanistic distinction between extracellular and intracellular lactate, we completely agree with the reviewer that extracellular lactate can exert immunosuppressive effects via receptor-mediated signaling (e.g., GPR81) or extracellular pH modulation. However, multiple lines of evidence in our current study demonstrate that exogenous lactate can also mimic intracellular lactate to directly drive histone lactylation (H4K12la) in microglia. To decouple extracellular signaling from intracellular metabolic-epigenetic regulation *in vitro*, we treated HMC3 microglia with 2-Deoxy-D-glucose (2-DG) to block endogenous glycolytic lactate production. As shown in Figure 8M, 2-DG treatment completely abrogated the inflammation-induced upregulation of H4K12la. Crucially, the addition of exogenous lactate was sufficient to effectively rescue the intracellular H4K12la levels. This biochemically proves that extracellular lactate can be internalized by microglia and directly utilized as a substrate for intracellular histone lactylation.

These *in vitro* dynamics are faithfully recapitulated *in vivo*. Our immunofluorescence analysis (Figure 4J-L) revealed that systemic lactate administration significantly increased H4K12la deposition specifically within the nuclei of $Iba1^+$ microglia in EAE lesions, indicating that the supplemented lactate enters these target cells to enhance epigenetic reprogramming. Furthermore, while extracellular lactate undoubtedly shapes the inflammatory microenvironment, our genetic model highlights the indispensable role of the intracellular pathway. In *Ldha* inducible conditional knockout (icKO) mice (Figure 3), the targeted impairment of endogenous microglial lactate production led to a profound loss of H4K12la and exacerbated EAE pathology. If neuroprotection were solely dependent on extracellular lactate acting via surface receptors, the mere loss of microglial LDHA would not have resulted in such a dramatic abrogation of the protective phenotype.

Mechanistically, as widely reported in immunometabolism literature, we propose that activated microglia upregulate monocarboxylate transporters (MCTs) during neuroinflammation, allowing them to efficiently uptake available extracellular lactate. This exogenous supplementation essentially replenishes the intracellular lactyl-CoA pool, reinforcing the intrinsic Lactate–H4K12la–HDAC1 epigenetic axis. We have carefully revised the Discussion section to explicitly acknowledge the pleiotropic (extracellular) effects of lactate, while clarifying how exogenous lactate bolsters the specific intracellular metabolic-epigenetic mechanism described in our study.

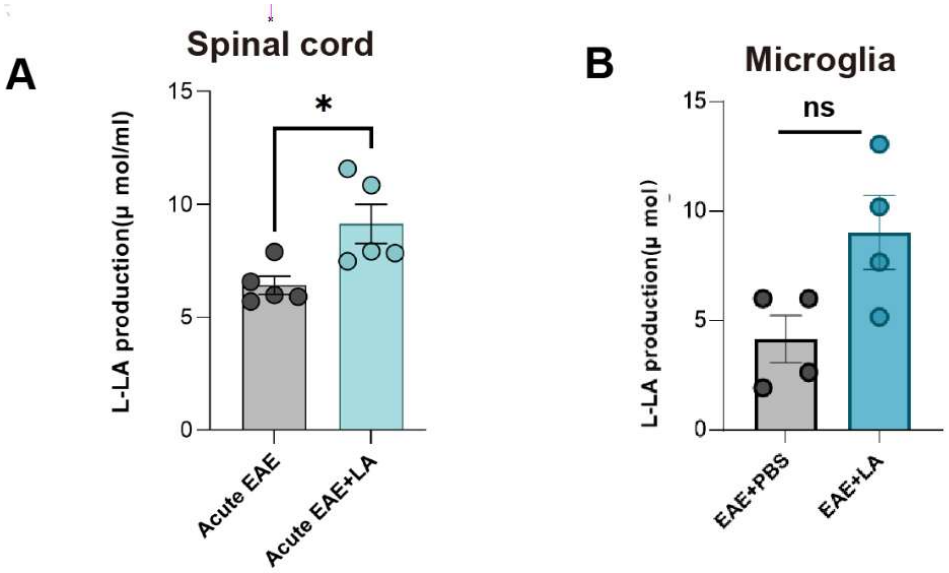


Figure10S(A-B) L-lactate concentrations in spinal cord tissue (A) and isolated $CD11b^{+}$ cells (B) following systemic administration in EAE mice.

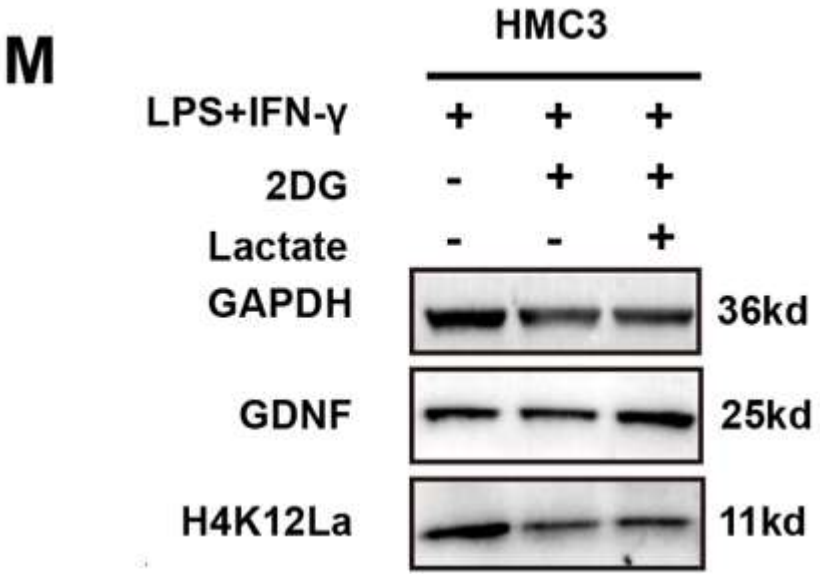


Fig. 8M Western blot demonstrating 2-DG-mediated inhibition and subsequent lactate rescue of GDNF and H4K12la expression

3. Lactate can be also used as a fuel for energy production, could that explain downregulation of glycolysis and oxidative phosphorylation?

Response: We thank the reviewer for this insightful point. We fully agree that lactate's role as an energy substrate is indeed an important factor contributing to the metabolic changes we observed in our scRNA-seq data.

When exogenous lactate is available, cells can preferentially utilize it as an efficient energy substrate [1,2]. This increased reliance on lactate-derived metabolism exerts feedback inhibition on upstream glycolysis, representing a well-recognized mechanism of metabolic flexibility [3,4]. Furthermore, as demonstrated in our scRNA-seq analysis (Fig. 6E), the global downregulation of high-flux metabolic pathways (including both glycolysis and OXPHOS) reflects a relative dampening of energy-intensive metabolic programs. This is consistent with a reduction in metabolic strain as microglia shift from a hyper-active inflammatory state to a more quiescent, tissue-supportive phenotype under lactate supplementation [5,6]. Accordingly,

this metabolic compensation effectively explains the downregulation of the metabolic genes we detected.

However, our study aims to demonstrate that lactate's role extends fundamentally beyond being merely a fuel source [7]. It acts concurrently as a critical epigenetic signaling molecule, providing the necessary precursor (Lactyl-CoA) to drive specific H4K12la modifications. The most compelling evidence that "fuel alone is insufficient" comes from our microglia-specific *Ldha* inducible conditional knockout (icKO) mouse model (Fig. 3). In these mice, we specifically blocked the enzymatic conversion of pyruvate to lactate. Despite the continuous availability of other metabolic fuels (such as glucose and glutamine) for energy metabolism *in vivo*, the loss of microglial LDHA and the resulting loss of the protective H4K12la epigenetic mark (Fig. 3K, M) led to a significant exacerbation of EAE disease severity (Fig. 3C).

This genetic evidence clearly demonstrates that the conversion of lactate to H4K12la is a rate-limiting, mechanistically indispensable step for mediating the neuroprotective phenotype. The tight coupling between molecular loss (H4K12la erasure) and phenotypic loss (EAE exacerbation) proves that metabolic compensation alone cannot recapitulate the protective effects.

Therefore, we propose a dual-role model in which the downregulation of energy pathways is indeed a consequence of metabolic compensation and reduced inflammatory strain, the core mechanism actively driving neuroprotective gene expression (*Gdnf, Fgf1*) is histone lactylation. We have expanded on this dual role (metabolic fuel vs. epigenetic signal) in the Discussion section of the revised manuscript to more clearly distinguish between these parallel processes and to emphasize the central importance of the Lactate-H4K12la-HDAC1 axis in microglial reprogramming.

Reference:

[1] Rabinowitz, J. D., & Enerbäck, S. (2020). Lactate: the ugly duckling of energy metabolism. Nature Metabolism, 2(7), 566-571.
[2] Brooks, G. A. (2018). The Science and Translation of Lactate Shuttle Theory. Cell Metabolism, 27(4), 757-785.
[3] Smith, R. L., et al. (2018). Metabolic Flexibility as an Adaptation to Energy Resources and Requirements in Health and Disease. Endocrine Reviews, 39(4), 489-517.
[4] Monsorno, K., Buckinx, A., & Paolicelli, R. C. (2022). Microglial metabolic flexibility: emerging roles for lactate. Trends in Endocrinology & Metabolism, 33(3), 186-195.
[5] Baik, S. H., et al. (2019). A Breakdown in Metabolic Reprogramming Causes Microglia Dysfunction in Alzheimer's Disease. Cell Metabolism, 30(3), 493-507.e6.
[6] Lauro, C., & Limatola, C. (2020). Metabolic Reprograming of Microglia in the Regulation of the Innate Inflammatory Response. Frontiers in Immunology, 11, 493.
[7] Ieronymaki, E., et al. (2025). Lactate: A key regulator of the immune response. Immunity.

4. The authors use overexpression of HDAC1 in a microglial cell line as a model to demonstrate that histone lactylation is responsible for the modulation of expression of neurotrophic factors. However, HDACs are not specific for histone lactylation, they have been mostly described as deacetylases (hence the name). Did the authors check histone acetylation in their model, and how do they know that not acetylation is responsible for these effects?

Response: We thank the reviewer for this insightful and technically important comment. We fully agree that HDACs, including HDAC1, have been classically characterized as histone deacetylases, and that it is therefore essential to distinguish between effects mediated by histone acetylation versus histone lactylation in our experimental system.

To directly address this concern, we performed additional experiments to functionally decouple histone acetylation from histone lactylation. Specifically, we modulated intracellular

acetyl-CoA availability using the ACLY inhibitor BMS-303141, which has been shown to markedly suppress global histone acetylation without directly targeting lactylation pathways. As shown in our Supplementary Figure S10 (D-J), treatment with BMS-303141 in LPS+IFN-γ-stimulated HMC3 microglial cells resulted in a pronounced and specific reduction of histone acetylation (H4K12ac), while histone lactylation (H4K12la) remained preserved. Importantly, under conditions in which histone acetylation was substantially abrogated but histone lactylation remained intact, the expression level of the downstream neurotrophic factor FGF1 was not diminished. This uncoupling experiment clearly indicates that the induction of this reparative program is not strictly dependent on histone acetylation.

In contrast, when HDAC1-GFP was forcefully overexpressed in these BMS-treated cells, we observed a spatially restricted depletion of H4K12la specifically within HDAC1-positive nuclei, and this targeted erasure of lactylation was accompanied by a concomitant loss of FGF1 expression (Fig. S10F, J).

These findings are consistent with emerging evidence that HDAC1 can function as a potent histone deacetylase in specific inflammatory contexts. Our results unequivocally indicate that, in our model, the HDAC1-mediated suppression of neurotrophic gene expression is predominantly linked to its deacetylase activity erasing H4K12la, rather than its classical deacetylase function. By experimentally uncoupling these two epigenetic modifications at both the metabolic (BMS-303141) and enzymatic (HDAC1 overexpression) levels, we provide robust functional evidence supporting a lactylation-dependent mechanism. We have incorporated these data into the revised manuscript (Supplementary Fig. S10) and expanded the Results and Discussion sections to explicitly acknowledge the classical role of HDACs, thereby more precisely defining the epigenetic specificity of HDAC1 in regulating microglial neurotrophic programs.

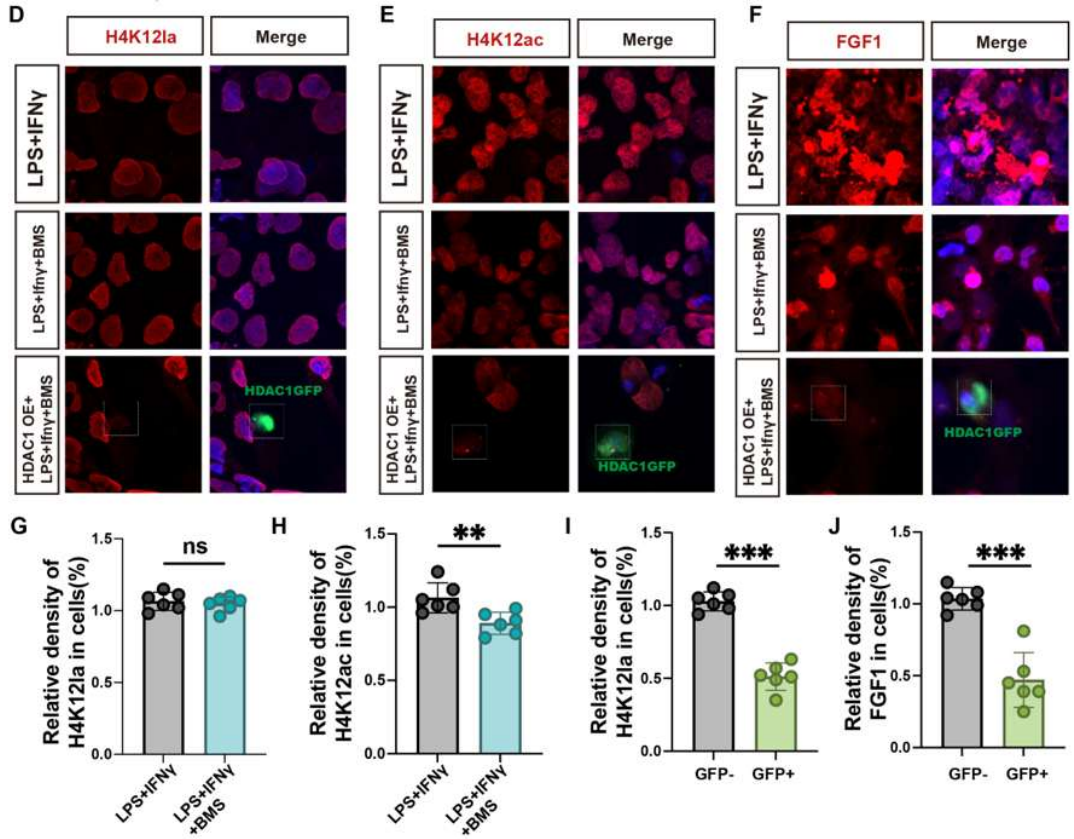


Fig. S10 Verification of lactate penetration and HDAC1-mediated delactylation

(D-F) Representative immunofluorescence images for H4K12la (D), H4K12ac (E), and FGF1 (F) in red. HMC3 cells were stimulated with LPS+IFNγ alone or in combination with the ACLY inhibitor BMS-303141. The bottom row displays HDAC1-GFP (green)

overexpression in cells pre-treated with BMS-303141.
(G-H) Quantification of relative H4K12la (G) and H4K12ac (H) density following BMS treatment (n = 6 biological replicates).
(I-J) Comparison of H4K12la (I) and FGF1 (J) signal density between GFP-negative and GFP-positive (HDAC1-overexpressing) nuclei (n = 6 biological replicates). Scale bars 50 μm. Data represent mean ± SEM. Shapiro-Wilk tests were used to assess normality. Statistical significance was determined by Mann-Whitney U test based on distribution. $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not significant.

5. One potential manner to discriminate whether HDAC effects depend on lactylayion or acetylation is to repeat the experiment in the presence of a strong glycolysis inhibitor: if HDAC overexpression still inhibit the neurotrophic factors even in the absence of lactate, this may be due to acetylation.

Response: We thank the reviewer for this insightful and technically important comment. We fully agree that it is crucial to rigorously discriminate whether HDAC1 exerts its suppressive effects primarily through histone deacetylation or delactylation.

To examine the dependency of this neurotrophic program on glycolysis and lactate, we utilized the strong glycolysis inhibitor 2-DG. As shown in Figure 8M, 2-DG treatment effectively blocked endogenous lactate production, which directly abrogated H4K12la accumulation and completely abolished the induction of GDNF. Crucially, the addition of exogenous lactate successfully rescued this 2-DG-induced suppression, confirming that lactate-driven lactylation is the essential driver of this specific neurotrophic expression.

To further and most directly address the reviewer's core question of whether the suppressive effects of HDAC1 overexpression are due to deacetylation, we implemented a targeted decoupling strategy. As shown in our Supplementary Figure S10 (D-J), BMS-303141 treatment markedly suppressed global histone acetylation (H4K12ac) without affecting histone lactylation (H4K12la), and the expression of the neurotrophic factor FGF1 remained robustly induced. Strikingly, when HDAC1 was overexpressed in these acetylation-depleted cells, it specifically erased the remaining H4K12la marks and concomitantly abolished FGF1 expression.

This specific decoupling experiment directly answers the reviewer's concern. It provides definitive functional evidence that even in a cellular context where histone acetylation has already been pharmacologically minimized, HDAC1 still effectively suppresses neurotrophic programs primarily through its delactylase activity (erasing H4K12la). We have incorporated these results into the revised manuscript and expanded the Results and Discussion sections to explicitly clarify the rationale and specificity of this mechanism.

6. There are many CNS infections and inflammatory processes characterized by high lactate. Can the authors speculate whether similar effects as described here are to be expected in those diseases?

Response: We thank the reviewer for this important question. We agree that under certain pathological conditions, such as acute CNS infections, an elevated level of lactate in the cerebrospinal fluid (CSF) is a recognized biomarker. A systematic review and meta-analysis has indicated that CSF lactate concentration can be used to distinguish bacterial from viral meningitis, and its elevation typically reflects a surge in glycolytic metabolism and tissue hypoxia driven by both the pathogen and the host immune response [1]. In the context of such acute infections, lactate accumulation is largely considered an indicator of metabolic crisis and tissue damage. However, a growing body of evidence suggests that the function of lactate is highly context-dependent. A recent review has highlighted that lactate is a key immunoregulatory molecule, with effects contingent on its concentration, duration of exposure, the cell type it acts upon, and the specific pathological setting (e.g., cancer, infection, or autoimmunity) [2].

This functional duality is particularly evident in the context of CNS autoimmunity. A study conducted in the EAE model found that lactate limits CNS autoimmunity by stabilizing

hypoxia-inducible factor-1α (HIF-1α) in dendritic cells (DCs) [3]. This research provides direct evidence for a protective role of lactate in autoimmunity and uncovers a novel immunometabolic regulatory axis, which contrasts with its role as a damage marker in acute infections.

Our study's central thesis that lactate exerts neuroprotection by inducing long-lasting epigenetic reprogramming (specifically histone lactylation) and functional modulation in microglia is consistent with recent findings, which elucidated that lactate can drive sustained innate immune reprogramming by fueling the TCA cycle and regulating histone lactylation[4]. Concurrently, lactate metabolism and histone lactylation have been shown to play a central regulatory role in the CNS [5]. Together, this evidence suggests that in the context of chronic autoimmunity or adaptive immune regulation, lactate can function as a signaling molecule and an epigenetic modulator.

To more clearly illustrate this context-dependency, we have summarized the key distinctions below:

Context	Role of Lactate	Primary Mechanism	Key Reference
Acute CNS Infections	Damage Marker	Glycolytic surge, tissue hypoxia	Wang Q, et al. *BMC Neurol*. 2023 [1]
CNS Autoimmunity	Immunomodulator	HIF-1α stabilization, limiting pathogenic T cells	Sanmarco LM, et al. *Nature*. 2023 [3]
Trained Immunity	Signaling Molecule / Epigenetic Modulator	Driving histone lactylation, reprogramming immune cells	Cai H, et al. *Nat Commun*. 2025 [4]

Based on current evidence, we consider the role of lactate in the CNS to be highly context-dependent. In acute CNS infections, elevated lactate predominantly reflects metabolic distress rather than engaging protective lactylation-dependent reprogramming. In contrast, during chronic neuroinflammation or autoimmune conditions characterized by sustained low-to-moderate lactate levels, lactate-induced histone lactylation is more likely to occur and may mediate effects similar to those described in our study. Moreover, emerging work in neurodegenerative diseases such as Alzheimer's and Parkinson's disease suggests that dysregulated lactate metabolism and microglial epigenetic remodeling are common features[6,7,8], raising the possibility that lactylation-based mechanisms may also be relevant, although direct causal evidence is still limited and warrants further investigation. We have added a paragraph to the discussion section of our manuscript to more comprehensively elaborate on the complexity of lactate's role, citing relevant literature to support our view.

Reference

[1] Wang Q, et al. The value of elevated cerebrospinal fluid lactate for the diagnosis of postoperative bacterial meningitis: a systematic review and meta-analysis. *BMC Neurol*. 2023;23(1):393. doi: 10.1186/s12883-023-03428-8.

[2] Llibre A, Kucuk S, Gope A, Certo M, Mauro C. Lactate: A key regulator of the immune response. *Immunity*. 2025;58(3):535-554. doi: 10.1016/j.immuni.2025.02.008.

[3] Sanmarco LM, Rone JM, Polonio CM, et al. Lactate limits CNS autoimmunity by stabilizing HIF-1α in dendritic cells. *Nature*. 2023;620(7975):881-889. doi: 10.1038/s41586-023-06409-6.

[4] Cai H, et al. Lactate activates trained immunity by fueling the tricarboxylic acid cycle and regulating histone lactylation. *Nat Commun*. 2025;16(1):1481. doi: 10.1038/s41467-025-58563-2.
[5] Wang Y, et al. Lactate metabolism and histone lactylation in the central nervous system. *J Neuroinflammation*. 2024;21(1):111. doi: 10.1186/s12974-024-03303-4.
[6] Qin Q, Wang D, Qu Y, et al. Enhanced glycolysis-derived lactate promotes microglial activation in Parkinson's disease via histone lactylation. npj Parkinsons Dis. 2025;11(1):3. doi: 10.1038/s41531-024-00858-0.
[7] Wei L, Yang X, Wang J, et al. H3K18 lactylation of senescent microglia potentiates brain aging and Alzheimer's disease through the NFκB signaling pathway. J Neuroinflammation. 2023;20(1):286. doi: 10.1186/s12974-023-02879-7.
[8] Zhao Y, Xu H. Microglial lactate metabolism as a potential therapeutic target for Alzheimer's disease. Mol Neurodegener. 2022;17(1):36. doi: 10.1186/s13024-022-00541-z.

Reviewer #2 (Remarks to the Author):

In the manuscript by Jiahong Li, Su Meng, Jiajian Li et al, the team uses the EAE model of multiple sclerosis to address a potential role of lactate metabolism and histone lactylation in neuroinflammation. Though the topic is of relevance and interest, unfortunately, due to several fundamental flaws in the approach and analyses I cannot recommend publishing the manuscript.

The main issues are

1. The grouped together analyses of myeloid cells, that is presented as though the data reflect changes in microglia. The staining of IBA1 (as for example presented in fig 2) and quantification of other proteins/modifications within IBA1 positive cells in this case does not necessarily reflect a change in microglia. In peak EAE there is huge number of infiltrating cells from the blood, of which many also express IBA1. Therefor all the differences reported most likely reflect a difference in cell composition, rather than a difference in microglia. This is also a problem for all analyses performed on samples of isolated 'microglia' where CD11B beads were used. As is also demonstrated in supplemental figure S3, in the different stages of EAE (and control) different populations will be captured by this approach and the outcomes do not allow to allocate these effects to microglia.

Response: We appreciate the reviewer's critical comments and the opportunity to improve the clarity and robustness of our study. We fully agree that distinguishing resident microglia from peripherally-infiltrating macrophages is a central challenge in neuroinflammation research, and that CD11b magnetic isolation or IBA1 staining alone captures a mixed myeloid population during peak EAE. To rigorously address this concern and definitively allocate our findings to microglia, our study was specifically designed using a two-tiered approach that progresses from an unbiased bulk screening of the broader myeloid compartment to single-cell high-resolution validation.

We acknowledge that during peak EAE, the $CD11b^+$ fraction and global $IBA1^+$ cells encompass both resident microglia and infiltrating myeloid cells. As noted in the Results section (see subsection: "Glycolytic activation selectively couples to H4K12la in CNS myeloid cells"), we initially employed CD11b magnetic bead sorting not to isolate ultra-pure microglia, but as an unbiased screening approach to capture the broader CNS myeloid compartment. The bulk proteomics (Fig. 2) and bulk RNA-seq (Fig. 7), alongside IBA1 histological staining,

served as our initial discovery platforms to identify global inflammation-associated metabolic and epigenetic remodeling within the lesion microenvironment.

To fundamentally resolve whether these molecular changes occurred intrinsically within resident microglia, we relied on scRNA-seq (Fig. 6) as our definitive, cell-type-resolved validation strategy. As suggested, we utilized well-established, unambiguous molecular markers to computationally separate these lineages with high confidence. Specifically, resident microglia were stringently defined by high expression of *Tmem119*, *P2ry12*, and *Cx3cr1*, and low expression of *CD45*. In contrast, infiltrating macrophages were characterized by high expression of *Ccr2*, *Ly6c2*, and high *CD45*, with negligible expression of core microglial markers like *Tmem119*. Crucially, all of our key conclusions regarding lactate-driven transcriptional and metabolic reprogramming were analyzed and validated specifically within this unambiguously identified microglial cluster. Our scRNA-seq data clearly demonstrate that lactate intervention intrinsically downregulates high-flux metabolic genes and pro-inflammatory programs while preserving homeostatic signatures within microglia. This provides definitive evidence that the effects we observed are intrinsic changes within microglia, not merely an artifact of a shift in the ratio of microglia to infiltrating macrophages.

We are grateful to the reviewer for prompting us to articulate this logic more transparently. We have revised the Results and Discussion sections to explicitly acknowledge the limitations of CD11b enrichment and IBA1 staining during peak EAE. We have also clarified that our microglia-specific conclusions are firmly grounded in the high-resolution scRNA-seq data, ensuring that our findings are accurately attributed to the resident microglial population.

2.*The mouse experiments presented in figure 4, related to the conditional knockout of Ldha. These experiments lack the appropriate controls... To address these issues the experiment needs to be repeated with as a minimum a 3rd group that is homozygous Cx3cr1:CreERT2 animals that were treated with tamoxifen.*

Response: We thank the reviewer for emphasizing the importance of appropriate genetic and induction controls in the conditional *Ldha* knockout experiments. We fully agree that potential confounding effects arising from Cre toxicity, tamoxifen treatment, or alterations in *Cx3cr1* dosage must be rigorously addressed.

We would like to clarify that all conditional knockout mice used in this study were generated as heterozygous for the *Cx3cr1*-CreERT2 allele (*Ldha*$^{fl/fl}$; *Cx3cr1*$^{CreERT2/+}$), thereby retaining one functional copy of the *Cx3cr1* gene, rather than the homozygous genotype suspected by the reviewer. We apologize if this was not stated clearly in the original manuscript, and we have now explicitly clarified the exact genotype in the Methods section and in the schematic shown in Figure 3A.

Regarding the reviewer's suggestion to include homozygous *Cx3cr1*-CreERT2 mice treated with tamoxifen as an additional control. we carefully considered this approach but decided against it based on established microglial biology. Previous studies (e.g. PMIDs 23817416 and 30617416) have definitively demonstrated that complete *Cx3cr1* deficiency intrinsically exacerbates EAE and alters microglial behavior, entirely independent of any floxed allele. As a result, homozygous *Cx3cr1*-CreERT2 animals do not serve as a neutral control; rather they introduce a well-established biological confound that fundamentally complicates the interpretation of disease phenotypes. For this reason, we intentionally avoided this homozygous genotype in our experimental design.

Instead, to directly and rigorously control for the potential effects of Cre expression, tamoxifen administration, and partial *Cx3cr1* haploinsufficiency, we performed the appropriate validation using the gold-standard control: a group of heterozygous *Cx3cr1*$^{CreERT2/+}$ mice (lacking *Ldha* flox alleles) treated with tamoxifen alongside wild-type controls.

As presented in the new Supplementary Figure S2(M, N), this Cre-only control group exhibited EAE clinical scores and area under the curve (AUC) outcomes that were statistically indistinguishable from the wild-type control group. Importantly, all groups were treated with tamoxifen using the identical dosing and timing regimen.

Together, these results definitively confirm that the significantly aggravated EAE pathology observed in the *Ldha* icKO group (Figure 3C, D) is specifically attributable to targeted the microglial deletion of *Ldha,* rather than Cre toxicity or *Cx3cr1* haploinsufficiency. We have incorporated these new data and clarifications into the revised manuscript to further improve transparency and experimental rigor.

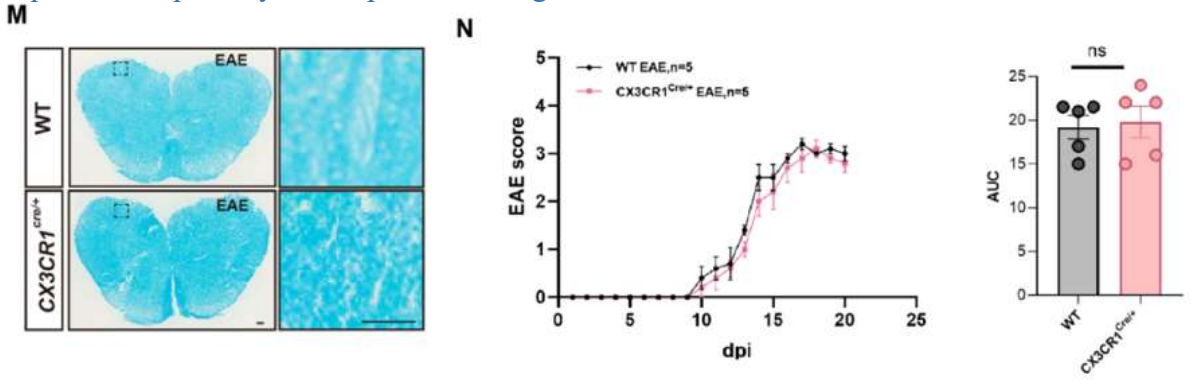


Fig. S2(M-N) Representative LFB staining (M), clinical EAE scores, and AUC quantification (N) in wild-type and *Cx3cr1*$^{CreERT2/+}$ control mice (n = 5 mice per group).

3. Many experimental details are lacking in the figures, results and in particular the methods sections, which is not providing sufficient detail to interpret or in future repeat the experiments (For example (but list is not exhausitive). This makes it difficult to assess the quality and validity of the work

- For the IIDD patients (particular MS) whose csf was used to measure lactate levels: please provide in the table their disease activity status. Was there ongoing demyelination and inflammation in the CNS at the time of sampling?
- Where in the spinal cord are the images taken, inside a lesion? Outside? At what level? Thoracic? Lumbar? Is the whole spinal cord used to isolate cells?
- the method used to generate the scRNAseq data are not provided, was it 10x genomics, something else?
- the methods used to analyze the scRNAseq data are very poorly described, were doublets removed, was ambient RNA correction performed? These steps are critical for obtaining a high quality dataset, but they are not indicated
- what were the parameters used for clustering analysis of the scRNAseq data? What do the clustering dendrogram and elbow plots look like that were used to make the decision to only generate 2 clusters?
- for all mice used in the manuscript: provide the clinical EAE score and day after immunization at the time of their termination
...

Response: We sincerely thank the reviewer for these valuable comments. They have helped us identify areas where additional detail was needed to enhance the clarity, reproducibility, and validity of our work. We agree that the original figures, results, and methods sections lacked sufficient experimental details, which made it challenging to fully assess the quality of the work. In response, we have revised the manuscript as follows:

Clinical Data and Disease Activity: We updated Supplementary Tables S1 and S2 to include the disease stage, the specific phase at sampling, and EDSS scores for each Multiple Sclerosis

patient. These tables now provide laboratory parameters such as CSF white blood cell counts and oligoclonal band status. These data characterize the disease activity and confirm the presence of ongoing CNS inflammation or demyelination at the time of sampling.

Table S1. Patient demographics and disease characteristics

Patient ID	Diagnosis	Age (years)	Sex	Disease Duration (months)	Disease Phase
961775	MS	31	F	18	Chronic
765478	MS\AD	37	F	84	Chronic
555720	MS	52	M	156	Chronic
1029042	MS	54	F	2	Acute
1022264	MS	42	M	0	Acute
855352	MS	39	F	108	Acute
969801	MS	46	F	90	Chronic
1017739	MS	61	F	360	Chronic
962732	MS	29	F	24	Chronic
940839	MS	25	M	0	Acute
953829	MS	30	F	24	Chronic
645402	MS	51	F	216	Chronic
1081366	MS	41	F	5	Acute
962942	MS	44	F	1	Acute
1077514	MS	48	F	12	Chronic
1080721	MS	30	F	12	Chronic
934722	MS	23	F	60	Chronic
820942	MS	46	M	36	Chronic
969842	MS	46	M	60	Chronic
997692	MS	53	F	48	Chronic

Table S2. Laboratory parameters of individual patients

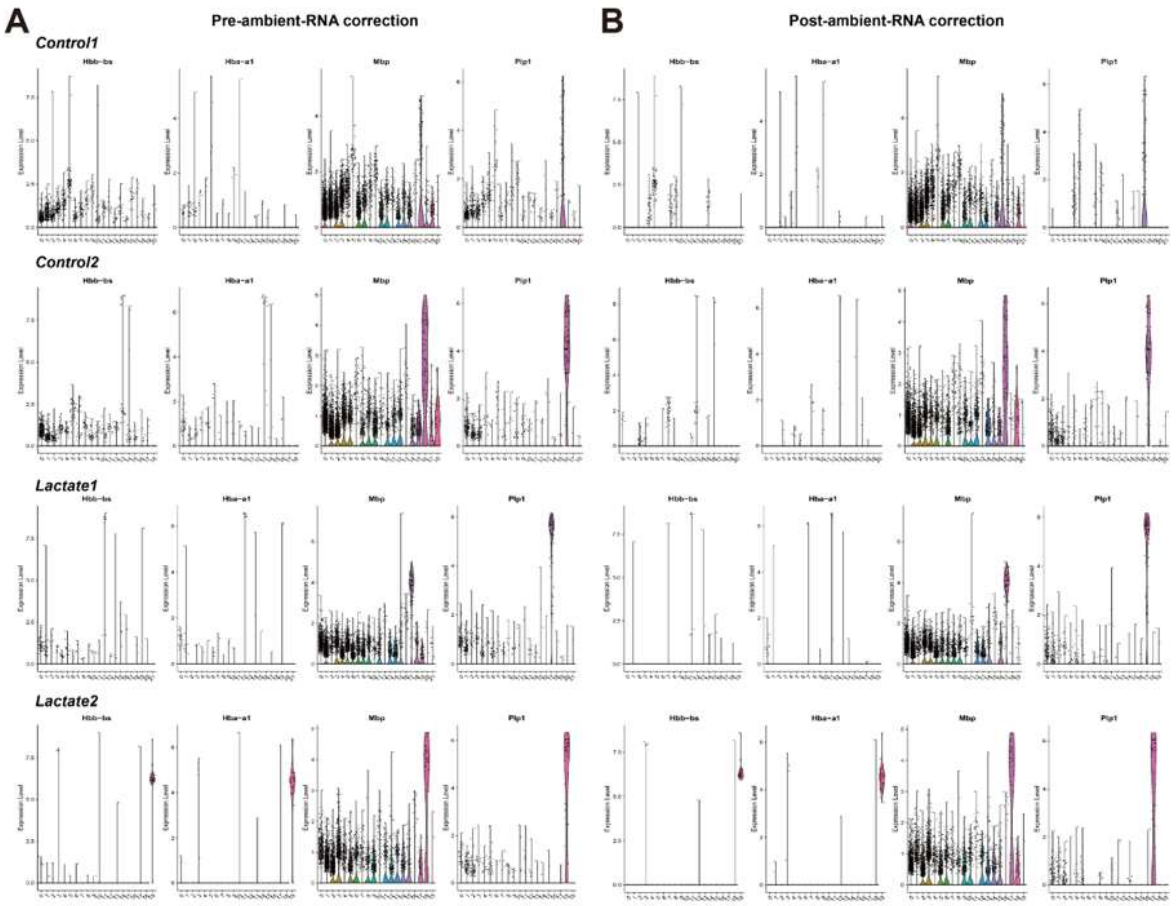
Patient ID	CSF OCB	CSF WBC ($\times 10^6$/L)	CSF Lactate (mmol/L)	Blood WBC ($\times 10^9$/L)	Neutrophil ($\times 10^9$/L)	Lymphocyte ($\times 10^9$/L)	IL-6 (pg/mL)	IL-10 (pg/mL)	CRP (mg/L)	EDSS Score
961775	Positive	4.0	1.5	8.1	5.38	2.03	3.07	1.18	2.0	0
765478	Weakly positive	2.0	2.0	5.34	4.33	0.45	5.29		2.0	6
555720	Weakly positive	2.0	1.6	12.55	9.17	2.11	2.29	3.04	4.0	7.5
1029042	Negative	0.0	2.4	4.07	2.44	1.21	23.16	3.45	4.0	
1022264	Weakly positive	9.0	2.0	7.08	4.33	2.14	2.83	1.83	1.0	2
855352	Negative	1.0	1.1	4.15	1.98	1.7	3.68	5.58	2.0	3.5
969801	Positive	4.0	1.5	7.89	6.96	0.71	4.34		2.0	2
1017739	Weakly	0.0	1.0	10.55	6.5	3.28	2.11	2.22	2.0	3

	positive									
962732	Positive	8.0	1.1	10.44	9.07	0.96	8.84	5.62	1.0	2
940839	Positive	7.0	1.3	8.84	5.17	2.95			2.0	2
953829	Positive	6.0	1.6	3.83	1.69	1.75	1.5		1.0	3.5
645402	Positive	1.0	1.6	4.52	2.39	1.81	1.95		1.0	8
1081366	Negative	1.0	1.4	4.71	2.52	1.68	2.82		2.0	2
962942	Negative	0.0	1.6	5.62	3.3	1.87	4.7	2.57	1.0	
1077514	Positive	2.0	1.7	10.99	9.88	0.68	2.12	1.31		6
1080721	Positive	7.0	1.2	7.24	4.58	2.27	5.89		5.0	2
934722	Negative	3.0	1.3	5.96	3.6	1.96			1.0	0
820942	Negative	23.0	1.6	5.77	4.83	0.43	7.82		5.0	3.5
969842	Positive	3.0	1.1	7.05	6.91	0.90	4.02		2.0	
997692	Negative	1.0	1.6	10.59	8.73	1.6	1.08	1.96	2.0	

Animal Experiment Details: Regarding the anatomical details of the animal experiments, the representative immunofluorescence images shown in the figures were acquired from the lumbar segment of the spinal cord, specifically inside the inflammatory lesions within the white matter to capture maximal immune cell infiltration. For the isolation of cells used in subsequent bulk and single-cell analyses, we harvested the whole spinal cord to ensure sufficient cell yield and to obtain a global representation of the pathological changes. Additionally, for all mice used in the manuscript, we have now explicitly provided the clinical EAE scores and the days post-immunization at the time of their termination in the revised Figure Legends and Methods section.

scRNA-seq Data Generation: We have clarified **in the Methods section** that single-cell RNA sequencing was performed using the 10x Genomics platform, with libraries prepared according to the manufacturer's protocol and sequenced on an Illumina NovaSeq 6000.

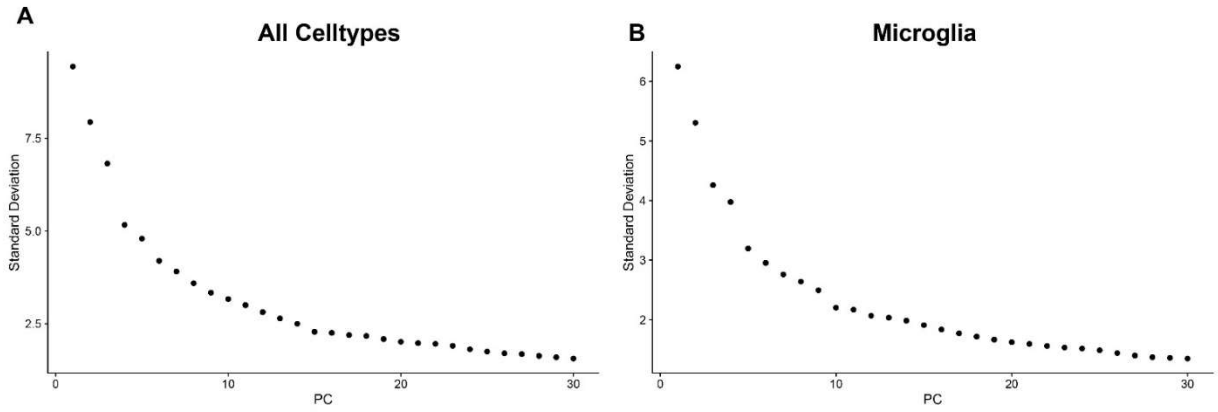
Data Analysis Improvements: To address data-quality concerns, we fully re-processed all raw scRNA-seq data. We incorporated an explicit ambient-RNA correction step using SoupX (contamination fraction estimated by autoEstCont). We then recalculated QC metrics on the corrected counts and retained cells with nFeature_RNA $\geq$ 200, nFeature_RNA $\leq$ 5,000, and percent.mt $\leq$ 10%. Putative doublets were identified and removed using DoubletFinder. Batch effects were corrected with Harmony prior to integration. After this re-analysis, we obtained 51,902 high-quality cells. Diagnostic plots and before/after comparisons (e.g., clearance of canonical ambient markers) are provided in Supplementary Figure S3.



FigureS3(A-B) Evaluation and correction of ambient RNA contamination. Violin plots show the expression levels of hemoglobin genes (Hbb-bs, Hba-a1) and myelin genes (Mbp, Plp1) in each cluster before (A) and after (B) correction using SoupX. Note the effective removal of background noise in non-relevant clusters.

Clustering Parameters and Subclustering: Principal components were evaluated with JackStraw (dims 1:30) and ElbowPlot (ndims = 30); the elbow stabilized at ~PC20 (*see Author Response Image A below*). Accordingly, we used dims = 1:20 (Harmony space) for graph construction (FindNeighbors) and visualization (RunUMAP), and set resolution = 0.8 for FindClusters, yielding 28 clusters. We subsequently annotated 19 major cell types, clearly identifying the microglial population (Supplementary Figure S4A-B).

Because our initial microglia partitioning was under-resolved (previously 2 clusters), failing to reflect expected transcriptional heterogeneity, we performed a focused re-clustering of the microglial compartment, again supported by JackStraw/Elbow diagnostics (*see Author Response Image B below*). This produced 13 transcriptionally distinct microglial states, which we consolidated into 8 biologically interpretable microglial subpopulations by comprehensive marker-based annotation (Figure 6B and Supplementary Figure S4C).



These updates have been incorporated into the revised **Methods** section. The re-analysis did not alter our core conclusions but provided a more granular view of cell populations, which we have updated in the **Results** and **Figures** sections. We believe these changes fully address your concerns and improve the manuscript's scientific rigor.

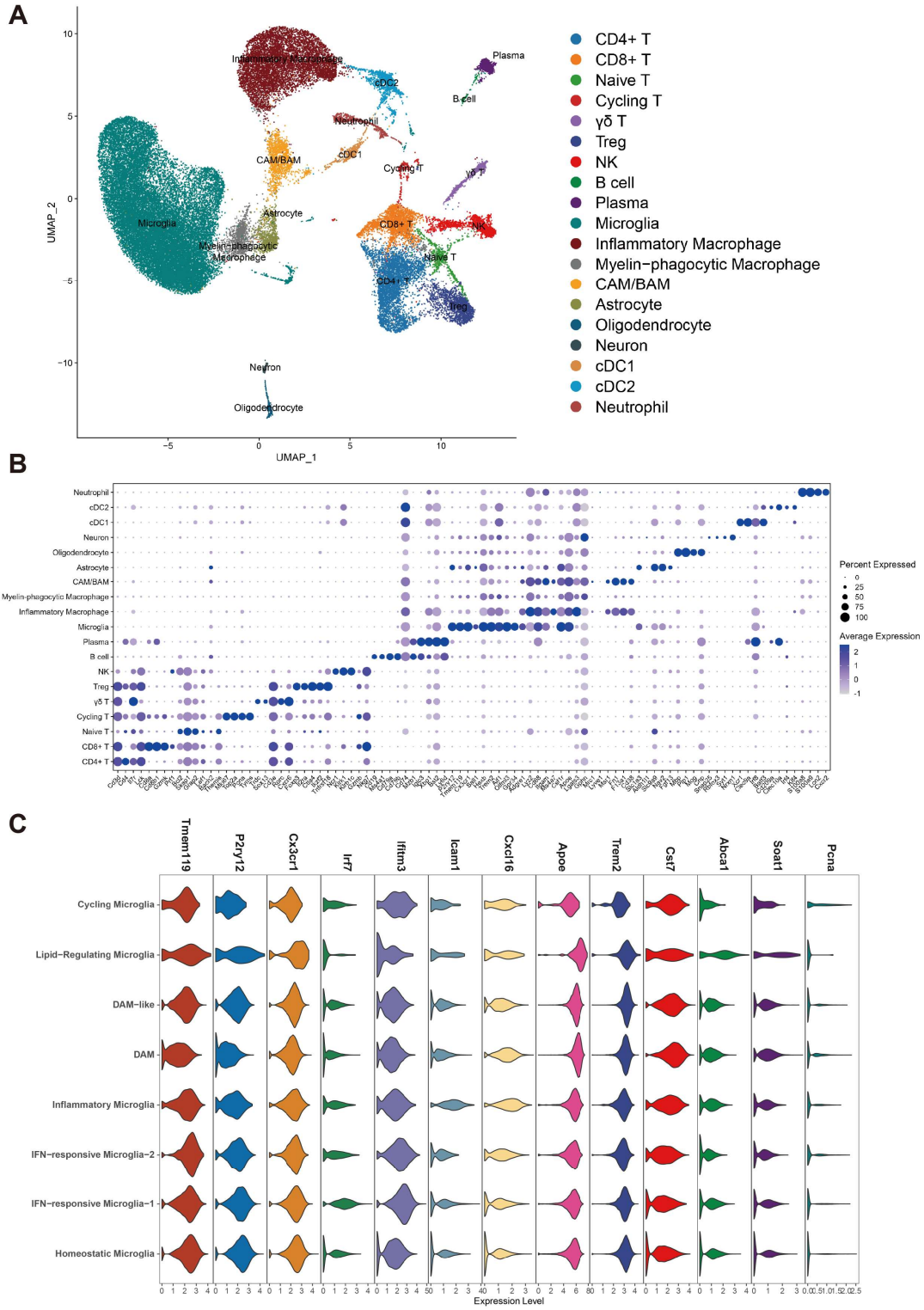


Fig. S4 High-resolution global cell atlas and unambiguous annotation of CNS myeloid cells.
(A) Integrated UMAP projection of 51,902 high-quality cells from all samples, annotated into 19 distinct cell types.
(B) Canonical marker gene expression dot plot used for cell type annotation. Dot size represents the percentage of cells expressing the gene, and color intensity indicates average expression. Note the distinct separation between resident microglia (*Tmem119, P2ry12, Cx3cr1*) and infiltrating macrophages (*Ccr2, Ly6c2*), as well as CNS-resident lineages (*Olig1* for Oligodendrocytes, *Aqp4* for Astrocytes, *Map2* for Neurons).
(C) Violin plots showing the expression of signature genes used to define the 8 microglial subpopulations described in Figure 6B.

4. The analysis and presentation of the single cell RNAseq data does not meet the standards of the field. Any basic overview of the QC of the data should be presented in supplement (eg

RNA counts, mito, scrublet score). The fact that the UMAP show many 'strings', the overall lack of clear specificity in expression of cell type markers (see dotplot in figure s2) and eg. the closeness of 'neurons' to the microglia (while the macrophages who should be more similar in expression pattern are far away) suggesting that there are still many doublets in the data, or that the data is not clean and contains high levels of ambient RNA.
If the whole spinal cord was homogenized, where are the neurons and oligodendrocytes? I appreciate that there is an influx of immune cells, but I expected more of the CNS resident cells in the total object.
Where in the UMAP (in which cells) are the relevant proteins for lactate metabolism expressed?
For the subclustering of microglia: were the CAMs/BAMs removed or included in the analysis? Why was a resolution chosen that resulted in only 2 clusters? There is a clear difference between the 2 groups, where the control animals have a group of cells on the right-side of the UMAP that should be further explored. I expect these to represent a separate cluster that warrants further investigation.
It is not helpful to make the distinction between homeostatic and activated microglia as is done. What does activated mean in this context? Are there any functional processes altered in these microglia (again here an increased clustering resolution might be helpful in identifying relevant microglia populations)?

Response: We are deeply grateful to the reviewer for the exceptionally thorough and constructive critique, which has substantially improved the rigor and clarity of our single-cell RNA-seq analysis. We fully acknowledge that the original presentation of the scRNA-seq data fell short of current field standards. We have therefore completely re-processed and re-analyzed the entire dataset following the reviewer's insightful suggestions. Below we address each point in detail.

1. Data quality control, ambient RNA, doublets, and UMAP "strings"

We agree that the initial submission did not provide sufficient QC information We have now re-processed every sample with an explicit ambient-RNA correction step using SoupX (contamination fraction estimated by autoEstCont). Doublets were identified and removed using DoubletFinder. Cells were subsequently filtered using stringent criteria (nFeature_RNA 200–5,000, percent.mt ≤ 10%). Batch effects were corrected using Harmony. Detailed QC metrics (nCount_RNA, nFeature_RNA, percent.mt) and before-after comparisons (including canonical ambient markers such as hemoglobin and immediate-early genes) are now provided in Supplementary Figure S2.

After this rigorous pipeline, we obtained 51,902 high-quality cells that resolved into 28 clusters and 19 clearly defined cell types (Supplementary Figure S3A).

Regarding the residual "string-like" structures in the new UMAP, these are now largely restricted to biologically meaningful continua (e.g., differentiation/activation gradients between cDC1–cDC2 and among T-cell subsets) and are no longer observed between unrelated lineages such as neurons and microglia. Macrophages now cluster separately from microglia, consistent with their distinct ontogeny and transcriptomic profiles.

2. Cell-type annotation and marker specificity

We thank the reviewer for highlighting the insufficient specificity of the original marker dotplot. All 28 clusters have been re-annotated de novo using canonical and recently published cell-type-specific markers, with particular attention paid to distinguishing microglia, inflammatory macrophages, myelin-phagocytic macrophages, and CAM/BAM populations. The updated, high-resolution dotplot is presented in Supplementary Figure S3B.

3. Presence and annotation of CNS-resident cells (neurons, oligodendrocytes)

In the revised dataset (51,902 high-quality cells), neurons and oligodendrocytes indeed represent a minor fraction (~0.23% and ~0.50%, respectively) of the total CNS cell population

recovered from whole spinal cord in peak-stage EAE. This extremely low abundance is a well-documented and reproducible feature of EAE/CNS autoimmune models and is attributable to several established technical and biological factors:

(i) Disease-induced cell loss and severe dissociation bias: At the peak of EAE, widespread axonal injury, neuronal death, and oligodendrocyte apoptosis occur in spinal cord white matter lesions. Moreover, mature myelinating oligodendrocytes and spinal projection neurons are among the most mechanically fragile CNS cell types and are preferentially lost during tissue dissociation and Percoll density-gradient centrifugation — a phenomenon repeatedly reported in spinal cord EAE scRNA-seq studies [1, 2].

(ii) Massive peripheral immune cell infiltration: At disease peak in the spinal cord (the stage and tissue we sampled), infiltrating leukocytes (T cells, monocytes/macrophages) routinely constitute >80 – 90% of total viable cells recovered, dramatically diluting resident populations.

(iii) Enrichment protocol: The standard Percoll protocol we used is optimized to maximize recovery of infiltrating leukocytes and microglia while minimizing red blood cell/debris contamination, but inevitably reduces yield of large, myelin-rich oligodendrocytes and neurons. We sincerely hope this explanation adequately addresses your concern.

4. Microglia subclustering and inclusion of CAM/BAM

We completely agree that the previous distinction into only two broad states ("homeostatic" vs "activated") was overly simplistic and uninformative. We have now performed focused re-clustering of the entire microglial compartment (after removal of obvious CAM/BAM, which are now annotated separately) using PCs 1–20 and resolution = 0.8. This yielded 13 initial clusters that were merged, based on shared marker profiles, into 8 biologically meaningful subpopulations: Homeostatic, IFN-responsive-1, IFN-responsive-2, Inflammatory, classical DAM, DAM-like, Lipid-regulating, and Cycling microglia (Figure 6B and Supplementary Figure S3C). Notably, the IFN-responsive, inflammatory, DAM, DAM-like, and Cycling populations were markedly reduced in relative proportion in the lactate-treated group compared with controls (Figure 6C), highlighting lactate's regulatory effect on pathogenic microglial states.

5. Expression of proteins relevant to lactate metabolism

To directly address the reviewer's question, we visualized key genes involved in both anaerobic and aerobic glucose metabolism (*Pgk1, Pkm, Ldha* for glycolysis/lactate production; *Cox5a, Atp5md, Ndufa1* for oxidative phosphorylation). These genes are broadly expressed in microglia, macrophages, and T cells (Supplementary Figure S5A), but within the microglial compartment they show highest expression within DAM microglia clusters (Supplementary Figure S5B). Quantitative analysis confirmed significant downregulation of these genes in microglia of lactate-treated mice (Figure 6D), fully supporting our conclusion that lactate drives metabolic reprogramming of the most metabolically active and inflammatory microglial subset away from glucose dependency.

6.Summary

We have substantially revised the QC pipeline, cell-type annotation, microglial subclustering, and metabolic gene analyses. These updates resolve the concerns raised and considerably strengthen the scRNA-seq portion of the manuscript. We are extremely grateful for the time and expertise the reviewer has invested in improving our work.

Other points.

- The title refers to several concepts that are not (confidently) addressed in the manuscript, including trained immunity, microglia and importantly also MS. As detailed above, the authors analysed a pool of myeloid cells whose exact cellular composition was different in the different groups analysed. Trained immunity is a concept that partially describes a

functional state that can only be uncoverd by a second (immune) stimulus. The experiments the authors did in my opinion do not address this aspect of trained immunity. For MS: in the manuscript the authors have studied EAE (apart from measuring lactate levels as depicted in figure 2). This is not the same as MS (if anything EAE is more of a model for MOGAD, though it is an acceptable model for several aspects of MS) and therefore that should not be in the title

Response: We sincerely thank the reviewer for this critical and constructive comment. Upon careful reflection, we fully agree with the reviewer's concerns regarding the terminology used in the manuscript, particularly the strict definition of "trained immunity", and the nuances of the EAE model. We have carefully revised the manuscript based on the points raised and have made several important changes to ensure the accuracy and clarity of the study's content. Below are our detailed responses to each concern:

(i). On the use of "Trained Immunity"

We fully acknowledge the reviewer's concern regarding the use of the term "trained immunity." As the reviewer correctly points out, trained immunity is formally defined as a long-term functional reprogramming of innate immune cells, typically triggered by stimuli like BCG or β-glucan, which confers enhanced non-specific protection against secondary infections [1, 2]. This process is underpinned by stable epigenetic and metabolic rewiring that can persist for months [3].

While our findings reveal a novel lactate-driven epigenetic mechanism involving H4K12 lactylation [4], we agree that it does not fully meet the stringent criteria of classical trained immunity, especially concerning the long-term memory and non-specific protection aspects. Recent work has begun to link histone lactylation to innate immune memory [5], but our study focuses more on a dynamic, context-dependent reprogramming of microglia in response to the inflammatory microenvironment, rather than a long-lasting trained state. Therefore, to avoid conceptual overstatement, we have removed all mentions of "trained immunity" throughout the manuscript.

Regarding the title, while we recognize that our mechanistic experiments were performed in the EAE model, the foundational clinical observation of this study—specifically the elevated CSF lactate levels driving our hypothesis—was derived directly from a well-characterized cohort of human Multiple Sclerosis (MS) patients (Figure 1 and Supplementary Tables S1-S2. Therefore, to accurately reflect both the clinical origin of our research and the experimental system used, we have adjusted the title to: "Lactate–H4K12la–HDAC1 Axis Reprograms Microglial Function to Alleviate Neuroinflammation in Models of Multiple Sclerosis."

(ii). On the distinction between "Microglia" and Mixed Myeloid Cells

We appreciate the reviewer's point about the potential contamination of infiltrating myeloid cells in our initial bulk analyses, especially since our earlier methods involved CD11b magnetic bead separation. We agree that this method captures a mixed population of both resident microglia and infiltrating monocyte-derived macrophages, particularly in a model like EAE where the blood-brain barrier is compromised [6].

Revision: We have revised the manuscript as noted in the Results section (see subsection: "Glycolytic activation selectively couples to H4K12la in CNS myeloid cells") to explicitly clarify that the $CD11b^+$ fraction represents the broader "CNS myeloid compartment" and was used as an initial screening approach. To confidently attribute our findings specifically to microglia, we relied on our scRNA-seq data. As detailed in the revised Results section (see subsection: "scRNA-seq reveals lactate-driven suppression of inflammatory microglial states"), we used a panel of well-established molecular markers—*Tmem119, P2ry12,* and *Cx3cr1* for homeostatic microglia [7, 8] versus *Ccr2, Ly6c2*, and high levels of CD45 for infiltrating macrophages [9]—to unambiguously separate these populations. This multi-layered analysis, which is consistent with state-of-the-art approaches for dissecting myeloid complexity in the

CNS [9, 10], definitively proves that resident microglia are the primary cell type exhibiting the observed epigenetic and transcriptional reprogramming.

(iii). On the use of "Multiple Sclerosis (MS)" in the context of an EAE model

We appreciate the nuanced point regarding the relationship between EAE, MS, and MOGAD, and agree that this distinction is crucial. The recognition of MOGAD as a separate clinical entity with distinct immunopathology has indeed refined our understanding of demyelinating diseases [11]. We acknowledge that direct extrapolation from any single animal model to human MS must be approached with caution, as no model perfectly recapitulates the full spectrum of the human disease [12, 13].

However, as emphasized above, the rationale for investigating the lactate-lactylation axis in our experimental model is intrinsically anchored in our original clinical findings from human MS patients, where we observed significantly elevated CSF lactate levels. This clinical observation is consistent with other reports linking altered metabolism to MS pathology [16].

Revision: We have carefully reviewed the manuscript to state clearly that EAE is utilized as a relevant experimental model of autoimmune neuroinflammation to dissect the biological consequences of the lactate accumulation we observed in our MS cohort. We have also made adjustments in the Discussion to frame our findings as revealing fundamental immunometabolic mechanisms that are relevant to CNS inflammatory conditions, thereby bridging our initial clinical observations in MS with a broader biological context, rather than making a direct and oversimplified claim of equivalency.

References

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- How do the levels of lactate in the csf correlate to levels in the blood? Both in patients and the animal model. This is also relevant given the confounding factor of infiltration of the peripheral immune system at the peak of EAE

Response: We thank the reviewer for raising this important question regarding the relationship between lactate levels in the CSF and blood, particularly in the context of peripheral immune infiltration at the peak of EAE. This issue is highly relevant for interpreting both endogenous lactate signaling and the effects of exogenous lactate supplementation in our study. While we did not collect paired blood samples to assess systemic lactate levels in our clinical MS cohort, the physiological relationship between these two compartments has been well established.

CSF and blood lactate levels remain largely independent because ionized lactate has limited blood-brain barrier permeability [1, 2]. Elevated CSF lactate in MS patients reflects active local accumulation rather than passive diffusion from systemic circulation.

Our experimental findings support this framework. Systemic lactate administration successfully augmented the CNS lactate pool through regulated transport mechanisms or altered barrier permeability during neuroinflammation [3, 4]. As shown in Supplementary Figure S10A, i.p. administration significantly increased lactate concentrations in the inflamed spinal cord. This elevation was sufficient to modulate microglial states and restore H4K12la levels.

Our genetic data address the potential confounding effect of peripheral immune infiltration. Microglial-specific *Ldha* deletion (Figure 3) exacerbated EAE severity despite the massive influx of peripheral myeloid cells. If infiltrating cells or systemic circulation were sufficient to support histone lactylation, the loss of locally produced microglial lactate would have been compensated. This lack of compensation demonstrates that the internal microglial lactate pool is the indispensable driver of the H4K12la-FGF1 reparative axis [5, 6]. Exogenous lactate exerts its therapeutic benefit by reinforcing this local metabolic-epigenetic signaling.

References

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- The quantification of fluorescence intensity as presented in fig 2F is not an appropriate estimation of protein levels. I recommend depicting the data similar as for astrocytes (panel 2H) and, given the caveats associated with this type of analysis, refrain from using intensity as a quantitative measure.

Response: We thank the reviewer for this important methodological point. We fully agree that for global modifications like Pan-Kla, fluorescence intensity can be a less reliable surrogate for protein abundance due to its sensitivity to staining efficiency, imaging settings, and tissue penetration.

In response to your constructive recommendation, we have completely removed the fluorescence intensity-based analysis for microglial Pan-Kla (originally presented in Figure 2F). In the revised manuscript, this data is now presented in Figure 1D and 1E, where microglial lactylation is quantified exclusively as the density of Pan Kla^+ $Iba1^+$ cells per unit area (cells/mm^2).

This revised metric perfectly matches the density-based quantification approach used for astrocytes (now Figure 1F and 1G, previously 2H) and neurons (now Figure 1H and 1I, previously 2J). By reflecting the proportion of microglia exhibiting detectable protein lactylation rather than relying on signal amplitude (which is inherently variable), this density metric provides a much more robust and biologically interpretable readout.

Importantly, all cell counts were performed using uniform detection thresholds and identical acquisition parameters across experimental groups, and quantification was carried out in a blinded manner. Following this revision, the data in Figure 1E continue to strongly support our conclusion that microglial protein lactylation is significantly elevated in the EAE group, while substantially strengthening the methodological rigor and consistency of our data presentation.

- At the end of the legend of Figure 1 the authors write 'promoting effective neuroprotection'. In the manuscript there is no evidence provided that demonstrates neuroprotection. Increased expression of a few genes does not constitute the same.

Response: We appreciate the reviewer's rigorous definition regarding neuroprotection and fully agree that gene expression alone alterations are insufficient to claim physiological protection.

In response to this valid point, we have revised the legend of Figure 1 to avoid implying direct neuroprotection at this initial stage. Because Figure 1 is intended as a conceptual schematic summarizing the proposed pathway, the wording has been changed to indicate that lactate promotes a "microenvironment supportive of neurorepair and tissue preservation," rather than claiming definitive neuroprotection within the schematic itself.

However, we respectfully clarify that the evidence for neuroprotection in our study is not derived from gene expression alone, but is firmly established by independent *in vivo* structural and functional readouts presented in subsequent figures. Specifically, Luxol Fast Blue (LFB) staining demonstrates significant preservation of myelin structure in the lactate-treated group compared to controls (as detailed in Figures 4 and 5, and conversely demonstrated in the *Ldha* icKO model in Figure 3). These robust histological findings are accompanied by significantly improved clinical EAE scores, indicating the preservation of overall neurological function.

Together, these complementary outcomes (clinical behavior, myelin integrity, and neuronal survival) meet the rigorously accepted criteria for neuroprotection. The upregulation of neurotrophic factors, such as GDNF and FGF1, serves as the underlying molecular mechanism driving the concrete structural and functional protection we observed histologically.

We believe that the revised wording in Figure 1, combined with the comprehensive histological and clinical data presented later in the manuscript, accurately reflects the scope and strength of our findings. We sincerely appreciate the reviewer's comment, which has helped us improve the conceptual precision and clarity of our manuscript.

- In figure legend of 2B it is written that n=8 animals were used, the figure itself only has 5 datapoints. Where are the missing 3 samples per group?

Response: We thank the reviewer for carefully checking the consistency between the figure and the legend. The discrepancy in the original Figure 2B was due to a typographical error in the figure legend, not in the underlying data.

The correct sample size for the spinal cord lactate measurements shown in Figure 2B is n = 5 biological replicates per group, which corresponds exactly to the five data points displayed in the figure. All animals included in this experiment are shown, and no samples were excluded from the analysis at any stage. Statistical analyses were performed solely on these five biological replicates.

We apologize for the confusion caused by this labeling error. In the revised manuscript, this data panel has been repositioned as Figure 1B, and the indication of "n = 8" has been corrected to "n = 5" in the corresponding figure legend. We have also carefully reviewed the entire manuscript to ensure that sample sizes are now consistently and accurately reported throughout. We deeply appreciate the reviewer's attention to detail.

- The bulk sequencing data is only partially depicted and should be shown as a whole. What was the number of genes detected and differentially expressed? What do the comparisons look like in a volcano plot? (or 4-way plot or something else that is a visual representation of the actual comparisons)

Response: We thank the reviewer for this constructive recommendation. We fully agree that providing a comprehensive, global overview of the bulk RNA-sequencing data is essential for data transparency and a better understanding of the global transcriptional shifts.

To directly answer your question, in the primary acute phase comparison (Acute EAE + La vs. Acute EAE), a total of 33 genes were reliably detected. Using a threshold of Q value < 0.05 and foldchange > 2, we identified 33 significantly differentially expressed genes (DEGs), all of which were downregulated.

While the number of DEGs is relatively small under these stringent criteria, they represent key modulations in inflammatory pathways. To provide a clear visual representation, we have now incorporated Volcano plots in the revised Supplementary Figure S8A–C.

- Many of the western blots depicted are oversaturated, shorter exposures should be provided. As ECL was used to develop the signal, and this was used to quantify the protein levels, this is a serious issue. ECL does not result in a linear signal, so preferable 2 different exposure times are quantified to make sure any differences are presented accurately. Again, how the measurements were done is not clearly indicated in the methods.

Response: We agree that oversaturated signals and the no-linear response of ECL detection can compromise accurate protein quantification.

We apologize for the confusion caused by our image presentation in the previous draft. We would like to clarify that our original densitometric quantifications were, in fact, conducted utilizing shorter, unsaturated exposure images that strictly fall within the linear dynamic range of our ECL system. However, during the preparation of the figures, we inadvertently selected longer, oversaturated exposures for the visual representation to make the bands appear more prominent.

To address your valid concern and ensure absolute transparency, we have now corrected this display oversight. We have replaced the oversaturated representative immunoblots in the revised figures (including Figures 1, 2, and 8) with the corresponding shorter, unsaturated exposure images. These revised images now accurately reflect the actual raw data from which our protein quantifications were derived.

Furthermore, as requested, we have significantly expanded the Methods section (under the subheading *"Protein Extraction and Western Blot Analysis"*) to explicitly detail our densitometric workflow. We now clearly describe the use of ImageJ software, the procedures

for background subtraction, normalization to appropriate loading controls (β-actin), and the criteria used to ensure all quantified signals are within the linear dynamic range. We believe these revisions fully address your concerns and ensure the rigor of our quantitative analyses.

- The reference to M1 and M2 like microglia markers should be removed from the manuscript. As the authors are aware (their reference #36) this is an outdated concept that does not contribute to our understanding of microglia function in the CNS.

Response: We sincerely thank the reviewer for this important point. We fully agree that the M1/M2 binary classification is an outdated concept that oversimplifies the complex and multidimensional nature of microglial states in the CNS, as critically discussed in the field [1].

Accordingly, we have revised the manuscript to remove all references to 'M1-like' and 'M2-like' microglia throughout the manuscript, including the main text, figure legends, and supplementary materials. Importantly, this revision is not limited to terminology alone. We have also revised the way microglial states are described and interpreted.

Rather than assigning microglia to discrete activation categories, we now describe our findings using marker-specific and pathway-level language, focusing on transcriptional programs and functional modules associated with inflammatory signaling, metabolic remodeling, and neurotrophic or homeostatic support. While markers such as NOS2 and Arg1 are still reported where experimentally measured, they are no longer used to define binary activation states. Instead, they are interpreted as components of broader, context-dependent gene expression patterns.

This framework is consistent with our single-cell and bulk transcriptomic analyses, which reveal graded and overlapping microglial states rather than mutually exclusive phenotypes. We have explicitly revised the Results and Discussion sections to reflect this spectrum-based view of microglial activation and to avoid overinterpretation based on individual markers.

We believe these changes align the manuscript more closely with current conceptual standards in the field and improve the accuracy of how microglial functional heterogeneity is presented and discussed.

References:

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Minor points:

- The authors fail in the paper to use the appropriate nomenclature that is common for gene names vs protein names from different species. Not all instances where genes are indicated are in italic and protein names should be written in all caps.

Response: We thank the reviewer for pointing out this important issue regarding gene and protein nomenclature. We agree that consistent and species-appropriate naming is essential for clarity and accuracy.

In the revised manuscript, we have systematically reviewed the entire text, including the main text, figure legends, methods, and supplementary materials, to ensure strict adherence to standard nomenclature conventions. Specifically, gene symbols are now consistently italicized, while protein names are written in non-italicized uppercase letters.

Where applicable, we have also ensured that species-specific conventions are respected (e.g., human gene symbols in uppercase italics and mouse gene symbols in capitalized italics), following commonly accepted guidelines such as HGNC and MGI nomenclature standards. Figure labels and annotations have been corrected accordingly to maintain consistency between text and figures.

These revisions are purely nomenclatural and do not affect the data, analyses, or conclusions of the study, but they improve the precision and readability of the manuscript. We appreciate the reviewer's careful attention to this detail.

- Fig 6a indicates tSNE, while the authors have used a UMAP approach in the actual data analysis and visualization in figure 6, panels C/D vs E: it is confusing that the same colors mean different things in these panels. Eg blue is activated microglia in C/D, while it refers to all microglia from control in E

Response:

We sincerely thank the reviewer for these careful and helpful observations regarding Figure 6, which we agree affected the clarity and consistency of data presentation.

1. tSNE vs UMAP inconsistency

We apologize for the misleading label in the original Fig. 6A. Although all analyses and visualizations in the manuscript were performed using UMAP, the schematic in panel 6A was mistakenly labeled as "tSNE". We have now corrected the label to UMAP throughout the revised Figure 6 and its legend. Importantly, this correction does not affect any of the underlying analyses or results.

2. Confusing color coding in panels

We fully agree that using the same color (blue) for different meanings across panels was highly confusing. In the revised Figure 6, we have completely redesigned the color scheme for "bar plot of microglial subpopulation proportions" (Figure 6C) to ensure consistency and clarity. Each color now corresponds to one unique biological meaning across all panels.

These changes are purely graphical and do not alter the data, analyses, or conclusions of the study. The updated Figure 6 is now provided in the revised manuscript and clearly explained in the figure legend. We are very grateful to the reviewer for spotting these issues; the revisions have significantly improved the clarity and professionalism of the presentation.

- Line 161 references to fig 3A, this should be 4A

Response: We thank the reviewer for spotting this error. We apologize for the incorrect figure citation. We have corrected in the revised Results section (see subsection: "Exogenous lactate ameliorates neuroinflammation via microglial reprogramming, independent of restricting infiltration") to accurately reference Figure 4A instead of Figure 3A.

- Figure S2D: the hierarchical tree is cut off on the left side and the legend representing the colors (and connected information on what is depicted: z-scores, expression values??) is missing

Response: We apologize for the formatting oversight regarding the cropped image and the missing legend. Please note that due to the comprehensive re-analysis of the scRNA-seq data and the addition of new quality control plots (which now occupy the new Figure S2), this heatmap panel has been reorganized. In its updated position (now presented as Figure S4C / S5), we have revised the figure to display the complete hierarchical clustering tree on the left. Additionally, we have added a color scale bar to the figure, explicitly indicating that the colors represent the Z-score of the relative expression levels.

- The colors in figure s2c do not contribute as the gene names are already listed on the top of the figure panel

Response: We appreciate the reviewer's suggestion to improve figure clarity. We agree that color-coding by gene is redundant since the gene names are already labeled at the top. Following the aforementioned reorganization of our supplementary figures, these revised violin plots are now presented in Figure 6D. As suggested, we have adopted a uniform color scheme for these violin plots (e.g., colored uniformly by experimental group), thereby removing the unnecessary visual complexity.

Reviewer #3 (Remarks to the Author):

Li et al. identify histone H4 lysine 12 lactylation (H4K12la) as a key mechanism to re-educate microglia and mediate neuroprotection in neuroinflammation... The findings are novel and may have therapeutic implications for MS. However, the manuscript requires extensive revision prior to publication.

Response: We thank the reviewer for the positive feedback and constructive suggestions.

Major Criticisms:

1. The authors use the term „trained immunity" to describe the reprogramming of microglia in response to lactate. However, most of the data is derived from the EAE model in which lactate accumulation as a response to inflammation may sustain throughout the observation period. More experiments are required to support the hypothesis that microglia are „trained" to support neuroprotection by epigenetic reprogramming. This could involve in vitro treatment of microglia cells with L-lactate and monitoring of H4K12la over time after decontinuation of the treatment (potentially in the presence or absence of an HDAC1 inhibitor such as valproid acid). Furthermore, if their hypothesis holds true, local short-term administration of L-lactate to the CNS prior to EAE induction should be sufficient re-educate microglia to mediate neuroprotection once disease is induced.

Response: We thank the reviewer for raising this important conceptual point and for clearly delineating the distinction between sustained activation and bona fide trained immunity.
We fully agree that the term "trained immunity" implies a memory-like phenomenon characterized by persistence after withdrawal of the initial stimulus, which is not directly addressed by our current experimental design, particularly within the EAE model where lactate accumulation persists during the inflammatory phase, reflects a continuous metabolic modulation rather than the strict temporal dynamics required to define trained immunity.

Our data therefore support a model of lactate-driven metabolic-epigenetic reprogramming under inflammatory conditions, rather than formal trained immunity in the strict sense. To ensure scientific precision and avoid overinterpretation, we have fully accepted your suggestion and removed the term "trained immunity" throughout the revised manuscript, including the Title, Abstract, and Discussion sections. We have replaced it with terms such as "metabolic-epigenetic reprogramming" or "functional modulation," which accurately describe our findings regarding the Lactate-H4K12la axis without implying a specific memory mechanism.

Within the boundaries of our current data, we show that lactate accumulation during neuroinflammation induces a reproducible epigenetic state in microglia characterized by increased H4K12 lactylation, suppression of pro-inflammatory programs, and induction of neurotrophic factors. This state is functionally relevant during disease progression, even if it does not yet meet the formal criteria of trained immunity.

We believe that clarifying this distinction strengthens the conceptual rigor of the manuscript and more accurately positions our findings within the current framework of immunometabolic regulation in the CNS. We thank the reviewer for prompting this important refinement.

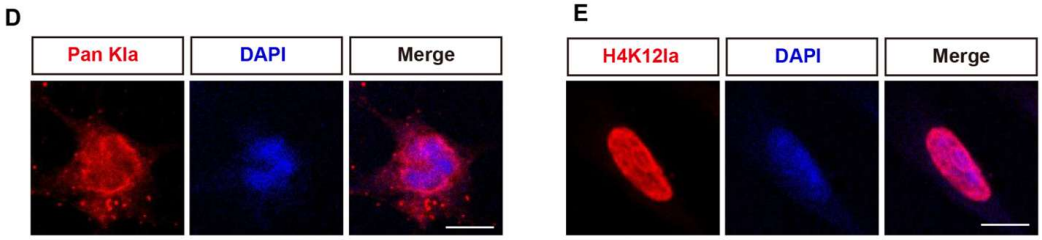
2. Fig. 2ff: The authors provide representative images of Pan Kla and H4K12la in tissue sections. Staining of histone marks should be confined to the nucleus, but appear to also be present in the cytoplasm. The authors should provide additional data to evaluate subcellular localization (i.e. enlarged images with DAPI counterstain, immunoblot of nuclear vs. cytoplasmic fraction).

Response: We appreciate the reviewer's careful examination of the subcellular localization of lactylation signals and for raising this important technical point.

We clarify that the apparent difference in localization arises from the distinct biological targets of the two antibodies used. H4K12la is a specific histone mark confined to the nucleus. The Pan-Kla antibody targets global lysine lactylation on all proteins rather than being limited to histones. The seminal study by Zhang et al. in Nature 2019 established lysine lactylation as a broad post-translational modification affecting numerous non-histone proteins such as cytoplasmic metabolic enzymes. The cytoplasmic signal observed with Pan-Kla staining is biological and reflects the lactylation of cytoplasmic proteins.

In contrast, H4K12la is a site-specific histone modification and should be confined to the nucleus. To directly address the reviewer's concern, we have provided new high-magnification confocal images with DAPI counterstaining in Figure S1(D,E). These images demonstrate that the H4K12la signal strictly colocalizes with DAPI in the nucleus while the Pan-Kla signal exhibits both nuclear and cytoplasmic distribution consistent with the broad biological nature of protein lactylation. These data clarify the specificity and subcellular distribution of these modifications.

We appreciate the reviewer's comment, which helped us improve both the clarity and rigor of the data presentation.



3. Fig. 3: Li et al. aimed to enrich microglia to perform proteomic and transcriptomic analyses. However, they used magentic enrichment of CD11b+ cells from the CNS. During acute EAE the CD11b+ fraction contains a large proportion of infiltrating myeloid cells. Therefore, all subsequent analyses may be biased by variations in cellular composition of the sample rather than by disease-induced changes in microglia. They should repeat these experiments using flow-cytometry enriched CD45^{dim} CD11b+ cells.

Response: We sincerely thank the reviewer for this precise and important technical point. We fully agree that for bulk analyses, the CD11b-enriched population during acute EAE can be confounded by infiltrating myeloid cells, and this is a critical issue that must be addressed. The reviewer's suggestion to use FACS-sorted CD45^{dim}CD11b+ cells is, without question, one of the gold-standard methods for improving microglial purity.

(i) Study rationale and positioning of bulk analyses: In our study, the bulk proteomic (now presented in Fig. 2) and transcriptomic analyses (now presented in Fig. 7) were intended as an initial, hypothesis-generating characterization of the CNS myeloid compartment, rather than as definitive microglia-specific measurements. We acknowledge that this approach is inherently limited by potential differences in cellular composition across disease stages. Accordingly, we have revised the manuscript to explicitly state this limitation and to avoid overinterpretation of

these data as microglia-exclusive (**see subsection: "Glycolytic activation selectively couples to H4K12la in CNS myeloid cells"**).

(ii) High-Purity Computational Sorting: To directly and rigorously address the issue of cellular attribution raised by the reviewer, we relied on single-cell RNA sequencing (scRNA-seq, Fig. 6) as our primary cell-type-resolved validation strategy. Within our scRNA-seq data, we used a panel of well-established, canonical marker genes to clearly and confidently separate resident microglia (*Tmem119+, P2ry12+, CD45low*) from infiltrating macrophages (*Ccr2+, Ly6c2+, CD45high*) with high confidence.
While we fully acknowledge that repeating the bulk analyses using FACS-sorted $CD45^{dim}$CD11b+ microglia would further increase cellular purity, we believe that the depth and resolution of the single-cell dataset already provide a more direct and comprehensive framework to assign molecular changes specifically to microglia and to disentangle intrinsic transcriptional reprogramming from shifts in cell composition. We have therefore chosen to emphasize the single-cell data as the principal evidence for microglia-specific effects.

(iii) Cross-Validation of Key Findings: Critically, the key pathways and molecular signatures we identified in the initial bulk analyses (e.g., genes related to lactate metabolism and epigenetics) were subsequently and explicitly confirmed to be predominantly enriched within the stringently defined microglia cluster in our single-cell data. In other words, the single-cell data provide definitive, cell-type-specific attribution for our bulk findings, demonstrating that the changes we observed were indeed driven by intrinsic alterations within microglia, and not simply by a shift in cell proportions.

(iv) Complementary Experimental Strategy: We believe that this two-step strategy—using bulk analysis for initial discovery and single-cell sequencing for precise validation—is a powerful and efficient research paradigm. It allowed us to first capture the overall biological signal and then use single-cell technology to deeply investigate the cellular origin and heterogeneity of that signal.

Revisions: In response to the reviewer's comment, we have revised the Results section (specifically the subsections "Glycolytic activation..." and "scRNA-seq reveals...") to (i) clearly frame the CD11b-based bulk analyses as an initial exploration of the broader myeloid compartment, (ii) explicitly acknowledge the limitations of CD11b enrichment during EAE, and (iii) clarify that our microglia-specific conclusions are grounded in the high-resolution scRNA-seq analyses. We believe these revisions make our line of reasoning clearer and more robust.

4. Fig. 5: The authors treat animals with a dose of 500 mg/kg lactate per day. In a previous study Sanmarco et al. (ref. 20) used a dose of 125 mg/kg in the same animal model. The authors should provide data on lactate levels in the CNS after lactate supplementation and compare this to elevated lactate levels during neuroinflammation.

Response: We thank the reviewer for this important question regarding dose selection and CNS lactate exposure, and for highlighting the need to relate exogenous lactate supplementation to endogenous lactate accumulation during neuroinflammation.

We appreciate the reviewer's insightful comment regarding the dosage comparison with previous studies. While Sanmarco et al. utilized a lower dose of 125 mg/kg to stabilize the HIF-1α protein, our study investigates histone lactylation[1], which is an epigenetic modification strictly driven by the availability of the substrate Lactyl-CoA. Driving chromatin remodeling often requires a higher metabolic threshold than signal transduction activation. High-impact studies in neurology have established the safety and necessity of higher lactate doses to elicit robust CNS metabolic changes. For instance, Carrard et al. (*Molecular Psychiatry*, 2021)

successfully administered 2 g/kg of lactate to modulate adult hippocampal neurogenesis and produce antidepressant effects[2]. Our selected dose of 500 mg/kg is well within this established therapeutic window and ensures sufficient expansion of the intracellular lactate pool to shift the enzymatic equilibrium towards histone modification.

Importantly, to directly address the request regarding CNS lactate levels, we have performed additional measurements of lactate concentrations in both the spinal cord parenchyma and isolated microglial cells following systemic administration. As presented in the new Supplementary Figure S10A, i.p. administration of lactate (500 mg/kg) significantly elevated the overall lactate concentrations in the spinal cords of EAE mice compared to vehicle-treated EAE controls. Furthermore, we observed a corresponding increase in the intracellular lactate pool within isolated $CD11b^+$ cells in the new Supplementary FigureS10B.

These data demonstrate that the selected dose of 500 mg/kg does not create an artificial metabolic state but rather augments a physiologically relevant lactate increase that already occurs during neuroinflammation. This elevation is sufficient to support histone H4K12 lactylation and the associated transcriptional changes described in this study. We have clarified these points in the revised Results and Methods sections to improve transparency and facilitate comparison with prior work. These measurements confirm that the supplemented lactate reaches the CNS, placing the local levels within, and modestly above, the range observed during endogenous inflammatory lactate accumulation.

References

[1] Sanmarco, L. M., et al. (2023). Lactate limits CNS autoimmunity by stabilizing HIF-1α in dendritic cells. *Nature*, 620(7975), 881-889.

[2] Carrard, A., et al. (2021). Peripheral L-lactate mediates antidepressant effects of exercise by enhancing hippocampal neurogenesis. *Molecular Psychiatry*, 26(9), 4874-4889.

5. Fig. 5, related to previous comment: In this study the authors started lactate treatment during the preclinical phase (day 10 post-immunization). While with this approach effects on T cell priming may be negligible, it can profoundly effect inflammatory monocytes and other myeloid cells. Indeed the authors detect a significant reduction in neuroinflammatory cell infiltration upon lactate-treatment, but they don't further characterize the infiltrate using cell type-specific antibodies such as CD3 or Mac-3. To assess whether lactate treatment efficiently blocks progression via reprogramming of microglia rather than influx of peripheral myeloid cells, the authors should provide data from EAE animals treated from the peak of disease onwards (approx. day 17 post-immunization).

Response: We appreciate the reviewer's suggestion to assess the therapeutic efficacy of lactate starting from the peak of disease as it is critical for distinguishing between blocking immune influx and modulating cell function.

We agree that initiating lactate treatment during the preclinical phase may influence peripheral myeloid cells before their entry into the CNS. To specifically disentangle these possibilities, we performed a new set of experiments where lactate administration (500 mg/kg) was initiated at the therapeutic stage (from day 14 post-immunization), a time point when clinical symptoms have manifested and immune cell infiltration into the CNS is already well-established.

As shown in Figure 5, lactate treatment starting at the disease peak significantly improved clinical scores (Fig. 5B-C) and preserved myelin integrity (Fig. 5D-E). Importantly, flow cytometric analysis confirmed that this late-stage treatment did not significantly alter the absolute numbers of infiltrating T cells (CD3+) or monocyte-derived macrophage populations (Ly6C+CD45hi) (Fig. 5H-K).

To investigate the underlying mechanism driving tissue recovery despite persistent infiltration, we performed immunofluorescence staining on spinal cord lesions. We observed

that late-stage lactate treatment significantly upregulated H4K12la levels specifically within Iba1-positive cells in the lesions (Fig. 5L-M). Crucially, this restoration of the H4K12la mark was accompanied by the induction of the neurotrophic factor FGF1, which we have identified as a key downstream effector of the lactate-H4K12la axis in our mechanistic studies (as shown in Supplementary Fig. S10 and Fig. 8).

These findings indicate that lactate can ameliorate disease progression even when immune infiltration is already established. Its beneficial effects are primarily associated with the functional and epigenetic reprogramming of CNS myeloid cells via the H4K12la-FGF1 axis, rather than a mere reduction in leukocyte recruitment.

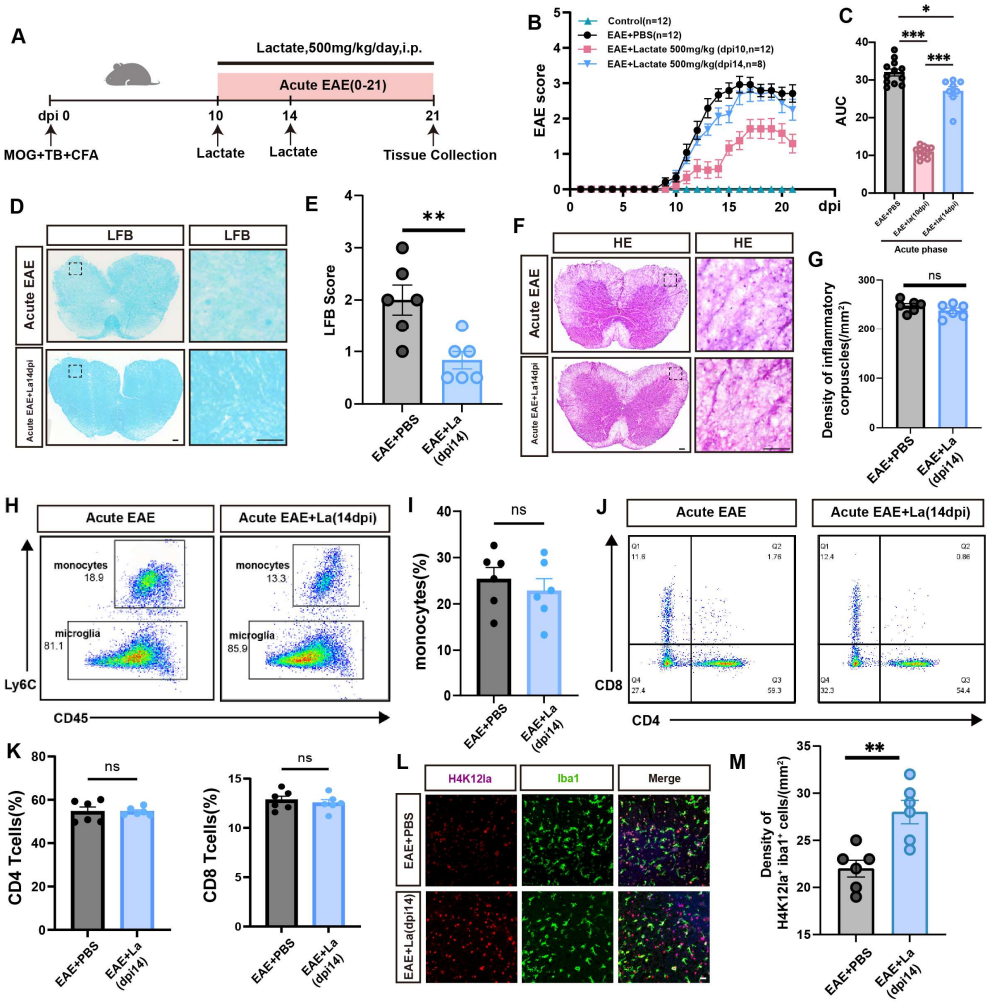


Figure 5. Lactate treatment initiated at peak disease ameliorates EAE severity via microglial reprogramming without altering immune infiltration.

(A) Schematic of the therapeutic regimen: lactate (500 mg/kg/day, i.p.) or PBS was administered starting from the peak of disease (day 14 post-immunization).

(B) Clinical EAE scores of mice treated with PBS (n=12), Lactate starting at day 10 (n=12), and Lactate starting at day 14 (n=8). Note that day 14 treatment effectively lowers scores compared to PBS.

(C) AUC quantification for EAE scores from (B).

(D, E) Representative LFB staining (D) and quantification of LFB scores (E) in spinal cord sections from PBS-treated and Lactate-treated (day 14) mice. Scale bars, 50 μm.

(F, G) Representative H&E staining (F) and quantification of inflammatory corpuscle density (G). "ns" indicates no significant difference in total infiltration density.

(H, I) Representative flow cytometry plots (H) and quantification (I) of infiltrating myeloid cells ($Ly6C^{+}CD45^{hi}$) in the spinal cord.

(J, K) Representative flow cytometry plots (J) and quantification (K) of CD4+ and CD8+ T cells in the spinal cord.

(L, M) Representative immunofluorescence images (L) and quantification (M) of H4K12la (red) in $Iba1^{+}$ cells (green). Lactate treatment significantly restores H4K12la levels even in the established inflammatory environment. Data are presented as mean ± SEM. Normality was assessed using the Shapiro-Wilk test. Statistical significance was determined by one-way ANOVA followed by Bonferroni's post-hoc test for multiple comparisons (B, C), and by Mann-Whitney U test for two-group comparisons (E, G, I, K, M). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns = not significant.

6. Fig. 7 and 8: The authors claim that histone deacetylases, especially HDAC1, are downregulated in response to lactate treatment in vivo. One the one hand this is not visible in the heatmaps provided in this manuscript. On the other hand the histological data provided in Fig. 7L and 7P does not allow the reader to assess how HDAC1 is regulated during the course of neuroinflammation without lactate treatment. The authors should quantify both total and microglial HDAC1 expression throughout different disease stages. Treatment of EAE animals with an HDAC1 inhibitor from the acute phase onwards and monitoring of H4K12la would further strengthen the functional link between lactate-H4K12la-HDAC1 and neuroprotection in vivo.

Response: We sincerely appreciate the reviewer's suggestion to characterize the dynamic expression of HDAC1 during the course of neuroinflammation and its functional relationship to lactate-induced H4K12 lactylation *in vivo*.

We agree that establishing a baseline for HDAC1 regulation is essential for interpreting the effects of lactate. Accordingly, we performed immunofluorescence staining and quantification of HDAC1 expression specifically in Iba1$^+$ microglia across Naive, Acute EAE, and Chronic EAE stages. As presented in the new Supplementary Figure S11 our analysis revealed that microglial HDAC1 protein levels are significantly upregulated during the Acute phase of EAE and remain persistently elevated into the Chronic phase. Comparing this with our treatment data (Fig. 7M, Q) demonstrates that lactate administration effectively reverses this pathological upregulation, restoring microglial HDAC1 to levels comparable to the healthy state.

Furthermore, as suggested by the reviewer, we have added a new set of in vivo experiments using the class I HDAC inhibitor MS-275 to strengthen the functional link. While we initially noted the systemic nature of such inhibitors, the results were highly informative. As presented in Figure 3N–Q, treatment of EAE mice with MS-275 from the acute phase significantly restored H4K12la levels in microglia and ameliorated EAE disease severity, mirroring the effects of exogenous lactate.

To complement these in vivo findings with higher cell-type precision, we also utilized the in vitro HMC3 model. As shown in Figure 8, pharmacological inhibition of HDAC1 using MS-275 under inflammatory conditions directly restored H4K12la levels and upregulated the neurotrophic factors GDNF and FGF1. Together, this combination of in vivo expression dynamics, in vivo functional inhibition, and in vitro validation provides robust evidence for the Lactate–HDAC1–H4K12la axis in regulating microglial neuroprotective programs.

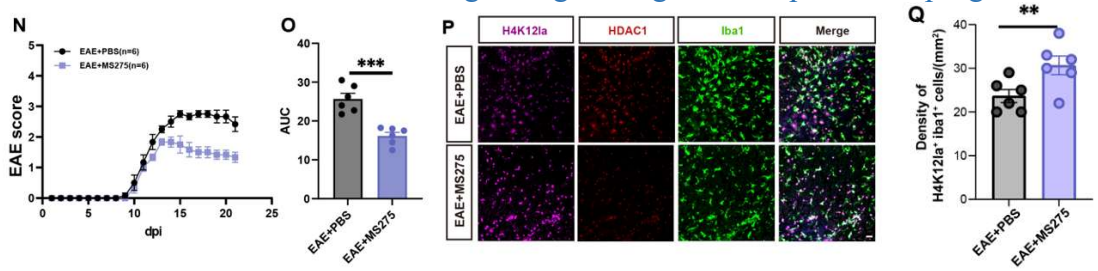


Figure3
(N-O) Clinical EAE scores (N) and AUC quantification (O) in EAE mice treated with MS-275 or PBS (n = 6 mice per group).
(P-Q) Representative immunofluorescence images (P) and quantification (Q) of H4K12la$^+$ Iba1$^+$ cell density in spinal cord lesions following MS-275 treatment.

7. Fig. 8: The authors claim that stimulation of HMC3 cells with IFNγ and LPS can mediate H4K12la accumulation in vitro. However, the authors should provide stainings of H4K12la in HMC3 cells to prove that this is the case. They should additionally include L-lactate treatment and a more disease-relevant stimulation regimen such as IFNγ and TFNα rather than LPS stimulation.

Response: We appreciate the reviewer's constructive suggestions regarding the in vitro experimental design and agree that validating the link between stimulation, histone lactylation, and phenotype is crucial.

First, to address this, we performed immunofluorescence staining of H4K12la in HMC3 cells and included an exogenous L-lactate treatment group. The new data, presented in the revised Figure 8J-K, confirm that stimulation leads to significant nuclear accumulation of H4K12la and that exogenous lactate directly mimics this effect.

Second, regarding the stimulation regimen, we fully agree that IFNγ+TNFα represents a clinically relevant inflammatory signal in neuroinflammatory diseases. We maintained the use of LPS+IFNγ was based on mechanistic considerations rather than an attempt to model disease complexity in vitro. Specifically, our work focuses on lactate-driven histone lactylation, which requires robust glycolytic activation. LPS+IFNγ is a well-established stimulus for inducing strong aerobic glycolysis and lactate production in microglia-like cells and has been widely used in immunometabolism studies to interrogate metabolism-dependent epigenetic regulation [1] [2].

Importantly, the in vitro HMC3 experiments are intended to provide mechanistic support for the metabolic-epigenetic pathway identified in vivo, rather than to fully recapitulate the complex disease environment. The clinical and disease relevance of our study is primarily supported by the human MS cohort, the EAE model, and the single-cell/bulk transcriptomic analyses of CNS-resident microglia. We have explicitly clarified this rationale in the revised Results section (see subsection: "H4K12la directly regulates neurotrophic gene expression via an HDAC1-sensitive mechanism")to avoid overinterpretation of the in vitro system.

References:

[1] Murray, P. J., et al. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41(1), 14-20.

[2] Baik, S. H., et al. (2019). A Mitochondrial-to-Glycolytic Metabolic Switch Is Required for BACE1-Mediated Microglial Function. *Cell Metabolism*, 29(6), 1324-1339.

8. Fig. 8, related to the previous comment: The authors mention that stimulation of HMC3 cells to induce H4K12la may drive expression of Gdnf and Fgf1, which they corroborate by fact that HDAC1 overexpression lowers Gdnf and Fgf1 levels while also lowering H4K12la accumulation. To support this functional link the authors should provide stainings of GDNF and FGF-1 on HMC3 cells. These experiments should be performed with the stimulation regimens mentioned above, and in the presence of absence of an HDAC1 inhibitor.

Response: We deeply appreciate the reviewer's suggestion to further validate the functional link between H4K12la and neurotrophic factor expression. We fully agree that verifying this mechanism through both gain- and loss-of-function approaches is essential.

To rigorously address your request, we employed a combination of pharmacological and genetic approaches to validate this epigenetic-transcriptional axis under the LPS+IFN-γ stimulation regimen.

First, to address the pharmacological loss-of-function aspect using the specific inhibitor mentioned by the reviewer, we treated cells with the class I HDAC inhibitor MS-275. Western blot analysis confirmed that pharmacological inhibition of HDAC1 effectively prevented the erasure of H4K12la and resulted in a robust, sustained expression of the neurotrophic factors GDNF and FGF1 at the global protein level (Figure 8L).

Second, to complement the global protein analysis with spatial and single-cell resolution, we utilized a genetic gain-of-function approach. Immunofluorescence staining revealed that HDAC1-GFP overexpression specifically erased H4K12la marks in situ and simultaneously suppressed the expression of GDNF within the transfected cells. The representative images and quantification are included in the revised Figure 8N–O.

While we relied on comprehensive Western blot profiling rather than immunofluorescence for the MS-275 pharmacological treatment group, the robust protein-level upregulation seen in our immunoblots (Fig. 8L), combined with the precise spatial co-localization of H4K12la erasure and GDNF suppression in the HDAC1-overexpression model (Fig. 8N-O), provides orthogonal and definitive evidence. Together, these data firmly establish that HDAC1-mediated delactylation negatively regulates microglial reparative programs.

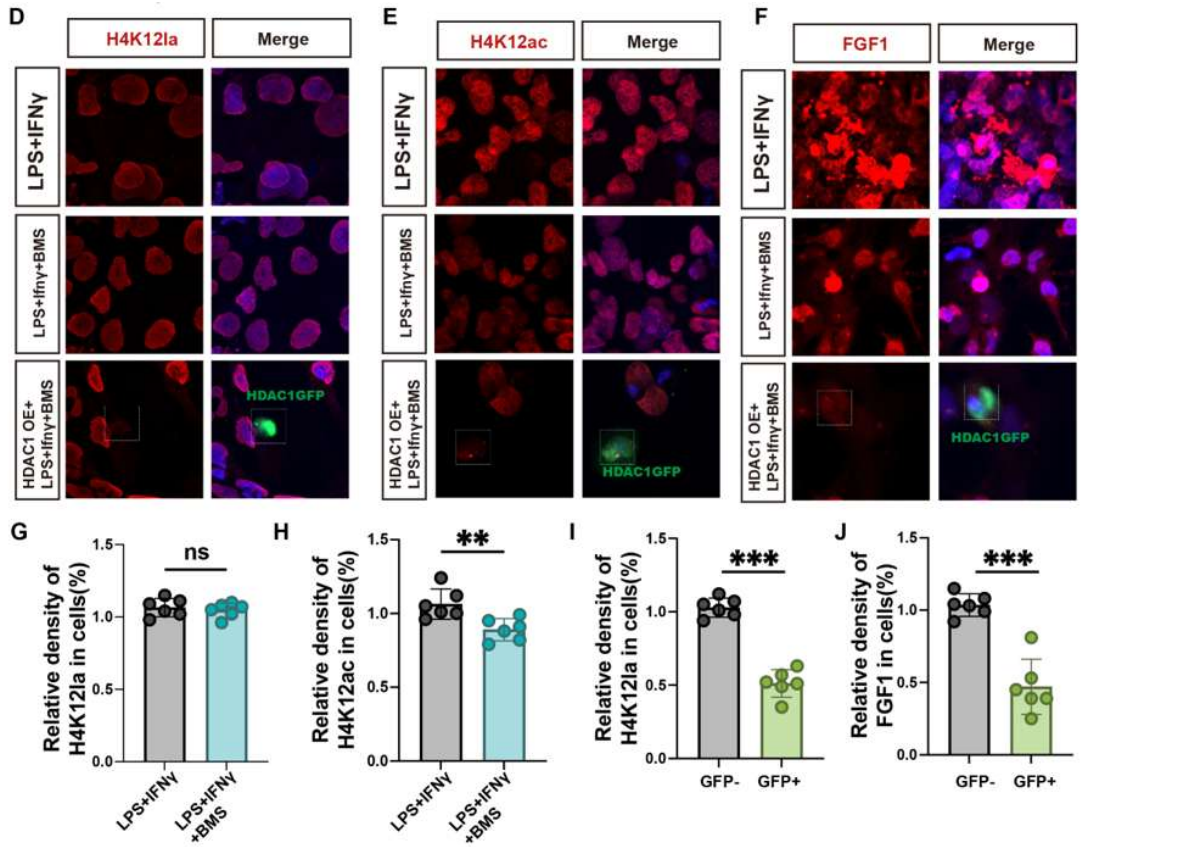


Fig. S10 Verification of lactate penetration and HDAC1-mediated delactylation

(D-F) Representative immunofluorescence images for H4K12la (D), H4K12ac (E), and FGF1 (F) in red. HMC3 cells were stimulated with LPS+IFNγ alone or in combination with the ACLY inhibitor BMS-303141. The bottom row displays HDAC1-GFP (green) overexpression in cells pre-treated with BMS-303141.

(G-H) Quantification of relative H4K12la (G) and H4K12ac (H) density following BMS treatment (n = 8 biological replicates).

(I-J) Comparison of H4K12la (I) and FGF1 (J) signal density between GFP-negative and GFP-positive (HDAC1-overexpressing) nuclei (n = 8 biological replicates). Scale bars 50 μm. Data represent mean ± SEM. Shapiro-Wilk tests were used to assess normality. Statistical significance was determined by Mann-Whitney U test based on distribution. $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not significant.

9. The statistical analysis must be revisited. The authors mostly rely on unpaired t-tests, yet, most of their data does not seem to follow a normal distribution. Therefore, they should use a Mann-Whitney U test (two experimental groups) or a Kruskal-Wallis test (three or more groups).

Response: We sincerely appreciate the reviewer's rigorous attention to our statistical analysis. We fully agree that applying the appropriate statistical test is fundamental to the validity of our conclusions. To address this, we systematically performed the Shapiro-Wilk test on every dataset to assess data distribution and have comprehensively updated our statistical analyses based on these normality assessments.

For datasets that violated normality assumptions, or involved small sample sizes (e.g., n=4 or 5, such as in Figure 1B), we transitioned to non-parametric tests as recommended. Specifically, we used the Mann-Whitney U test for two-group comparisons and the Kruskal-

Wallis test for comparisons involving three or more groups. Approximately 40% of our original experimental analyses were transitioned to these non-parametric methods.

For several key *in vivo* experiments (such as Figure 1F-J and Figure 2E-G), we actively increased the sample sizes to n=5-8 biological replicates. These expanded datasets successfully passed the Shapiro-Wilk test for normality. Consequently, we maintained the use of parametric tests (unpaired Student's t-tests or ANOVA) for these specific panels to ensure optimal statistical power. Additionally, for the *in vitro* HMC3 cell assays in Figure 8 (n=3 biological replicates), we retained parametric testing. This is because non-parametric tests lack the mathematical statistical power to detect significance at such small sample sizes (the minimum possible precise *p*-value is 0.1 for n=3).

Importantly, this rigorous statistical re-analysis yielded results that are completely consistent with our original findings across all comparisons. We have explicitly detailed these updates, including the specific tests used for each panel, in the revised Figure Legends and the "Statistical Analysis" section of the Methods.

Minor Criticisms:

1. Fig. 2C: The line depicting the linear regression analysis in the upper left graph appears to be driven by a single data point... The authors should consider performing an outlier analysis.

Response: We thank the reviewer for this keen observation and agree that the single data point in the upper right corner of the original Figure 2C appeared to drive the linear regression trend, potentially skewing the interpretation.

We performed outlier analysis using the ROUT method (Q = 1%). We refined the dataset to focus exclusively on Multiple Sclerosis (MS) patients and removed other IIDDS cases. This adjustment eliminates biological heterogeneity and ensures the correlation reflects the specific relationship between lactate and EDSS scores in MS. The positive association remains consistent after removing the outlier and focusing on the MS cohort. Supplementary Figure S1B (and Figure 1C) reflect these updates. Details of the outlier analysis are now included in the Methods section.

2. Fig. 2D: Quantification of Pan Kla normalized to an appropriate housekeeping protein alongside with the image of the immunoblot should be provided.

Response: We appreciate the reviewer's suggestion regarding Western blot normalization. We updated Figure S1C to include β-actin as an internal loading control for Pan-Kla signals. Pan-Kla and β-actin were detected on the same membrane. Quantification was performed using densitometric analysis of normalized band intensities.β-actin levels remained stable across experimental groups, justifying its use as a control. The revised figure presents representative immunoblot images and the corresponding quantification

3. *Fig.2J and I: Label should be „NeuN" instead of „Neun".*

Response: We apologize for this oversight regarding the nomenclature. The labels in Figure 1H and 1I (formerly 2I and 2J) were corrected to "NeuN". We standardized the nomenclature across the main text, figure legends, and methods to maintain manuscript-wide consistency.

4. Fig. 3: It is not clear at which time point EAE samples were collected to perform LC/MS. This should be specified in the figure legend.

Response: We appreciate the reviewer pointing out this omission regarding the sample collection timeline. We performed LC/MS analysis on CNS microglia collected at Day 21 and Day 55. These time points correspond to the peak acute phase and the stable chronic phase of EAE. We selected these intervals based on clinical scoring data showing maximal severity

around Day 21 and stabilization by Day 55. The Figure 2 legend now explicitly specifies this collection timeline.

5. Fig. S3: In the text the authors talk about Ly6C+ activated microglia. Ly6C is a marker for bone marrow-derived myeloid cells and is not expressed on microglia...

Response: We sincerely appreciate the reviewer for this critical correction regarding myeloid cell nomenclature.

We agree that Ly6C is a marker for bone-marrow-derived monocytes and is not expressed on resident microglia. The population previously labeled as "Ly6C+ activated microglia" was incorrectly annotated.

In EAE, the Ly6C+ population represents infiltrating inflammatory monocytes or macrophages. Our flow cytometry defined these cells by high CD45 expression and Ly6C positivity. Resident microglia were identified by a $CD45^{int}$ phenotype and remained outside the Ly6C+ gate. We updated the manuscript and Figure 5H–I to reclassify this population as "Ly6C+ infiltrating myeloid cells." This change improves terminological accuracy without affecting the study's conclusions. It clarifies that lactate treatment reduces pro-inflammatory cell accumulation alongside microglial reprogramming. We appreciate this important correction.

6. Fig. 4: Li et al. aim to limit Ldha knockout to microglia by inducing EAE two weeks after the last tamoxifen injection. This is rather short and therefore peripheral CX3CR1+ cells may still harbour reduced LDH levels. To support their hypothesis that loss of microglial Ldha mediates the observed effect, they should provide data from peripheral myeloid cells two weeks post-tamoxifen injection to show that their LDH levels are not affected.

Response: We appreciate this point. While we did not directly measure LDH levels in peripheral myeloid cells, existing data and the experimental design support the specificity of our microglial-targeted approach.

The 14-day interval between tamoxifen treatment and EAE induction relies on the rapid turnover of peripheral CX3CR1+ monocytes. Established literature (Goldmann et al., *Nat Neurosci* 2013) confirms that this period is sufficient for the full replacement of tamoxifen-targeted peripheral cells by non-recombined cells from the bone marrow[1].

Our internal findings further support this microglial-specific effect. Immunofluorescence in Figure S2D demonstrates the targeted depletion of LDHA protein specifically within Iba1+ microglia. Furthermore, the results in Figure 5H–K show that lactate remains therapeutically effective when administered at the disease peak, independent of any changes in the total number of infiltrating cells.

These consistent internal and external lines of evidence demonstrate that the exacerbated phenotype in *Ldha* icKO mice is driven by the loss of microglial LDHA rather than peripheral cell effects.

References

[1] Goldmann, J., et al.(2013). A new type of microglia gene targeting shows primary myeloid cells of the central nervous system are distinct from monocyte-derived macrophages. *Nature Neuroscience*, 16(11), 1618-1626.

7. Fig. 4: The authors show that Ldha icKO mice have a marked increase in neuroinflammatory cell infiltration. If their phenotype was solely explained by a loss of neuroprotective properties in microglia in response to LDH loss, infiltration by peripheral immune cells should not be modulated during the acute disease phase. The authors should discuss this finding.

Response: We appreciate the opportunity to clarify the link between microglial metabolism and peripheral infiltration. Microglia act as gatekeepers of CNS inflammation by regulating chemokine production and blood-brain barrier permeability.

Microglial LDHA deficiency shifts the transcriptional state toward pro-inflammatory signaling. This likely increases the expression of chemoattractants that facilitate early leukocyte recruitment during EAE.

Our late-stage lactate supplementation experiments (Figure 5) support this interpretation. Administering lactate at the disease peak improved pathology without changing the total number of infiltrating T cells or macrophages (Figure 5H–K). This dissociation suggests that once infiltration is established, disease outcome depends more on the functional state of myeloid cells than their absolute abundance.

We propose a two-phase model. During early disease, the loss of microglial lactate signaling promotes an environment that facilitates recruitment. Throughout disease progression, the absence of lactate-driven H4K12la prevents microglia from acquiring a reparative program, amplifying damage. We updated the Discussion to reflect this temporal distinction.

8. Fig. 4D: The authors should provide individual data points rather than a simple bar plot.

Response: We appreciate the reviewer's suggestion to enhance data transparency. We have revised Figure 4D to display individual data points overlaying the summary statistics. This modification provides a clearer visualization of the data distribution and sample size for the Area Under the Curve (AUC) analysis of EAE scores.

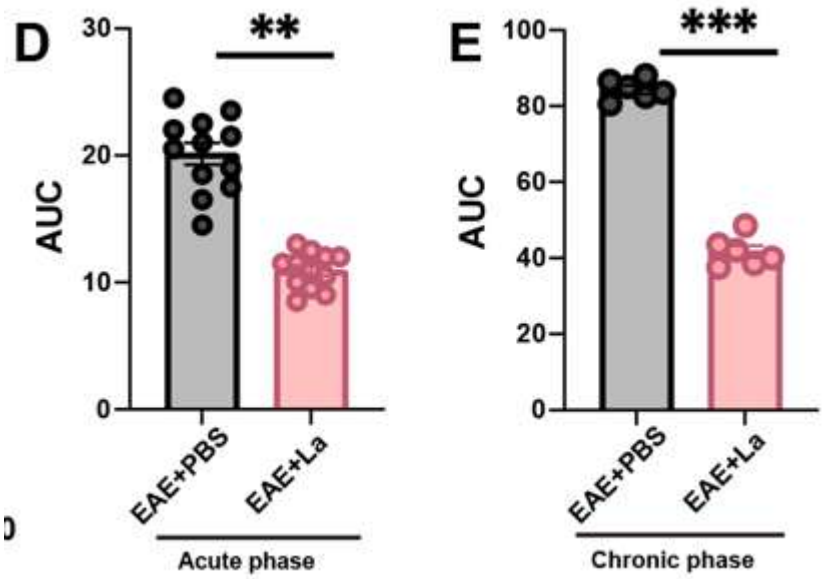


Fig. 4 Exogenous Lactate Supplementation Ameliorates EAE Severity and Enhances Microglial H4K12 Lactylation.

9. Fig. S4B: Font sizes in the figures are too small and should be increased to improve readability.

Response: We thank the reviewer for this helpful suggestion regarding figure presentation. We apologize for the small font size in the original Figure S4B. We increased the font size of all text elements in the new Figure S9 including axis labels, tick marks, legends, and annotations. We also reviewed all other supplementary figures and adjusted font sizes to ensure legibility at the final publication scale. High-resolution versions have been uploaded with the resubmission.

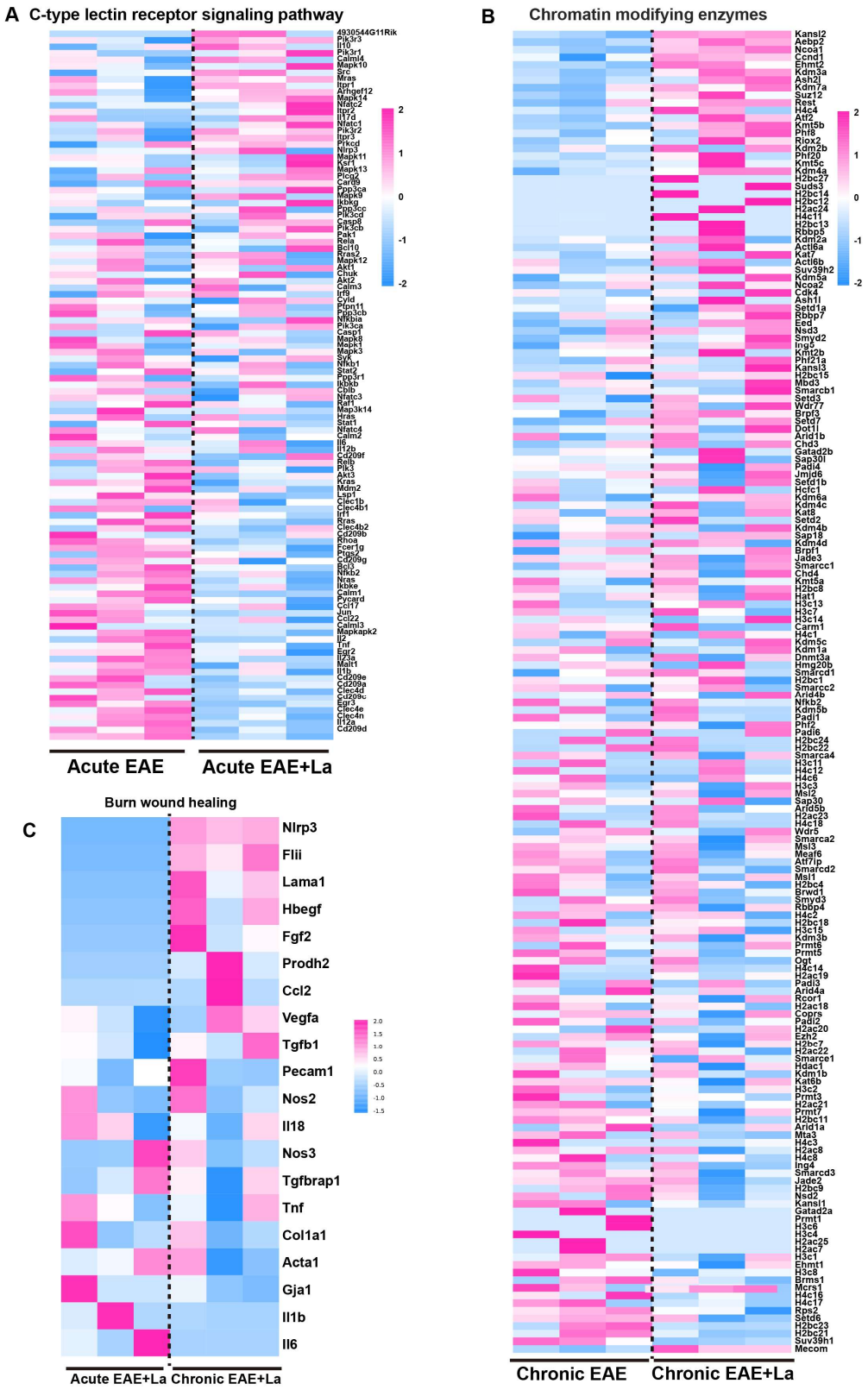


Fig. S9 Detailed Gene Expression Heatmaps for Key Pathways Modulated by Lactate Treatment in EAE.

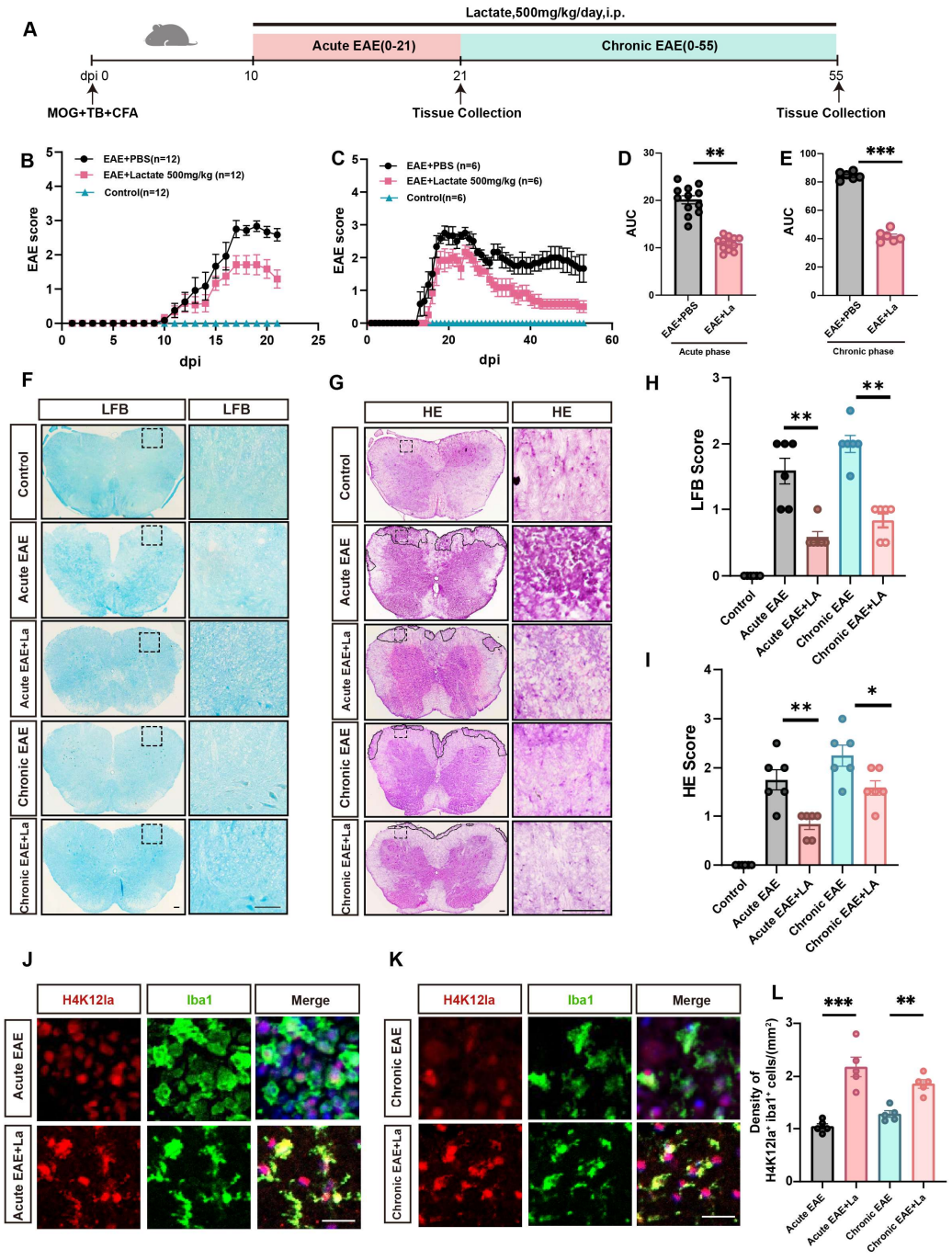
(A) Heatmap showing the expression of individual genes within the C-type lectin receptor signaling pathway" in microglia from Acute EAE and Acute EAE+La groups.

(B) Heatmap showing the expression of individual genes within the "Chromatin modifying enzymes" gene set in microglia from Chronic EAE and Chronic EAE+La groups.

(C) Heatmap showing the expression of individual genes within the "Burn wound healing" pathway, comparing microglia from the Chronic EAE+La group to the Acute EAE+La group to illustrate temporal evolution.

10. Fig. 5: The authors should use a consistent color code for the groups analyzed in Fig. 5B-5L.

Response: We agree with the reviewer that the use of inconsistent color schemes across panels in Fig.5B-5L reduced clarity. We unified the color scheme across Figure 5 (now Figure 4) to improve clarity. We applied consistent colors for EAE+PBS (black) and EAE+ Lactate (pink) across panels 4B–4L. These changes ensure each experimental group is visually distinct and easy to track. The figure legend now reflects this standardized color assignment.

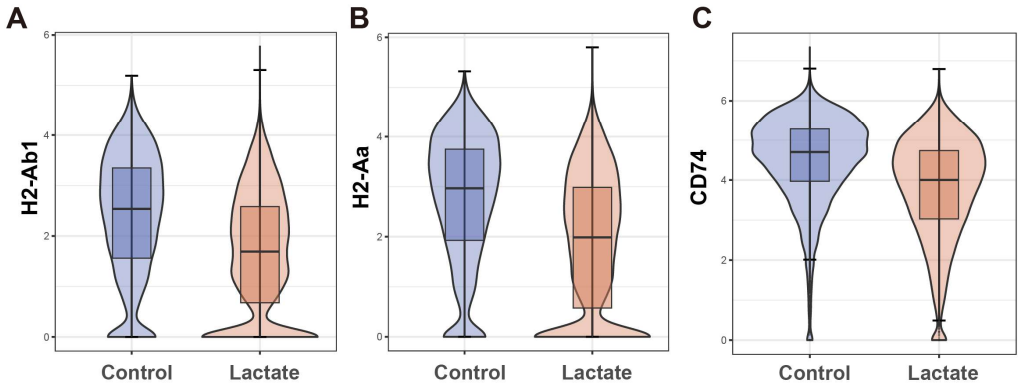


Now Fig. 4 Exogenous Lactate Supplementation Ameliorates EAE Severity and Enhances Microglial H4K12 Lactylation.

11. Fig. 7: When describing the phenotype of microglia in EAE, the authors should refrain from using the terms M1 and M2. Furthermore, they should include additional functional markers to characterize the microglia phenotype in addition to

Response: We removed the terms M1 and M2 throughout the manuscript. We now describe microglial states using functional programs such as inflammatory, antigen-presenting, and tissue-supportive.

We addressed the request for broader characterization by analyzing the suggested markers within our transcriptomic datasets. Our scRNA-seq (Figure 6D and Supplementary Figure S6) data show that lactate treatment significantly downregulates the expression of pro-inflammatory genes (e.g., *Ccl5*, *Tlr2*, *C3*, *Nfkbia*) and MHC II-related markers (e.g., *Cd74*, *H2-Ab1*, *H2-Aa*). Conversely, homeostatic and anti-inflammatory factors such as *Tgfbr1* and *Il10ra* were maintained or upregulated following treatment.We believe this high-resolution transcriptomic analysis provides a more robust and comprehensive view of the microglial phenotypic shift than individual immunostaining. These findings are now integrated into the revised Figure 6 and Figure S6 to better reflect microglial biology in EAE.



FigS6. Lactate treatment downregulates microglial MHC II-related markers.

12. Panels within each figure are not refered to in the correct order. Please adjust in the text or figures.

Response: We thank the reviewer for pointing out this issue. We reviewed the manuscript to ensure all figure panels appear in sequential order. We adjusted the text citations and rearranged specific figure layouts to follow a logical A-B-C sequence. The figure legends were updated to match these changes. We also corrected several cross-referencing errors between figure numbers. All panel citations now accurately reflect the final structure of the figures.

Response to Reviewers

We thank the Reviewers for their positive and thorough review of our manuscript and their insightful comments and suggestions. We have addressed all the issues raised and hope that the manuscript is now ready for publication. We proceed with a point-by-point reply (the comments of the reviewers are printed in *italics*).

Reviewer #1 (Remarks to the Author):

The authors responded to my comments.

Response:

We appreciate the reviewer's confirmation and time. We are pleased that our previous responses and revisions have satisfactorily addressed all of your comments.

Reviewer #2 (Remarks to the Author):

I appreciate the extensive efforts made by the authors to improve their manuscript and clarify the methodology and interpretation of the results. In particular the analysis and presentation of the scRNAseq data is much improved and now allows for biological interpretation.

My only remaining point relates to the inconsistent use of terminology for CNS myeloid cells. While in the rebuttal renaming to "CNS myeloid compartment" is mentioned, for example the figure title of figure 4 still refers to microglia (while IBA1 staining was used, which the authors agree that this is not specific).

Response:

We thank the reviewer for their positive feedback and for pointing out this remaining inconsistency. We have now thoroughly reviewed the entire manuscript, including all figures and legends, to ensure precise terminology. Specifically, we have corrected the title of Figure 4 from "microglia" to "CNS myeloid cells" to accurately reflect the cell population analyzed. We have also verified that the term "CNS myeloid cells" (or "macrophages/microglia" where appropriate) is used strictly and consistently throughout the text whenever non-specific isolation or staining methods (such as IBA1) were employed.

Reviewer #3 (Remarks to the Author):

Overall, the authors took the reviewers' comments seriously and amended the revised version of their manuscript accordingly. The quality of the study substantially improved and the functional link of lactate–H4K12la–HDAC1-mediated reprogramming of microglia in neuroinflammation.

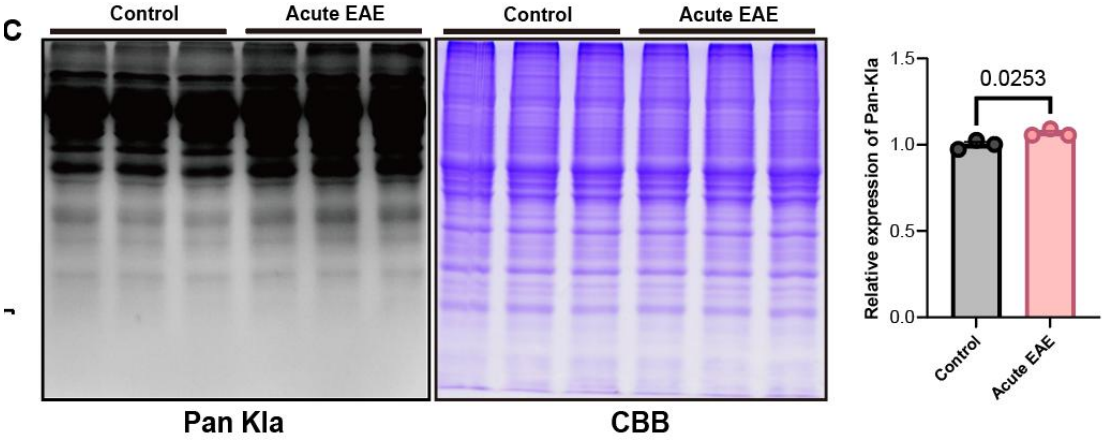
However, even after the inclusion of additional experimental data questions about the scientific rigor remain and need to be addressed prior to publication.

Major concerns

Major 1. As requested, the authors provide an immunoblot for a housekeeping protein to quantify Pan Kla expression (Fig. S1C). Yet, they did not provide the requested quantification of the Pan KIa expression in comparison to the housekeeping protein. Also, when having a closer look at the provided immunoblot image of the ß-actin bands, the membrane appears to be cropped right above and below the band. The authors should provide immunoblot images that allow the reader to judge the size of the individual bands and see potential unspecific bands.

Response:

We sincerely apologize for the lack of quantification and the heavily cropped presentation of the β-actin blot in our previous submission. To address your concerns with the highest scientific rigor, we have completely revised our loading control strategy by replacing the single housekeeping protein with Total Protein Normalization (TPN) using a Coomassie Brilliant Blue (CBB)-stained gel, which is highly recommended as a more reliable and unbiased control for global pan-modifications like lactylation. In the updated Figure S1C, we now provide the full-length, uncropped Pan Kla immunoblot alongside the corresponding full-length CBB-stained gel, allowing for full transparency regarding band sizes and specificities. Furthermore, we have performed the requested densitometric quantification of the entire Pan Kla lanes normalized to the total protein density. These quantification results have been added as a new bar graph in Figure S1C, definitively confirming a statistically significant elevation of global Pan Kla levels in acute EAE mice compared to controls ($p = 0.0253$). Complete, uncropped scans of all membranes are also included in the Source Data file as requested.



FigS1. Total protein normalization confirms significantly elevated global Pan Kla levels in acute EAE

Major 2. The authors clarified in the text that for the initial screening experiments CNS myeloid cells rather than pure microglia populations were used (Fig. 2 for proteomics, Fig. 7 for bulk RNA-seq). They argue that their findings are further supported by their in-depth single cell RNA

sequencing analysis, which allows them to specifically assess modulations in microglia (Fig. 6). While this is in general a valid approach, their single cell data lacks information about biological replicates and statistical comparisons. They use the same dataset to support their statement about the functional phenotype of microglia in response to lactate (Fig. S6), but also here do not provide any statistics. Some key findings should therefore be validated using microglia from individual bioreplicates.

Response:

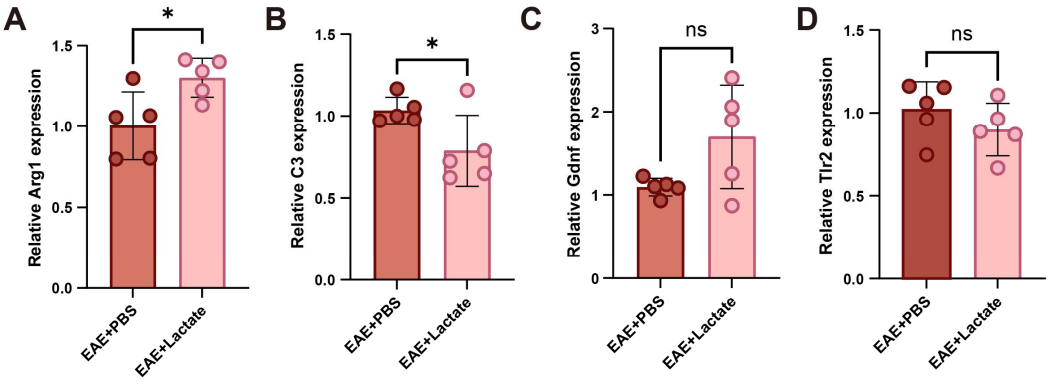
We thank the reviewer for this important critique and have addressed each point below.

(1) Clarification of scRNA-seq experimental design and biological replicates:

Our scRNA-seq dataset comprises two independent biological replicates per condition, with each replicate consisting of a pooled sample from two individual mice (4 mice per condition in total). Pooling was employed to reduce inter-individual variability and maximize cell recovery, consistent with standard practices in CNS single-cell transcriptomics. We have now explicitly described the sample composition, pooling strategy, and per-sample cell numbers in the revised Methods section.

We acknowledge that with n=2 pools per group, sample-level statistical inference is limited. We have therefore revised the figure legends for **Figure 6** and **Figure S6** to accurately reflect the sample composition, and we have performed independent RT-qPCR validation experiments (detailed below) to provide statistically powered confirmation of the key findings. We isolated highly pure microglial populations (gated as $CD45^{int}$CD11b+Ly6C-) from new, independent biological replicates (n = 5 individual mice per group) for RT-qPCR analysis. As shown in the newly provided figure, lactate treatment significantly upregulated the mRNA expression of the reparative marker *Arg1* ($p < 0.05$, Panel A) and significantly downregulated the expression of the pro-inflammatory marker *C3* ($p < 0.05$, Panel B) in purified microglia. Furthermore, while the neurotrophic factor *Gdnf* (Panel C) and the inflammatory receptor *Tlr2* (Panel D) did not reach strict statistical significance due to inherent in vivo biological variance among individual mice, they exhibited clear functional trajectories of upregulation and downregulation, respectively. These statistically powered changes and consistent expression trends at the individual mouse level perfectly mirror the transcriptomic modulations observed in our single-cell dataset, thereby providing robust, independent confirmation that lactate suppresses inflammatory states and

promotes reparative microglial programs.



FigS8. RT-qPCR validation of key phenotypic genes in isolated microglia

(2) Independent validation using purified microglia from biological replicates:

To directly address the reviewer's concern, we isolated pure microglial populations by FACS sorting ($CD45^{int}CD11b+Ly6C-$) from new independent biological replicates (n=5 per group) and validated the expression of key genes identified by scRNA-seq, including *Arg1*, *C3*, *Tlr2*, and *Gdnf*, by RT-qPCR. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests. These results confirm the transcriptional changes identified in our single-cell analysis and are incorporated into the revised manuscript as **Figure S8**. The consistency between the scRNA-seq findings and the RT-qPCR validation from independently sourced, purified microglia supports the reproducibility of our conclusions.

Major 3. As suggested, the authors performed additional experiments with exogenous lactate supplementation at the peak of disease (day 14 post-immunization) and claim that this treatment regimen significantly improved clinical scores. However, from Fig. 5B it becomes evident that the animals have not been stratified correctly to individual treatment arms since the dpi14 lactate group (blue) starts with a decreased disease severity prior to treatment start. Therefore, all additional readouts (AUC, LFB, etc.) are not reliable. They also argue that the immune cell infiltrate (T cells, monocytes) does not differ, but only provide frequencies rather than absolute cell counts. This part needs to be revisited.

Response:

We thank the reviewer for raising this critical point regarding the baseline disease severity in the dpi14 lactate treatment cohort. We have carefully revisited this experiment and taken the following corrective actions:

(1) Baseline stratification and re-evaluation: We acknowledge that the original Fig. 5B suggested a potential baseline imbalance between treatment arms at dpi14. We have now re-performed the experiment with strict baseline matching — animals were ranked by clinical score at dpi14 and allocated to treatment vs. vehicle groups using a stratified randomization approach to ensure identical mean scores at the time of treatment initiation. The updated **Fig. 5B and 5C**

clearly demonstrate comparable disease severity at baseline, followed by a significant therapeutic divergence after LA administration. Consequently, all downstream readouts have been updated based on this newly stratified cohort. To provide a precise and reliable quantitative assessment of the therapeutic effect, we now present statistical comparisons at specific critical time points (dpi 17, 20, and 21).

(2) Absolute cell counts: As requested, we now provide absolute cell counts for T cells and monocytes in the revised supplementary figures, allowing for a more comprehensive assessment of immune cell infiltration.

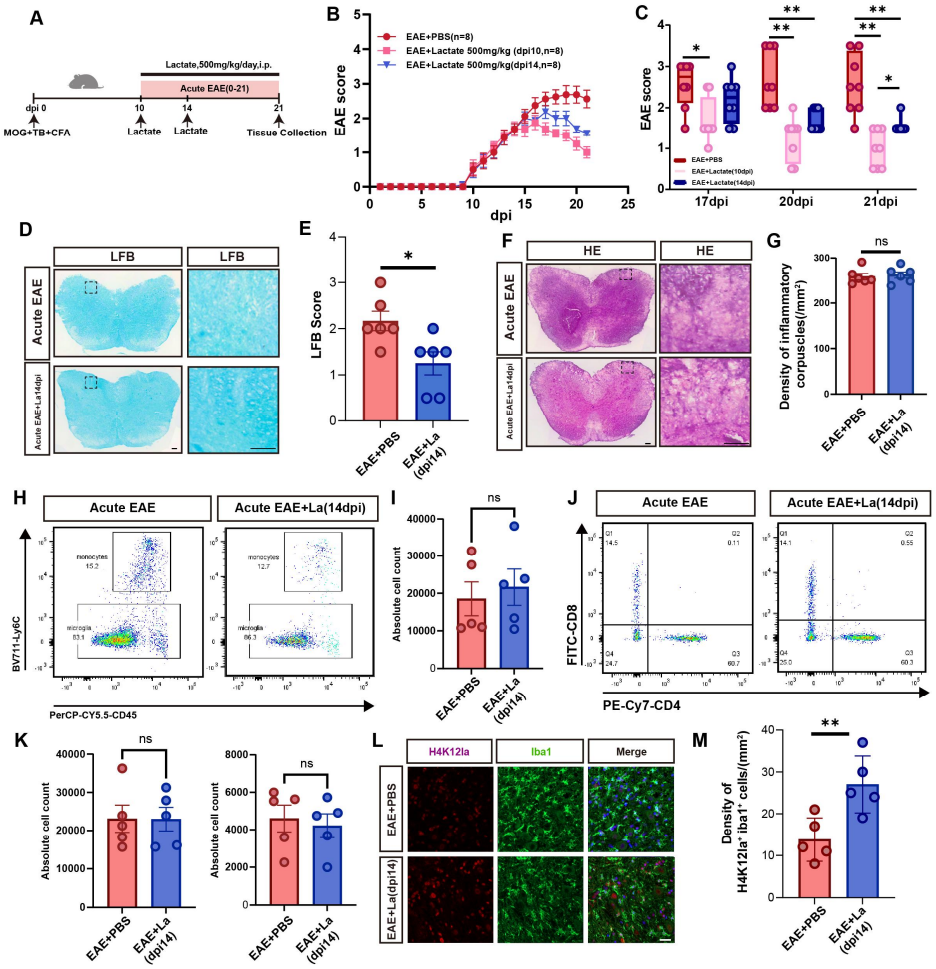


Figure 5. Lactate treatment initiated at peak disease ameliorates EAE severity via CNS myeloid cell reprogramming without altering immune infiltration.

Minor concerns

Minor 1. Fig. 2A and 7A: The schematic representation of the experimental workflow still suggests that the author analyzed microglia, when indeed CNS myeloid cells were used. Please adjust. Also, there is a typo in "Magnetic labeling/separation".

Response:

We apologize for the oversight. We have corrected the schematic representations in both **Figure 2A** and **Figure 7A** to accurately state "CNS myeloid cells" instead of "microglia." The typo in "Magnetic labeling/separation" has also been corrected. The updated schematics are included in the revised manuscript.

Minor 2. As suggested, the authors included an HDAC inhibitor (MS-275) to corroborate their findings and refer previous studies that identified HDAC1 as "eraser" (ll. 227-229). However, they fail to provide a reference for this statement.

Response:

We thank the reviewer for pointing out this omission. We have now added the appropriate high-impact references to support the statement that HDAC1 acts as an enzymatic "eraser" (delactylase) for histone lactylation. Specifically, we cited:
• Moreno-Yruela, C., et al. (2022). Class I histone deacetylases (HDAC1–3) are histone lysine delactylases. Science Advances, 8(3), eabi6696.
• Sheng, X., Lin, H., Cole, P. A. & Zhao, Y. (2026). Biochemistry and regulation of histone lysine L-lactylation. *Nature Reviews Molecular Cell Biology*, 27(2), 95–109.These references have been inserted at lines 227-229 of the revised manuscript.

Minor 3. The authors state that they corrected the typo in "NeuN" in Figure 1H and 1I (formerly 2I and 2J). However, this typo is still present in the provided version.

Response:

We sincerely apologize for this repeated error. We have now thoroughly checked and definitively corrected the spelling of "NeuN" in both **Figure 1H** and **Figure 1I.** The corrected figures are included in the revised manuscript.

Minor 4. To corroborate their findings in microglia-specific Ldha knockout mice, the authors were asked to provide supporting data to validate the restriction of their knockout to microglia. They argue that after 2 weeks all targeted peripheral cells have been re placed without providing additional data. Based on their suggested publication > 10% of peripheral Ly6C+ monocytes (from initially 40%) remain labeled with a marker fluorochrome after 2 weeks, indicating that the turnover is slower. They furthermore refer to immunohistochemistry data in Iba1+ cells. However, since in naive mice only sparse infiltrates by peripheral myeloid cells are present, this is not informative. Therefore, the authors should provide data of lactate levels in peripheral myeloid cells 2 weeks after tamoxifen injection.

Response:

We thank the reviewer for highlighting this important point. We agree that functionally validating the restriction of the *Ldha* knockout to microglia at the time of EAE induction is crucial for the interpretation of our results.

As explicitly requested, to address the concern regarding the turnover of peripheral myeloid cells, we performed additional experiments to evaluate the lactate metabolism in the peripheral myeloid

compartment. Specifically, we isolated CD11b^{+} myeloid cells (which encompass the peripheral monocyte/macrophage pool) from the spleens of tamoxifen-treated *Cx3cr1-CreERT2; Ldha$^{fl/fl}$* mice and control mice exactly 2 weeks after the last tamoxifen injection. We then measured the intracellular lactate levels in these cells.

The new data (now included as **Supplementary Figure S3A**) demonstrate that intracellular lactate levels in peripheral splenic CD11b^{+} myeloid cells were comparable between the *Ldha* icKO mice and controls, showing no significant difference.

These functional results confirm that, following the 2-week washout period, the peripheral myeloid cell pool has been sufficiently renewed. Even if a minor fraction of slowly turning-over cells remains as the reviewer pointed out, it is not sufficient to alter the overall lactate metabolism of the peripheral myeloid compartment. Therefore, this finding functionally validates that the exacerbated EAE phenotype observed in our model is specifically driven by the sustained *Ldha* deletion in long-lived CNS-resident microglia, rather than peripheral effects. We have updated the manuscript and supplementary figures to include these supporting data.

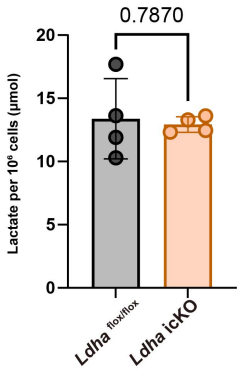


Fig S3A. Peripheral myeloid lactate metabolism is fully restored in *Ldha* icKO mice.

Minor 5. The authors adjusted the color code for their lactate treatment EAE (now Fig. 4 and 5). However, it is still misleading that in the schematic representation they use pink and blue to mark the acute and chronic phase of the disease, but also use these colors in B-L to label their treatment groups. Please revise.

Response:

We appreciate the reviewer's feedback on the color scheme. We have completely revised the color coding in **Figures 4 and 5** to eliminate any potential confusion. Specifically, the schematic representations (panel A) now use a distinct set of colors that are clearly differentiated from the treatment group colors used in the data panels (B-L). A revised color legend has been added to ensure clarity.

Minor 6. The authors state they have rearranged figure panels to appear in sequential order. However, in the revised version of the manuscript the supplementary figures are not referred to in a chronological order, which makes it difficult to read (i.e. Fig. S11 and S10 right after S2).

Response:

We apologize for the confusion caused by the disordered supplementary figure numbering. We have now carefully reviewed the entire manuscript text and renumbered all Supplementary Figures so that they are cited in strict chronological order of their first appearance in the text. The supplementary figure files have been renamed and reordered accordingly. We believe this revision significantly improves the readability of the manuscript.

Minor 7. The discussion lacks some references for their statements in ll. 546-557.

Response:

We thank the reviewer for the suggestion. We have substantially strengthened the referencing in the Discussion section (lines 546-557). We have incorporated several high-impact publications that provide supporting evidence for our statements, including:

• Certo, M., et al. (2021). Lactate modulation of immune responses in inflammatory versus tumour microenvironments. Nature Reviews Immunology, 21(3), 151-161.

• Ziogas, A., et al. (2025). Long-term histone lactylation connects metabolic and epigenetic rewiring in innate immune memory. Cell.

• Cai, H., et al. (2025). Lactate activates trained immunity by fueling the tricarboxylic acid cycle and regulating histone lactylation. Nature Communications, 16, 3230.

• Miron, V. E., et al. (2013). M2 microglia/macrophages drive oligodendrocyte differentiation during CNS remyelination. Nature Neuroscience, 16(9), 1211-1218.

These and additional references have been incorporated into the revised Discussion to provide comprehensive support for our interpretations. The complete updated reference list is included in the revised manuscript.

Response to Reviewers

We thank the Reviewers for their positive and thorough review of our manuscript and their insightful comments and suggestions. We have addressed all the issues raised and hope that the manuscript is now ready for publication. We proceed with a point-by-point reply (the comments of the reviewers are printed in *italics*).

Reviewer #3 (Remarks to the Author):

I would like to thank the authors for their thorough and thoughtful revision of the manuscript. They have invested considerable effort in addressing all of the comments raised during review process, and the resulting revised manuscript has significantly improved. I am satisfied with the revisions and support publication in Nature Communications.

Response:

We are deeply grateful to the reviewer for their highly positive evaluation, their recognition of our revision efforts, and their strong support for publication. We also sincerely appreciate the time and expertise they invested throughout the review process, which have been invaluable in improving the overall quality and rigor of our manuscript.