nature portfolio

Peer Review File

NR3C1-mediated epigenetic regulation suppresses astrocytic immune responses in mice

Corresponding Author: Professor Inkyung Jung

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)
In the present study, Park et al. describe the relevance of NR3C1 epigenetic regulation in astrocytes during development. The authors used transcriptomic, chromatin accessibility and chromatin interaction analysis to identify regulatory elements in astrocytes during mouse development in pre and postnatal stages. The authors then focus on NR3C1 and use conditional knockout and EAE mouse models to show that a NR3C1 knockout during astrocyte development epigenetically predisposes astrocytes to increased inflammatory responses in the context of EAE.

This is a nice, comprehensive study. Experiments are properly done, controlled and described. This manuscript should be of interest to the broad readership of Nature Communications. However, some questions should be addressed before this work is ready for publication.

Major Comments

1. The contribution of NR3C1 deletion in astrocyte development leading to predisposition to astrocytic immune responses in autoimmunity is not completely convincing. The NR3C1 KO could also affect immediate immune response in mature astrocytes. What happens if NR3C1 is depleted in late stage, ie after P30 just before EAE induction?

2. Does inflammation regulate NR3C1 expression in astrocytes? To address this question, can the authors compare NR3C1 expression of astrocytes in WT and EAE mice?

3. The authors should also provide staining's in the spinal cord for CD4 T cells and GFAP expression.

4. Does NR3C1 KO have an effect on the phenotype of CNS-infiltrating CD4 T cells/ microglia/myeloid cells or both? Is there any difference in effector CD4 T cell subsets (i.e., producing IFN-γ, IL-17A, GM-CSF)?

5. The authors focus on the role of NR3C1 in astrocytes, specifically examining gene expression in the corpus callosum. Given that the EAE model, primarily a spinal cord inflammatory model, do the authors consider whether astrocytes in other CNS regions might also be affected?

6. The authors may want to mention in their discussion a recent report on the role of the related receptor NCR3C2 in the regulation of astrocyte responses and EAE/MS (clark et al, Nature 2023), which suggests shared roles and therapeutic value for multiple members of this TF family.

Minor Comments

1. Figure 4h: IBA1 is only mildly and non-significantly overexpressed in NCR3C1 KO mice, this should be corrected.

Reviewer #2

(Remarks to the Author)
This manuscript explores a novel aspect of astrocyte biology, focusing on the role of NR3C1 in mediating astrocytic immune responses via epigenetic mechanisms during development and disease contexts. The study employs robust methodologies, including multi-omics approaches, conditional knockout models, and single-nucleus RNA sequencing, to reveal NR3C1's regulatory role in astrocytes. The manuscript is well-structured, and the experiments are meticulously designed. The findings are significant, providing insights into astrocytic vulnerability to immune dysregulation in the context of diseases like multiple sclerosis. However, there are several points that need clarification and additional discussion to strengthen the impact and reproducibility of the findings.

Specific comments:

1. The manuscript emphasizes NR3C1 as both a transcriptional activator and repressor. However, the molecular mechanisms underlying this dual functionality in astrocytes and how this feature is related to disease pathology remain vague. For example, it is not clear whether exacerbated immune responses upon EAE induction are mediated by dysregulation of activator or repressor function, or both. Consider experimentally elaborating on how combinatorial actions with other transcription factors (e.g., AP-1, NF-κB) are contextually regulated and whether these interactions are subtype- or developmental time-specific. In this regard, it would also be interesting to investigate whether p300, a transcription coactivator previously reported to be critical for memory astrocytes and EAE, has any role in NR3C1-dependent epigenetic priming in astrocytes.
2. The study suggests that NR3C1 depletion during the early postnatal stage predisposes astrocytes to immune dysregulation later in life. While this hypothesis is compelling, further evidence related to the duration of epigenetic priming events is required to substantiate this model. The current study only investigated P17 for the comparison of gene expression and chromatin accessibility with and without EAE induction. Considering that MS onset in humans occurs at 20–40 years of age (approximately 12 weeks in mice), longitudinal studies linking early epigenetic changes to late-stage outcomes would corroborate the authors' conclusion regarding the role of NR3C1 in MS pathology.
3. The ChIP-seq data in Fig. 6e only show log2 FC ratios of ChIP signal over input without any peak analysis, making it difficult to appreciate the quality of the ChIP-seq data. There do not appear to be significant differences in NR3C1 ChIP-seq signals between the upregulated genes and control genes. Given that this data is important for understanding the direct role of NR3C1, it is advised to provide more details on the ChIP-seq analysis.
4. The color scale used in Figure 2A lacks sufficient contrast. Consider enhancing the contrast or selecting a more distinct color gradient to improve readability.

Reviewer #3

(Remarks to the Author)
In this study, Park, Park et al., focused on investigating astrocyte developmental programs linking them with the susceptibility to neuroinflammatory diseases. As the contribution of astrocytes to various conditions, despite their important role in the central nervous system (CNS), is not yet well understood, the subject is of relevance to a broad readership. The authors performed a comprehensive study of gene regulatory programs during development of astrocytes from initial gliogenesis (embryonic day 16) to mature astrocyte stage (postnatal day 30) using a reporter mouse line (Aldh1l1-EGFP transgenic) and multi-omics, including transcriptome (RNA-seq), open chromatin (ATAC-seq) and chromosomal interactions (HiCAR).

They describe gene regulatory programs characteristic for the three distinct developmental stages (based on the stage-specific cis regulatory elements and their target genes defined using the activity-by-contact model). They identified novel transcription factors (TFs) involved in astrocyte development, and in particular, the important role of NR3C1 during early postnatal period. Deletion of NR3C1 in astrocytes resulted in exacerbated inflammatory responses in adults and enhanced symptoms of a widely used model (EAE) of autoimmune neuroinflammation in general and multiple sclerosis (MS) in specific. Interestingly, they demonstrated that the lack of NR3C1 predisposed for the epigenetic profile with an accessible chromatin at inflammatory genes during early development, which exerted its function only in adults and upon challenge, leading to activation of inflammatory pathways.

This is an interesting and well-conducted study that highlights the role of astrocytes in neuroinflammation and links molecular modulations/disturbances of developmental programs with the vulnerability to neuroinflammatory pathologies later in life.

Major points:
1. Given the anti-inflammatory effects of glucocorticoids (GCs), their wide use to treat acute relapses in MS, and the documented immune functions of astrocytes, the motivation to study NR3C1in EAE/MS (lines 184-187) should be adjusted to include these aspects.
2. Please describe the details of control groups of mice, especially in EAE experiments (a breeding scheme would be nice). What exactly is Cre- WT? I assume that they are Cre-negative littermate wild-type controls obtained from the same breeding used to generate Cre-positive KO animals? Has Cre-positive control been tested for any of the phenotypes (given potential impacts of Cre, PMID: 29336844). Since the Aldh1l1-creERT2 strain seems to have a mixed BL6/N and J background (with some contribution from FVB/N), controls are critical, especially as only two replicates were used for multi-omics profiling.
3. If I understood correctly, GSEA analyses were conducted on differentially expressed genes (DEGs), not all ranked genes for a given comparison. I would advise being more stringent with thresholds (wrt detection, fold-change and significance cutoffs), given that a heterogeneity between the individual animals is likely (due to the nature of EAE disease, random distribution of inflammation in the CNS, genetic background of animals) and since only two replicates were run. For example (Fig. 4d and e) 2,256 down- and 1,895 up-regulated DEGs (based on the unadjusted p-value < 0.05) were considered for GSEA, but despite many DEGs, the fact that TF was KO and expression measured in a pure cell type, it is surprising that very few GO terms were (barely) significant. Additionally, the rationale for GSEA on up- and down-regulated genes separately, when looking at functional consequences is unclear, as functions/pathways are most often controlled by both up- and down-regulation of the constituent genes). Analyzing DEGs separately makes sense in (later) analysis when the focus is on the role of NR3C1 as a repressor or activator, but for obtaining insights into processes regulated by KO, GSEA on combined DEGs and with more stringent selection is better suited, and would give a possibility to estimate activation state of significant GO terms (a quick analysis on combined up- and down-regulated genes with FDR < 5%, 1719 DEGs, gives many more highly significant GO terms). As QC, it would be nice to know that GRs, alone or in combination with co-factors, are among predicted upstream regulators. It would be important to double-check GSEA analysis in order to establish if the statement “Unexpectedly, the DEGs in NR3C1 cKO astrocytes did not exhibit significant enrichment for immune response pathways among either up-regulated or down-regulated genes (Fig. 4e)” truly holds given the large number of DEGs.
4. In MOG35-55-induced EAE on the BL6 background, the spinal cord is predominantly affected by inflammation, demyelination and axonal transection. What is the rationale for focusing on corpus callosum and cortex? Have the authors investigated any of the studied changes in spinal cord?
5. I could not find GSEA and the corresponding expression in MS lesions for the specific group of genes that displayed binding of NR3C1, accessible chromatin during development and get up-regulated during EAE (Fig. 6)? I might have missed it, but this list of genes and associated functions seem the most interesting. How do they behave in MS, and if possible, present active, chronic active and inactive lesions (NAWM if available) separately? “Furthermore, the subtle reduction of NR3C1 in the corpus callosum of MS patients suggests that the role of NR3C1 in brain immune responses extends beyond the mouse EAE model”. Any chance to connect NR3C1 levels with open chromatin in astrocytes in non-inflammatory controls, NAWM, inactive, chronic inactive and active lesions in MS from a publicly available dataset? It would be very interesting to see the outcome.
6. “Thus, our study highlights the importance of early genetic regulation and its long-term impact on health, offering potential insights for early interventions and therapeutic strategies to prevent mature-onset diseases.” How does this translate to human conditions (MS in the context of NC3R1 specifically)? Is there any evidence of genetically and/or environmentally-associated changes in NC3R1 that can occur in humans and lead to vulnerability later in life? On the other hand, what happens upon treatment with GCs in MS? Can these rounds of GCs have lasting effects given the documented epigenetic imprint of GCs in peripheral immune cells (PMID: 31064978)?

Minor points:
1. The relevance of GSEA in Fig. 3 is unclear, e.g., is the ‘Multiple Sclerosis’ term genes based on DEGs identified in the CNS tissue or blood? The authors should consider enrichment analysis across polygenic risk score loci, which might give more evidence for the involvement of astrocytic genes in the disease etiology.
2. Please provide the rationale of not including Klf2, Fosb and Nr4a1 in further analysis (Fig. 3), they are also mid-stage genes that seem to exhibit exclusive perturbation impact on astrocytes.
3. “Approximately half of the NR3C1 cKO mice reached a clinical score of 5, characterized by sudden death or severe paralysis.” How were these mice accounted for in the plots and statistical analysis? Please provide full statistics on the incidence and mortality.
4. Please provide short motivation for the use of the Aldh1l1-EGFP transgenic line, e.g., how comprehensive is the Aldh1l1 promoter. Expression is obviously stable throughout examined stages (Suppl. Fig 1), but are there any astrocyte populations that could have been missed due to the lack of Aldh1l1 expression?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my comments.

Reviewer #2

(Remarks to the Author)

The authors adequately addressed the key questions raised by the reviewers. Although not entirely satisfactory, the revised manuscript includes new experimental data and provides more mechanistic interpretations, thereby improving its overall rigor and clarity. This reviewer supports the publication of the revised manuscript in Nature Communications.

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed my comments.

We thank all three reviewers for their thoughtful critiques and constructive feedback. In response to the reviewers' comments, we have substantially revised the manuscript. We believe the revised manuscript has been significantly improved in rigor, clarity, and impact.

Our major improvements include:

1. **Determine the impact of NR3C1 deletion induced at early versus late stages of astrocyte development**: We conducted comparative analyses after depleting NR3C1 at postnatal day 0 (P0-induced) and day 30 (P30-induced). Molecular profiling (RNA-seq / ATAC-seq) revealed that only perinatal deletion, but not the late-stage deletion, leads to sustained epigenetic priming of immune response genes, underscoring the developmental importance of NR3C1.
2. **Determine the long-term persistence of epigenetic priming induced by NR3C1 depletion**: By performing RNA-seq and ATAC-seq on astrocytes from 12-week-old mice, we found that astrocytes from perinatal NR3C1 cKO mice retained long-lasting epigenetic marks that predispose them to immune reactivity.
3. **Characterize immune cell profiles upon NR3C1 deletion in the EAE model**: We performed additional immunohistochemistry, flow cytometry, and transcriptomic analyses using brain and spinal cord samples, and identified increased infiltration of myeloid and T cell populations in both tissues from NR3C1-deleted mice at the peak of EAE. These data also show that the inflammatory phenotypes previously observed in the cortex and corpus callosum are present in other regions of the central nervous system, such as the spinal cord.
4. **Examine the impact of co-binding between c-JUN and NR3C1 using ChIP-seq**: ChIP-seq for NR3C1 and c-JUN was performed to delineate the dual regulatory roles of NR3C1 (as an activator or suppressor). NR3C1 binding at candidate cis-regulatory elements (cCREs) varied depending on co-occupancy with c-JUN, revealing context-dependent transcriptional regulation.

These revisions collectively strengthen the conclusion that NR3C1 is a key developmental regulator of astrocytic immune competence, with long-lasting epigenetic implications for neuroinflammatory diseases such as multiple sclerosis. Please see below for our point-by-point responses to each reviewer in *blue Italic*.

Reviewer #1

In the present study, Park et al. describe the relevance of NR3C1 epigenetic regulation in astrocytes during development. The authors used transcriptomic, chromatin accessibility and chromatin interaction analysis to identify regulatory elements in astrocytes during mouse development in pre and postnatal stages. The authors then focus on NR3C1 and use conditional knockout and EAE mouse models to show that a NR3C1 knockout during astrocyte development epigenetically predisposes astrocytes to increased inflammatory responses in the context of EAE.

This is a nice, comprehensive study. **Experiments are properly done, controlled and described. This manuscript should be of interest to the broad readership of Nature Communications**. However, some questions should be addressed before this work is ready for publication.

We sincerely appreciate the reviewer's positive feedback recognizing the significance of our findings in advancing the understanding of astrocyte developmental processes. We have carefully addressed each of the reviewer's concerns in the detailed, point-by-point responses below.

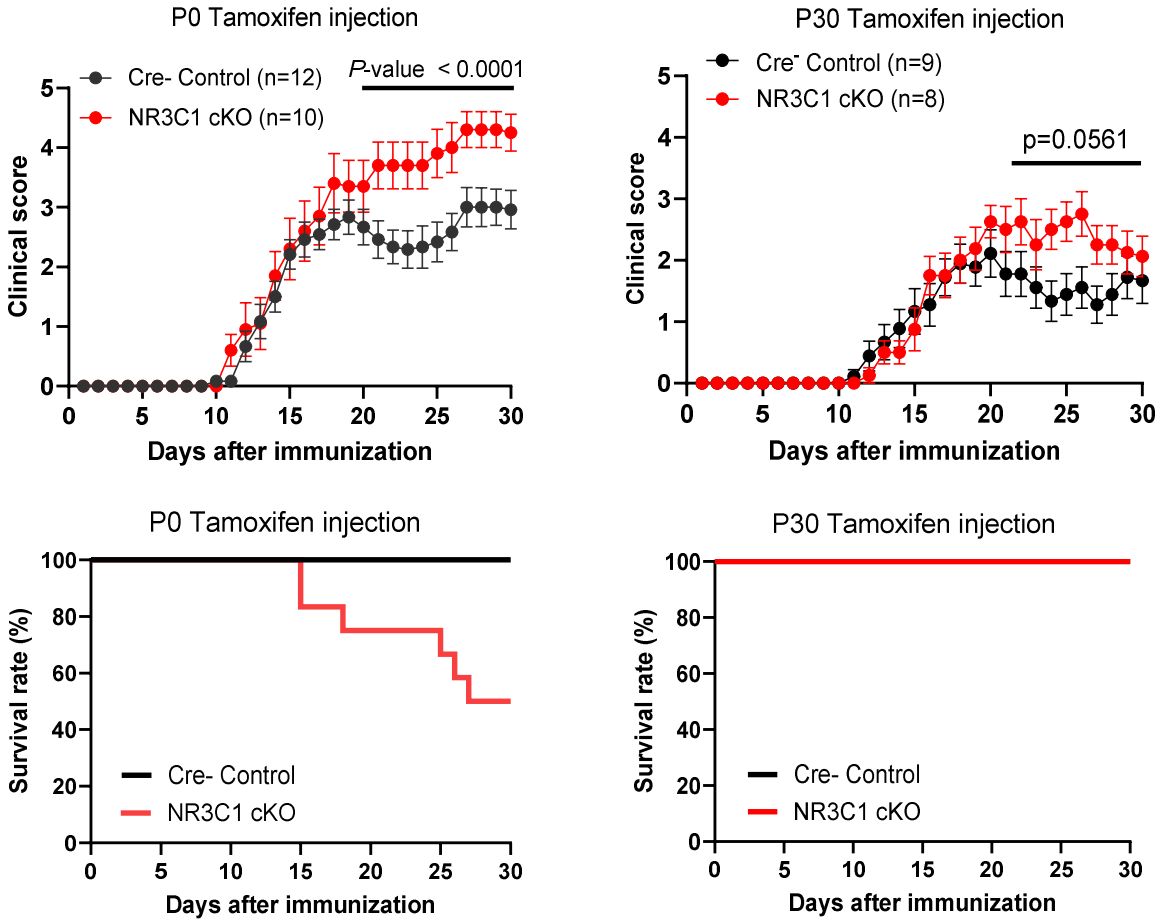
Major Comments

1. The contribution of NR3C1 deletion in astrocyte development leading to predisposition to astrocytic immune responses in autoimmunity is not completely convincing. The NR3C1 KO could also affect immediate immune response in mature astrocytes. What happens if NR3C1 is depleted in late stage, ie after P30 just before EAE induction?

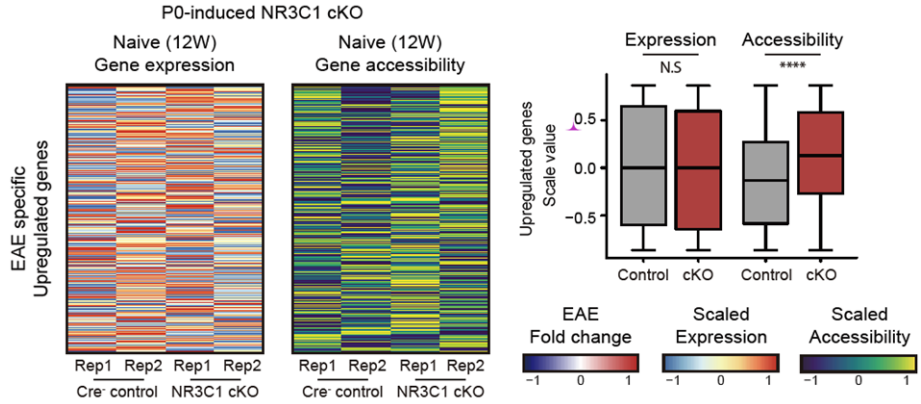
We thank the reviewer for this important comment. To address this concern, we performed additional EAE experiments using NR3C1 cKO mice with tamoxifen injection at P30 (P30-induced NR3C1 cKO). As shown in Responsive Figure. 1, these mice also exhibited increased EAE scores compared to controls. However, unlike the P0-induced NR3C1 cKO mice, none of P30-induced NR3C1 cKO mice reached a clinical score of 5. This contrasts sharply with our previous data, which showed that approximately half of the P0-induced NR3C1 cKO mice reached a clinical score of 5, characterized by sudden death or severe paralysis.

To further investigate the differential impact of NR3C1 deletion induced at early versus late stages of astrocyte development, we conducted comparative RNA-seq and ATAC-seq analyses of P0-induced and P30-induced NR3C1 cKO astrocytes. Our new data revealed that only astrocytes from the P0-induced NR3C1 cKO group, but not the P30-induced group, displayed sustained epigenetic priming of EAE-responsive upregulated genes (new Fig. 6e, new Supplementary Fig. 6f). The dysregulated cCREs identified in astrocytes from P0-induced NR3C1 cKO mice remained accessible up to 12 weeks of age, including those linked to EAE-specific upregulated

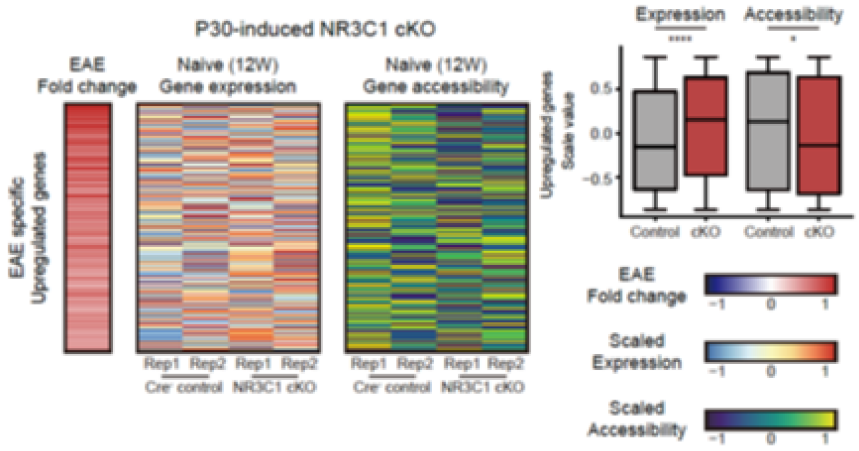
genes (new Fig. 6f). These findings suggest that while NR3C1 depletion in mature astrocytes may influence immediate immune responses, its loss during early development exerts a more profound and long-lasting impact by epigenetically predisposing astrocytes to heightened immune activation in EAE. We have revised the main text accordingly and included the new data in Fig. 6 and Supplementary Fig. 7.



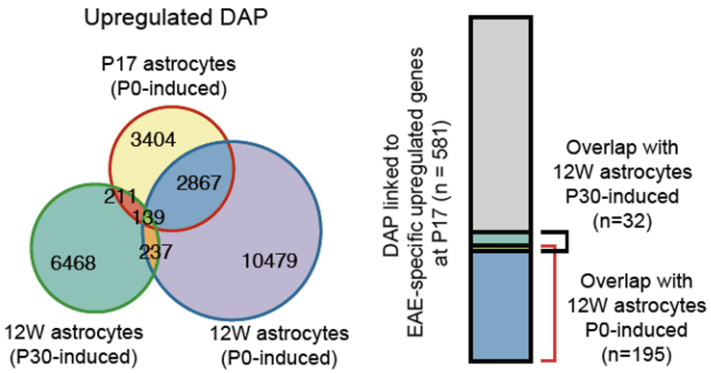
Responsive Figure. 1 The progression of EAE clinical score and survival rates in Cre$^-$ control and NR3C1 cKO mice following P0-induced (left) or P30-induced (right) NR3C1 cKO. Only P0-induced NR3C1 cKO mice exhibited mortality within 30 days after immunization (bottom left). Cre$^-$ control (P0-induced), n=12; NR3C1 cKO (P0-induced), n=10; Cre$^-$ control (P30-induced), n=9; NR3C1 cKO (P30-induced), n=8. Data are shown as mean ± SEM.



New Figure 6e. Heatmap showing gene expression and gene body accessibility of EAE-specific upregulated genes. Expression levels and accessibility of gene body in astrocytes from 12W naïve mice following P0-induced NR3C1 cKO were shown (left). Boxplots showing the gene expression and chromatin accessibility of EAE-specific upregulated genes (right). Two-sided Kolmogorov Smirnov test, accessibility P = 9.59×10^{-8}



New Supplementary Figure 6f. Heatmap showing gene expression and gene body accessibility of EAE-specific upregulated genes. Expression levels and accessibility of gene body in astrocytes from 12W naïve mice following P30-induced NR3C1 cKO were shown (left). Boxplots showing the gene expression and chromatin accessibility of EAE-specific upregulated genes (right). Two-sided Kolmogorov Smirnov test, expression P = 2.50×10^{-4}, accessibility P = 3.85×10^{-2}.



New Figure 6f. Venn diagram showing the overlap between differentially accessible peaks in three groups: astrocytes from P17 naïve NR3C1 cKO mice (P0-induced NR3C1 cKO), astrocytes from 12W naïve NR3C1 cKO mice (P0-induced or P30-induced NR3C1 cKO) (left). Bar graph showing the overlap of DAPs linked to EAE-specific upregulated genes with DAPs in astrocytes from 12W naïve NR3C1 cKO mice (P0-induced or P30-induced NR3C1 cKO) (right).

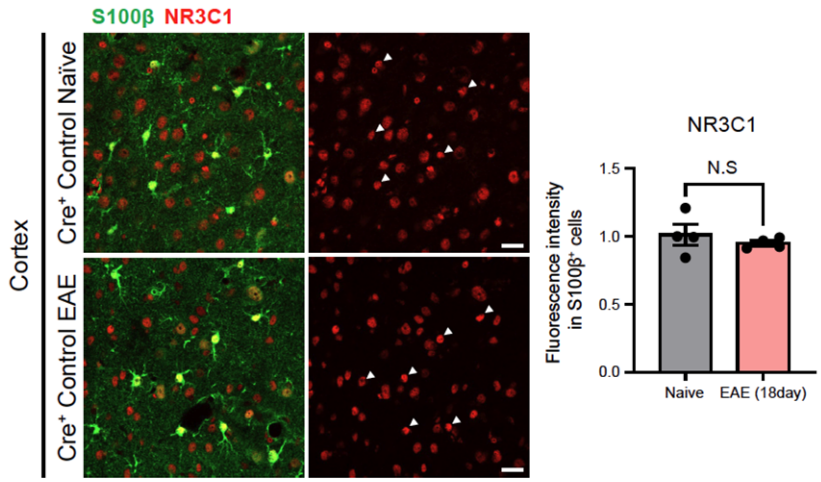
(pages 15-16, lines 325-345).

"Next, to determine whether NR3C1-dependent epigenetic predisposition persists into later stages, we performed RNA-seq and ATAC-seq using astrocytes from 12-week-old (W) mice following NR3C1 cKO at P0 (P0-induced) (Supplementary Fig. 6d). Interestingly, we found that gene expression patterns and altered gene accessibility persisted even at 12W (Fig. 6e). Of the 581 differentially accessible peaks linked to EAE-specific upregulated genes at P17, 33.6% retained their altered chromatin accessibility at 12W (Fig. 6f), strongly supporting the long-lasting effect of early NR3C1 depletion on the epigenetic priming of immune response genes in EAE.

We further assessed the developmental stage-specificity of this effect by comparing NR3C1 cKO astrocytes induced at P0 (early postnatal) versus P30 (mature stage). RNA-seq and ATAC-seq analyses at 12W revealed that P30-induced NR3C1 cKO failed to recapitulate the epigenetic alterations observed in P0-induced cKO (Supplementary Fig. 6e, f). Only 5.5% of the differentially accessible peaks linked to EAE-specific upregulated genes were retained in P30-induced NR3C1 cKO (Fig. 6f). Furthermore, the differentially expressed genes at 12W in P30-induced cKO mice exhibited distinct functional enrichment patterns compared to those observed in P0-induced cKO mice at the same time point (Supplementary Fig. 6g). These findings underscore a critical developmental window in which NR3C1 regulates long-lasting epigenetic programming of astrocytic immune-responsive genes. Collectively, our findings suggest that NR3C1 depletion in early postnatal stages alters the epigenetic state of inflammatory genes in astrocytes, thereby predisposing them for future activation."

2. Does inflammation regulate NR3C1 expression in astrocytes? To address this question, can the authors compare NR3C1 expression of astrocytes in WT and EAE mice?

To examine whether inflammation upon EAE affects NR3C1 expression in astrocytes, we compared its expression in astrocytes from naïve or EAE Aldh1l1-creERT2;GR fl/fl mice without tamoxifen injection using immunohistochemistry. We found that astrocytes under naïve conditions expressed NR3C1, and its expression level was not significantly changed at the peak of EAE (new Supplementary Fig. 4a). These data suggest that astrocytic inflammatory responses mainly depend on the chromatin accessibility that allows GR binding, rather than on GR expression itself. Our new analyses are now included in the revised manuscript as below.



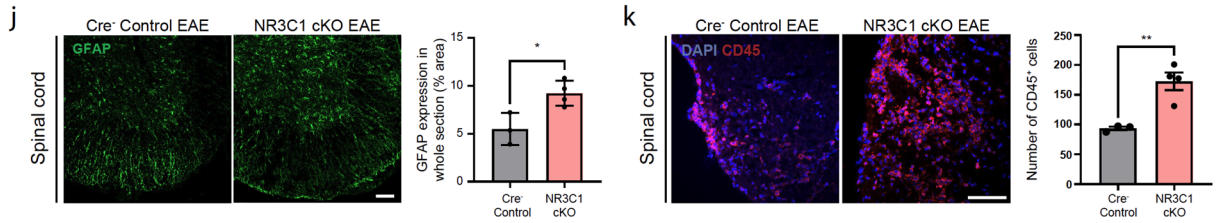
New Supplementary Figure 4a. Representative confocal images of NR3C1 expression in the cortex under naïve conditions (upper) and at the peak of EAE (bottom). Bar graphs showing normalized NR3C1 expression levels (right).

(page 11, lines 229-230).

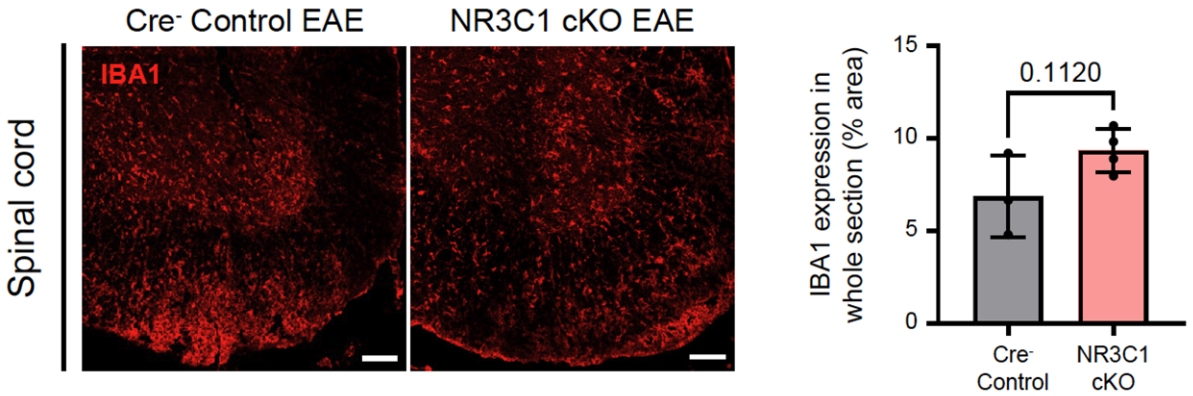
"We found that NR3C1 expression in astrocytes remained unchanged following EAE induction compared to non-EAE (naive) controls (Supplementary Fig. 4a)."

3. The authors should also provide staining's in the spinal cord for CD4 T cells and GFAP expression.

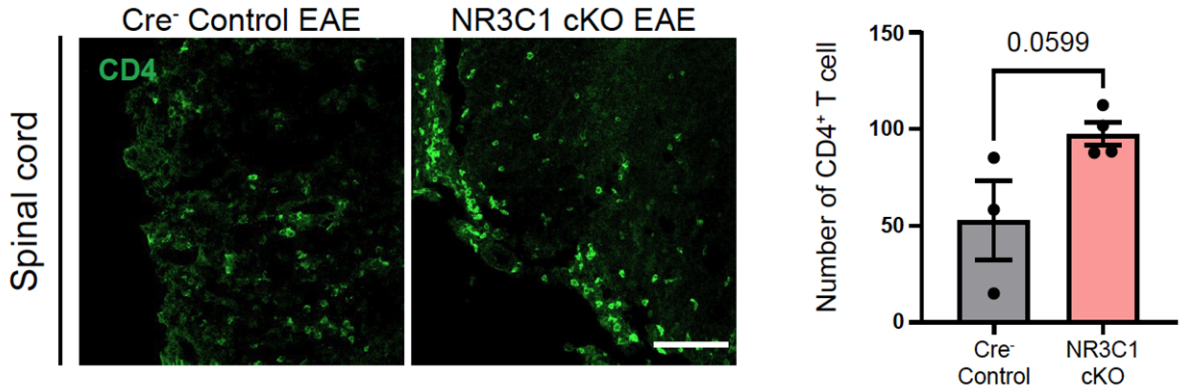
We appreciate the suggestion. In response, we conducted immunostaining of the spinal cord for CD4⁺ T cells and GFAP. Consistent with our findings in the corpus callosum, we observed increased expression of GFAP and IBA1, as well as infiltration of CD4⁺ T cells and CD45⁺ cells in the spinal cord of NR3C1 cKO mice (new Fig. 4j, k, new Supplementary Fig. 4d, f). These results have been added to the revised manuscript.



New Figure 4j, k. Representative confocal images of GFAP (j) and CD45 (k) expression in the spinal cord of Cre$^-$ control and NR3C1 cKO mice at the EAE peak (left). Bar graphs showing the area of GFAP expression (j) and the number of CD45$^+$ cells (k) in the spinal cord at the EAE peak (right). Mice used: n=3 for Cre$^-$ control and n=4 for NR3C1 cKO).



New Supplementary Figure 4d. Representative confocal images for IBA1 expression in the spinal cord from Cre$^-$ control and NR3C1 cKO mice (left). Bar graphs showing the area of IBA1 expression (right).



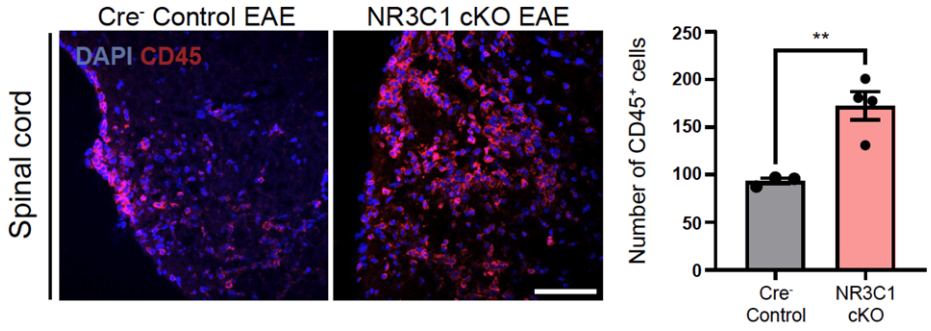
New Supplementary Figure 4f. Representative confocal images for CD4$^+$ T cell infiltration in the spinal cord from Cre$^-$ control and NR3C1 cKO mice (left). Bar graphs showing the number of infiltrated CD4$^+$ T cell (right).

(pages 11-12, lines 241-247).

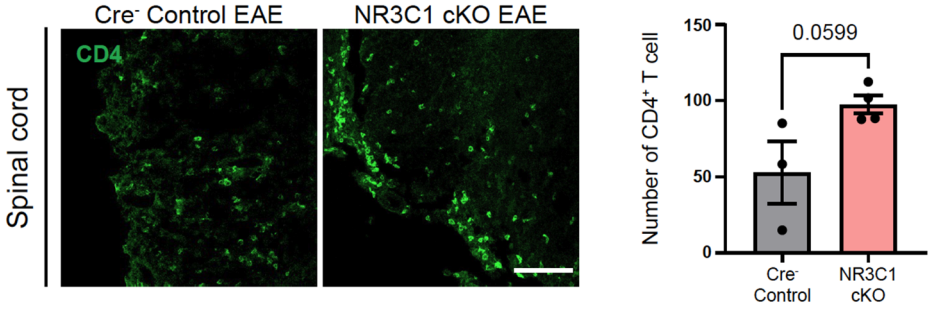
"We observed a similar increase in GFAP and IBA1 expression in the spinal cord and cerebellum of NR3C1 cKO mice following EAE induction (Fig. 4j, Supplementary Fig. 4d, e). Furthermore, NR3C1 cKO mice exhibited increased numbers of $CD45^+$ immune cells and $CD4^+$ T cells in the spinal cord (Fig. 4k, Supplementary Fig. 4b, c, f), indicating that NR3C1 regulates astrocyte-mediated immune responses across multiple CNS regions beyond the cortex. Taken together, these data suggest that astrocytic NR3C1 deficiency contributes to a more inflammatory immune environment."

4. Does NR3C1 KO have an effect on the phenotype of CNS-infiltrating CD4 T cells/ microglia/myeloid cells or both? Is there any difference in effector CD4 T cell subsets (i.e., producing IFN-γ, IL-17A, GM-CSF)?

Thank you for this insightful question. We observed an increased number of $CD45^+$ immune cells and $CD4^+$ T cells infiltrating the spinal cord at the peak of EAE in NR3C1 cKO mice (new Fig. 4k, new Supplementary Fig. 4f). Furthermore, we performed FACS analysis and observed elevated numbers of $CD4^+$ T cells with increasing trends in Foxp3+, IL-17A+, and IFN-γ expressing subsets (new Supplementary Fig. 4b, c), indicating that NR3C1 loss in astrocytes contributes to a more inflammatory immune environment. These findings have been added to the revised manuscript and are presented in the new figures.

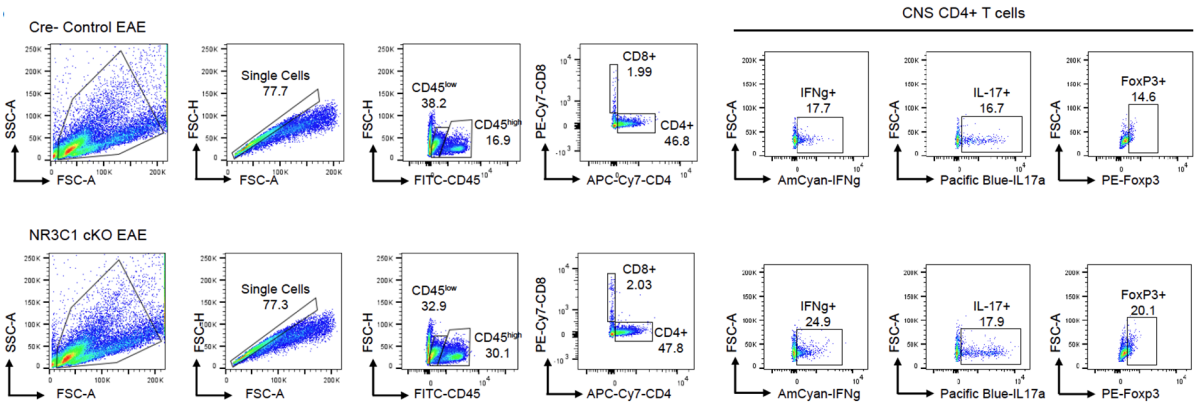


New Figure 4k. Representative confocal images for CD45 expression in the spinal cord from Cre^- Control EAE and NR3C1 cKO EAE mice (left). Bar graphs showing the number of $CD45^+$ cells (right).

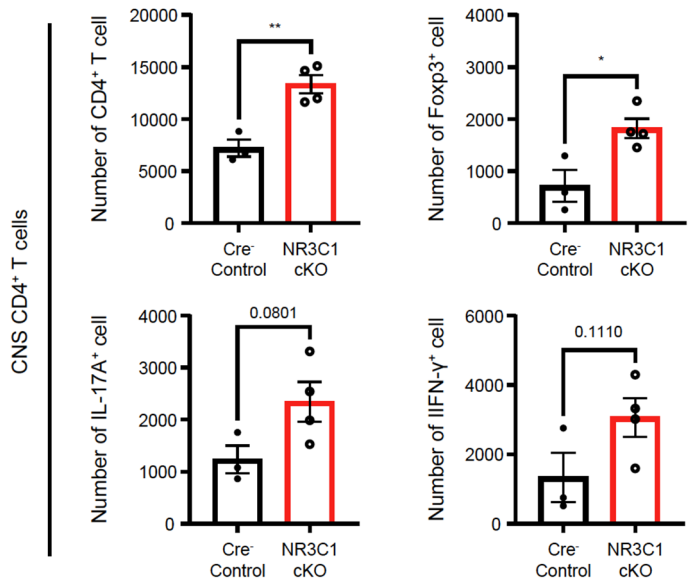


New Supplementary Figure 4f. Representative confocal images for $CD4^+$ T cell infiltration in

the spinal cord from Cre⁻ control and NR3C1 cKO mice (left). Bar graphs showing the number of infiltrated CD4⁺ T cell (right).



New Supplementary Figure 4b. FACS sorting schematic for CD4⁺ T cell and its subsets.

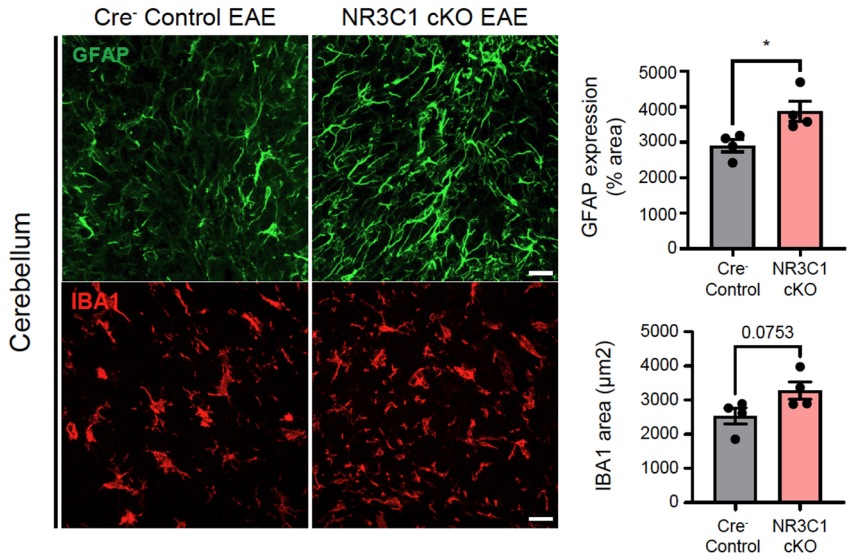


New Supplementary Figure 4c. Bar graphs showing the number of CD4+ T cell and its subsets (Foxp3+, IL-17A+, and IFN-γ+).

5. The authors focus on the role of NR3C1 in astrocytes, specifically examining gene expression in the corpus callosum. Given that the EAE model, primarily a spinal cord inflammatory model, do the authors consider whether astrocytes in other CNS regions might also be affected?

To observe the gene expression changes in NR3C1 cKO astrocytes, we focused on the cortex, where we initially identified NR3C1 as a critical developmental factor. We

then further validated exaggerated immune responses in the corpus callosum, a region known to be responsive during EAE. Based on the reviewer's suggestion, we now show that similar inflammatory responses occur in the spinal cord and cerebellum of NR3C1 cKO mice under EAE conditions (new Fig. 4j, k, new Supplementary Fig. 4b-e). Consistent with the increased immune cell infiltration observed in NR3C1 cKO mice, these additional data support the broader relevance of NR3C1-regulated astrocyte responses across multiple CNS regions and have been included in the revised manuscript.



New Supplementary Figure 4e. Representative confocal images for GFAP and IBA1 expression in the cerebellum from Cre$^-$ control (upper) and NR3C1 cKO mice (bottom). Bar graphs showing the area of GFAP and IBA1 expression (right).

(pages 11-12, lines 241-247).

"We observed a similar increase in GFAP and IBA1 expression in the spinal cord and cerebellum of NR3C1 cKO mice following EAE induction (Fig. 4j, Supplementary Fig. 4d, e). Furthermore, NR3C1 cKO mice exhibited increased numbers of CD45$^+$ immune cells and CD4$^+$ T cells in the spinal cord (Fig. 4k, Supplementary Fig. 4b, c, f), indicating that NR3C1 regulates astrocyte-mediated immune responses across multiple CNS regions beyond the cortex. Taken together, these data suggest that astrocytic NR3C1 deficiency contributes to a more inflammatory immune environment."

6. The authors may want to mention in their discussion a recent report on the role of the related receptor NCR3C2 in the regulation of astrocyte responses and EAE/MS (clark et al, Nature 2023), which suggests shared roles and therapeutic value for

multiple members of this TF family.

We appreciate the reviewer's suggestion. Given the structural and functional similarities between NR3C1 and NR3C2, we agree that both may play key roles in regulating astrocyte immune responses. We have expanded the Discussion section to include insights from recent studies, including Clark et al. (Nature, 2023), which highlight the potential functional interplay among nuclear receptors in modulating astrocyte identity and immune responses.

(page 20, lines 429-436).

"Also, NR3C1 belongs to the nuclear receptor subfamily 3, group C, which also includes mineralocorticoid receptor (MR, NR3C2), androgen receptor (AR), and progesterone receptor (PGR). These receptors share structural similarities and chromatin-binding sequences, often interacting in a context-dependent manner. Recent studies have highlighted that NR3C2 functions as a transcription factor during astrocyte development (Sojka, Caitlin, et al and an immune modulator (Clark et al., Nature, 2023). Given these findings, further studies are needed to determine potential functional interplay between these nuclear receptors and also other mid-stage specific TFs in shaping astrocyte identity and immune responses."

Minor Comments

1. Figure 4h: IBA1 is only mildly and non-significantly overexpressed in NCR3C1 KO mice, this should be corrected.

We examined IBA1 expression in multiple CNS regions, including the corpus callosum, spinal cord and cerebellum, and found that although IBA1 expression consistently showed an increased trend in NR3C1 cKO EAE mice compared to control EAE mice, the increase was mild and not statistically significant. This contrasts with GFAP expression, which showed strong upregulation across all CNS regions examined. This point has been added to the revised manuscript.

(page 11, lines 237-239).

"NR3C1 cKO mice with EAE also showed increased IBA1 expression compared to Cre^- control mice, (Fig. 4h) although the increase was less pronounced than that of GFAP."

Reviewer #2 (Remarks to the Author):

This manuscript explores a novel aspect of astrocyte biology, focusing on the role of NR3C1 in mediating astrocytic immune responses via epigenetic mechanisms during development and disease contexts. The study employs robust methodologies, including multi-omics approaches, conditional knockout models, and single-nucleus RNA sequencing, to reveal NR3C1's regulatory role in astrocytes. **The manuscript is well-structured, and the experiments are meticulously designed. The findings are significant, providing insights into astrocytic vulnerability to immune dysregulation in the context of diseases like multiple sclerosis**. However, there are several points that need clarification and additional discussion to strengthen the impact and reproducibility of the findings.

We sincerely thank the reviewer for their thoughtful and constructive feedback, which helped us improve the clarity and impact of our manuscript. We have addressed each of the comments below.

Specific comments:

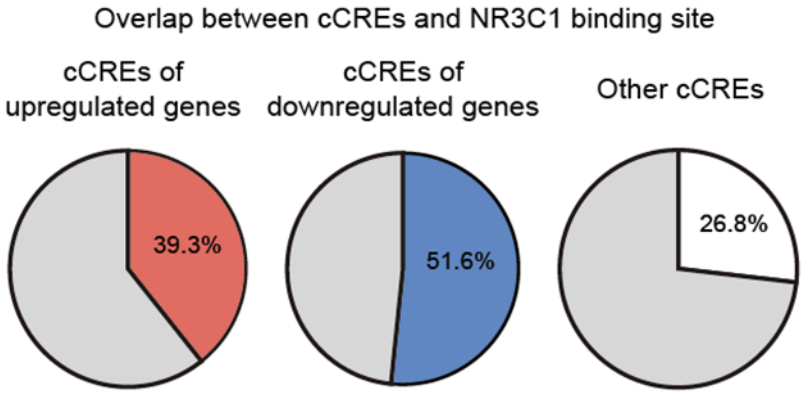
1. The manuscript emphasizes NR3C1 as both a transcriptional activator and repressor. However, the molecular mechanisms underlying this dual functionality in astrocytes and how this feature is related to disease pathology remain vague. For example, it is not clear whether exacerbated immune responses upon EAE induction are mediated by dysregulation of activator or repressor function, or both. Consider experimentally elaborating on how combinatorial actions with other transcription factors (e.g., AP-1, NF-κB) are contextually regulated and whether these interactions are subtype- or developmental time-specific. In this regard, it would also be interesting to investigate whether p300, a transcription coactivator previously reported to be critical for memory astrocytes and EAE, has any role in NR3C1-dependent epigenetic priming in astrocytes.

Thank you for the insightful suggestion. NR3C1 is known to act both as a transcriptional activator and repressor through several mechanisms: (1) binding to negative glucocorticoid response elements (nGREs), (2) interfering with other transcription factors such as NF-κB and AP-1, and (3) RNA-dependent inhibition of DNA binding.

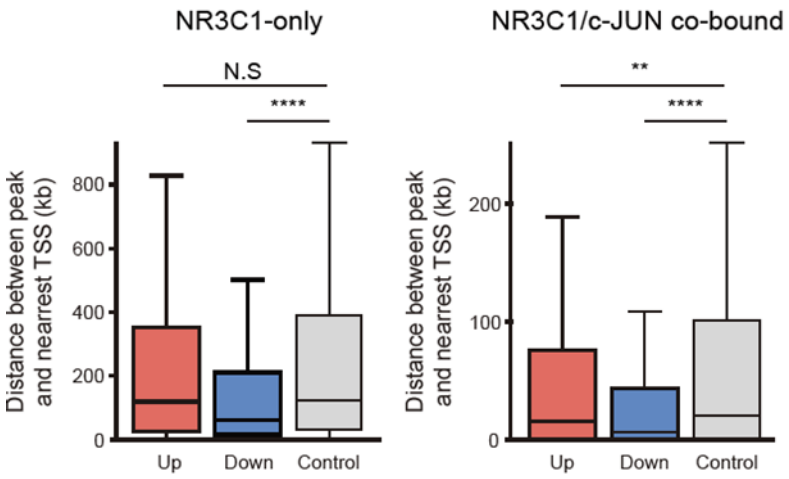
To explore the underlying combinatorial mechanisms in cooperation with other regulators, we conducted ChIP-seq for NR3C1, c-JUN (AP-1), and NF-κB following glucocorticoid treatment of primary mouse astrocytes. NF-κB and P300 ChIP-seq experiments yielded weak signals with a limited number of peaks, likely due to insufficient cell input. Because it is difficult to obtain numerous numbers of astrocytes, it was technically challenging to optimize those experiments for high quality. As a result,

these datasets were excluded from further analysis, and we focused on NR3C1 and c-JUN ChIP-seq datasets, which showed robust and interpretable binding profiles. NR3C1 ChIP-seq analyses revealed that 39.3% and 51.6% of cCREs linked to upregulated and downregulated genes were bound by NR3C1, respectively. In contrast, only 26.8% of other cCREs were marked by NR3C1, indicating strong regulatory role of NR3C1 in up/down-regulated genes in EAE (new Fig. 7b).

Furthermore, co-binding analysis showed NR3C1-only peaks were enriched near downregulated genes, while NR3C1–c-JUN co-bound regions were associated with both up- and downregulated genes (new Fig. 7c). These data suggest NR3C1 may function differentially depending on its interacting partners, such as c-JUN. We added these new results and figures to the manuscript.



New Figure 7b. Pie charts illustrating the proportion of NR3C1-bound cCREs associated with upregulated genes (left), downregulated genes (middle) and cCREs not linked to dysregulated genes (right) at the peak of EAE.



New Figure 7c. Boxplot showing the distance between dysregulated genes and NR3C1-only binding sites (left) or NR3C1/c-JUN co-bound regions. NR3C1-only, upregulated P = 9.04×10^{-2}, downregulated P = 2.82×10^{-23}; NR3C1/c-JUN co-bound, upregulated P = 5.36×10^{-3},

downregulated P = $1.23x10^{-25}$.

(pages 16-17, lines 347-366).

"**Context-dependent regulatory role of NR3C1 in shaping epigenetic predisposition**

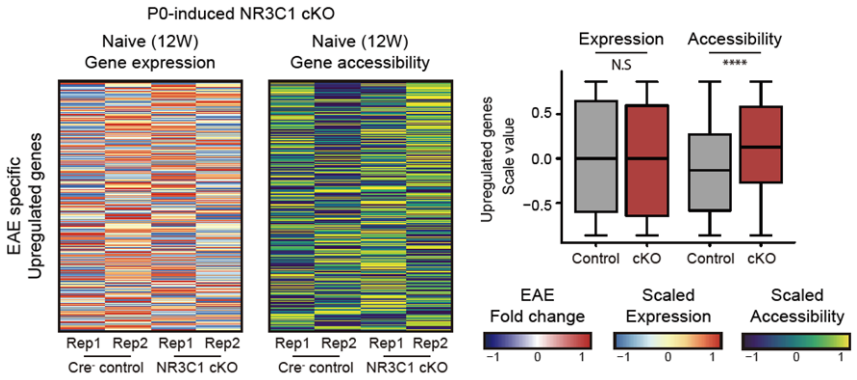
Previous studies have suggested that NR3C1 dimerization may activate gene expression, while its interaction with another TFs, such as AP-1 and NF-κB may repress gene expression, although both functions require NR3C1 binding to the genome[33,34]. To test this possibility and confirm that the observed epigenetic predispositions were directly driven by NR3C1 depletion, we performed NR3C1 and c-JUN ChIP-seq on primary mouse astrocytes (see Methods, Fig. 7a, Supplementary Fig. 7a, b). NR3C1 ChIP-seq peaks were significantly enriched in cCREs linked to both up- and down-regulated genes, indicating that the abnormal EAE-responsive gene expression is directly associated with NR3C1 binding (Fig. 7b).

To investigate the context dependent regulatory role of NR3C1, we categorized genomic regions into NR3C1-only and NR3C1/c-JUN co-bound sites, where c-JUN is a core component of the AP-1 complex. We found that downregulated genes were located near both NR3C1-only and co-bound regions, whereas upregulated genes were predominantly associated with NR3C1/c-JUN co-bound regions, compared to control regions (Fig. 7c, see Methods). These results suggest that NR3C1 may exert distinct regulatory effects in astrocytes depending on its binding partners[35]. We also performed motif analysis of cCREs linked to EAE-upregulated and -downregulated genes. Notably, cCREs associated with upregulated genes were enriched for STAT5, BCL6, and STAT6 motifs, implying the involvement of additional immune-regulatory factors cooperating with NR3C1 in astrocytes (Fig. 7d; Supplementary Tables 7, 8)."

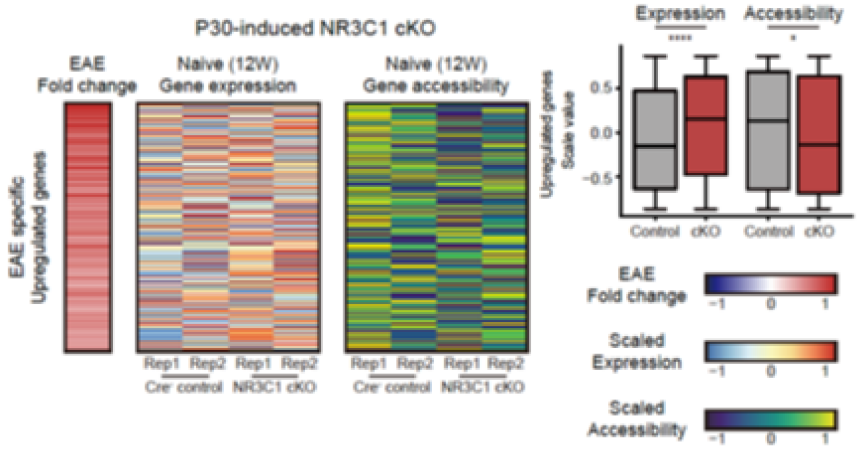
2. The study suggests that NR3C1 depletion during the early postnatal stage predisposes astrocytes to immune dysregulation later in life. While this hypothesis is compelling, further evidence related to the duration of epigenetic priming events is required to substantiate this model. The current study only investigated P17 for the comparison of gene expression and chromatin accessibility with and without EAE induction. Considering that MS onset in humans occurs at 20–40 years of age (approximately 12 weeks in mice), longitudinal studies linking early epigenetic changes to late-stage outcomes would corroborate the authors' conclusion regarding the role of NR3C1 in MS pathology.

We agree with the reviewer's concern regarding long-term effects of perinatal depletion of NR3C1 in adulthood. To address the question, we performed RNA-seq and ATAC-seq on astrocytes from 12-week-old mice. The primed chromatin and gene expression patterns established in the perinatal NR3C1 KO persisted into adulthood (new Fig. 6e), confirming the lasting impact of early NR3C1 depletion. Additionally, late-stage KO astrocytes (P30-induced) showed a markedly reduced priming effect compared to perinatal KO (P0-induced), underscoring the developmental window of susceptibility (new Supplementary Fig. 6f). These new findings are included in the

revised manuscript and figures.



New Figure 6e. Heatmap showing gene expression and gene body accessibility of EAE-specific upregulated genes. Expression levels and accessibility of gene body in astrocytes from 12W naïve mice following P0-induced NR3C1 cKO were shown (left). Boxplots showing the gene expression and chromatin accessibility of EAE-specific upregulated genes (right). Two-sided Kolmogorov Smirnov test, accessibility P = 9.59×10^{-8}



New Supplementary Figure 6f. Heatmap showing gene expression and gene body accessibility of EAE-specific upregulated genes. Expression levels and accessibility of gene body in astrocytes from 12W naïve mice following P30-induced NR3C1 cKO were shown (left). Boxplots showing the gene expression and chromatin accessibility of EAE-specific upregulated genes (right). Two-sided Kolmogorov Smirnov test, expression P = 2.50×10^{-4}, accessibility P = 3.85×10^{-2}.

(pages 15-16, lines 325-345).

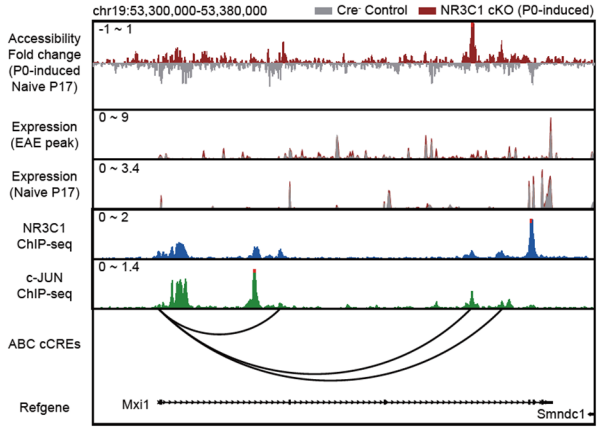
"Next, to determine whether NR3C1-dependent epigenetic predisposition persists into later stages, we performed RNA-seq and ATAC-seq using astrocytes from 12-week-old (W) mice following NR3C1 cKO at P0 (P0-induced) (Supplementary Fig. 6d). Interestingly, we found that gene expression patterns and altered gene accessibility persisted even at 12W (Fig. 6e). Of the 581 differentially accessible peaks linked to EAE-specific upregulated genes at P17, 33.6% retained their altered chromatin accessibility

at 12W (Fig. 6f), strongly supporting the long-lasting effect of early NR3C1 depletion on the epigenetic priming of immune response genes in EAE.

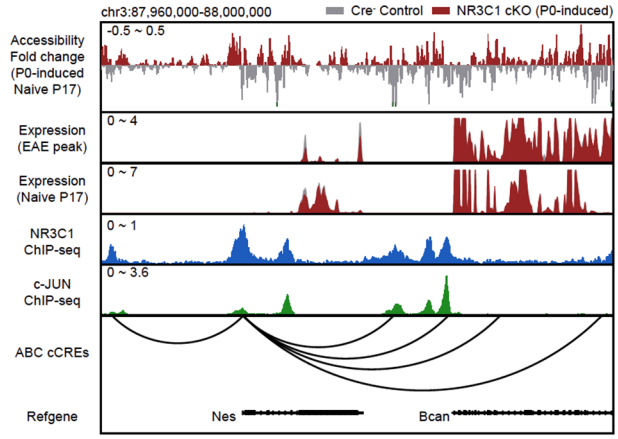
We further assessed the developmental stage-specificity of this effect by comparing NR3C1 cKO astrocytes induced at P0 (early postnatal) versus P30 (mature stage). RNA-seq and ATAC-seq analyses at 12W revealed that P30-induced NR3C1 cKO failed to recapitulate the epigenetic alterations observed in P0-induced cKO (Supplementary Fig. 6e, f). Only 5.5% of the differentially accessible peaks linked to EAE-specific upregulated genes were retained in P30-induced NR3C1 cKO (Fig. 6f). Furthermore, the differentially expressed genes at 12W in P30-induced cKO mice exhibited distinct functional enrichment patterns compared to those observed in P0-induced cKO mice at the same time point (Supplementary Fig. 6g). These findings underscore a critical developmental window in which NR3C1 regulates long-lasting epigenetic programming of astrocytic immune-responsive genes. Collectively, our findings suggest that NR3C1 depletion in early postnatal stages alters the epigenetic state of inflammatory genes in astrocytes, thereby predisposing them for future activation."

3. The ChIP-seq data in Fig. 6e only show log2 FC ratios of ChIP signal over input without any peak analysis, making it difficult to appreciate the quality of the ChIP-seq data. There do not appear to be significant differences in NR3C1 ChIP-seq signals between the upregulated genes and control genes. Given that this data is important for understanding the direct role of NR3C1, it is advised to provide more details on the ChIP-seq analysis.

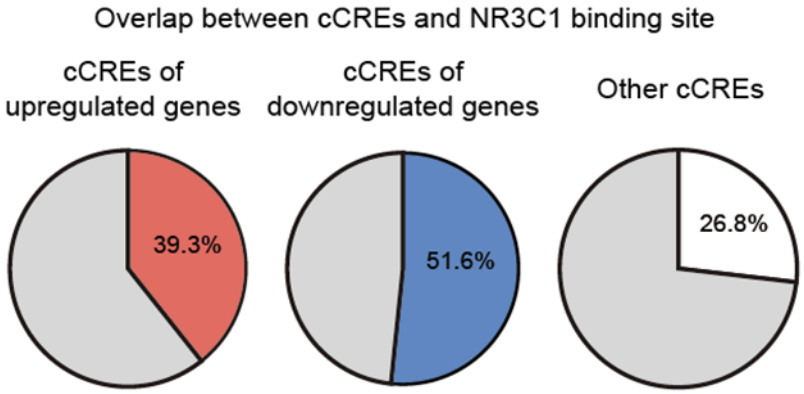
We appreciate this point. We now include a representative genome browser view (new Fig. 7a) showing NR3C1 and c-JUN binding at key cCREs, alongside corresponding gene expression and chromatin accessibility changes. We also include multiple genome browser views to demonstrate the quality and reproducibility of ChIP-seq results (new Supplementary Fig. 7a). Additionally, peak calling analysis supports strong enrichment of NR3C1 at cCREs associated with dysregulated genes compared to controls (new Fig. 7b, new Supplementary Fig. 7b) and combinatorial effect with c-JUN (new Fig. 7c), reinforcing its direct regulatory role in EAE-responsive astrocytic genes. These additions are described in both the Results and Methods sections.



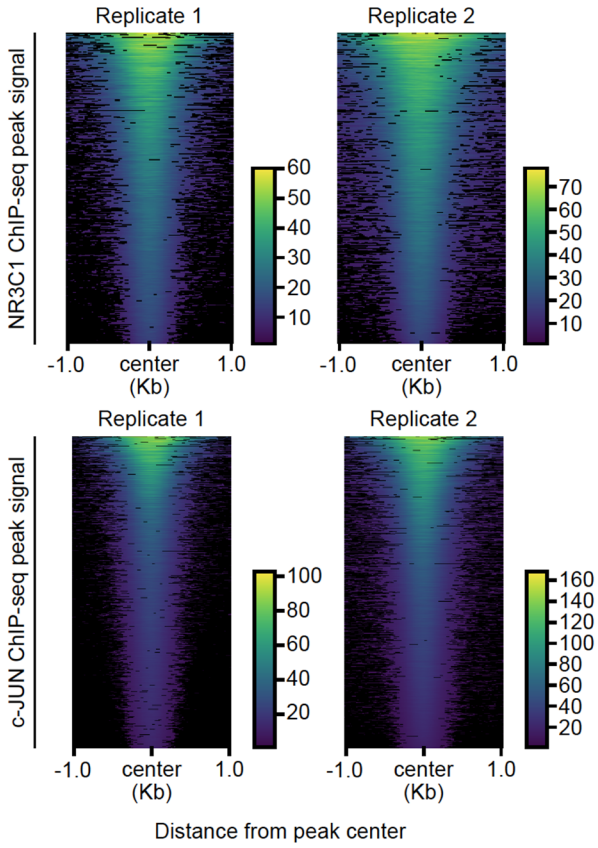
New Figure 7a, Genome browser snapshots of representative EAE responsive upregulated gene (Mxi1).



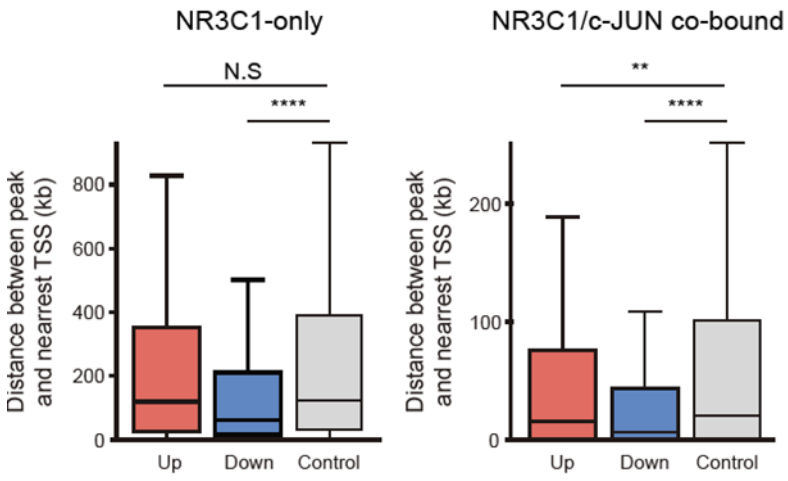
New Supplementary Figure 7a, Genome browser snapshots of representative EAE responsive downregulated gene (Nes).



New Figure 7b. Pie charts illustrating the proportion of NR3C1-bound cCREs associated with upregulated genes (left), downregulated genes (middle) and cCREs not linked to dysregulated genes (right) at the peak of EAE.



New Supplementary Figure 7b, Heatmap showing NR3C1 and c-JUN ChIP-seq signals on their peaks (NR3C1, n=25,111; c-JUN, n=47,071).



New Figure 7c. Boxplot showing the distance between dysregulated genes and NR3C1-only binding sites (left) or NR3C1/c-JUN co-bound regions. Mann-Whitney test, NR3C1-only, upregulated P = 9.04×10^{-2}, downregulated P = 2.82×10^{-23}; NR3C1/c-JUN co-bound, upregulated P = 5.36×10^{-3}, downregulated P = 1.23×10^{-25}.

(pages 16-17, lines 347-366) for main text.

"**Context-dependent regulatory role of NR3C1 in shaping epigenetic predisposition**

Previous studies have suggested that NR3C1 dimerization may activate gene expression, while its interaction with another TFs, such as AP-1 and NF-κB may repress gene expression, although both functions require NR3C1 binding to the genome[33,34]. To test this possibility and confirm that the observed epigenetic predispositions were directly driven by NR3C1 depletion, we performed NR3C1 and c-JUN ChIP-seq on primary mouse astrocytes (see Methods, Fig. 7a, Supplementary Fig. 7a, b). NR3C1 ChIP-seq peaks were significantly enriched in cCREs linked to both up- and down-regulated genes, indicating that the abnormal EAE-responsive gene expression is directly associated with NR3C1 binding (Fig. 7b).

To investigate the context dependent regulatory role of NR3C1, we categorized genomic regions into NR3C1-only and NR3C1/c-JUN co-bound sites, where c-JUN is a core component of the AP-1 complex. We found that downregulated genes were located near both NR3C1-only and co-bound regions, whereas upregulated genes were predominantly associated with NR3C1/c-JUN co-bound regions, compared to control regions (Fig. 7c, see Methods). These results suggest that NR3C1 may exert distinct regulatory effects in astrocytes depending on its binding partners35. We also performed motif analysis of cCREs linked to EAE-upregulated and -downregulated genes. Notably, cCREs associated with upregulated genes were enriched for STAT5, BCL6, and STAT6 motifs, implying the involvement of additional immune-regulatory factors cooperating with NR3C1 in astrocytes (Fig. 7d; Supplementary Tables 7, 8)."

(pages 34-36, lines 745-787) for methods.

"Glucocorticoid receptor stimulation of primary astrocyte

Primary astrocytes were isolated from postnatal day 0 (P0) mouse brain. Briefly, the cerebral cortices were dissected from P0 mice and enzymatically digested in trypsin-EDTA (Gibco) for 5 minutes at 37°C. Then, the cortices were mechanically dissociated through trituration in DMEM (Welgene) supplemented with 10% FBS (Welgene) and 1% penicillin/streptomycin (Gibco). After dissociation, the cell suspension was filtered through a 70μm cell strainer (SPL). The single-cell suspension was plated in T175 culture flask (SPL). After maintaining for 1 week, the flasks were shaken at 240 rpm for 10 hours to detach microglia and OPCs. The supernatant was removed, and the adherent astrocytes were detached using trypsin, and replated in culture plates for recovery. After one additional week of astrocyte culture, the glucocorticoid receptor was stimulated with 5 μM corticosterone (Sigma-Aldrich, 27840) for 24 hours and collected for further sequencing analysis. The astrocyte culture media consisted of 45% neurobasal medium (Gibco), 45% DMEM (Welgene), 10% FBS (Welgene), 1% penicillin/streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 292 μg/ml L-glutamine (Gibco), 1X SATO, and 5 ng/ml HBEGF (Sigma-Aldrich).

TF ChIP-seq and data analysis

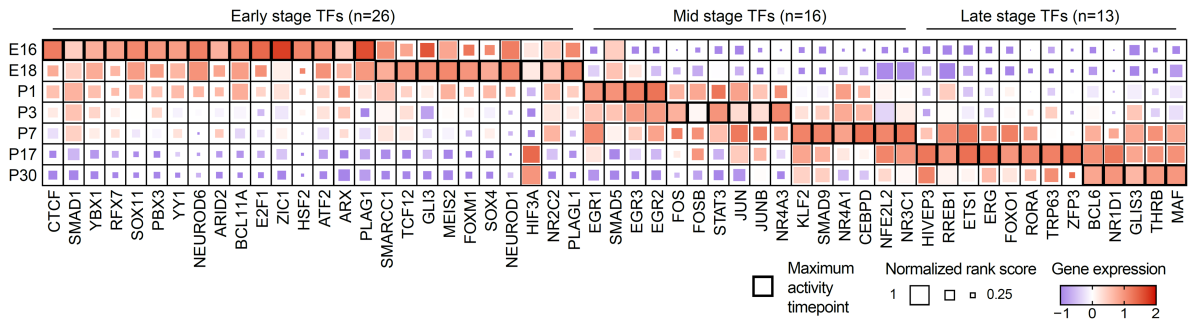
We collected 3 million of astrocytes to perform ChIP-seq experiment for each TF. The collected cells

were crosslinked twice: first with 2mM disuccinimidyl glutarate for 35 min, followed by 1% formaldehyde for 10 minutes, both at room temperature. The crosslinking was quenched using 1.25M glycine, and then cells were washed twice with PBS. The cells were then lysed in nucleus buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors), followed by sonication using a Covaris M220 (70 amplitude, 30-minute process time, alternating 30 seconds ON and 30 seconds OFF). Debris was removed by centrifugation, and DNA fragments were extracted and diluted ten times with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM pH 8.1 Tris-HCl, and protease inhibitor). To conjugate TF-targeting antibody and protein A Sepharose (ThermoFisher), we added 2ug of TF antibody into 10ul beads (for NR3C1, Thermo Fisher, PA1-511A; for c-JUN, Cell Signaling Technology, #9165). The antibody-conjugated protein A Sepharose was added to the diluted sample for overnight immunoprecipitations at 4 °C. Then, the beads were washed with TSE I buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl), TSE II buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 10 mM Tris-HCl (pH 8.1) and 1 mM EDTA), three times with TE buffer (10 mM pH 8.0 Tris-HCl and 1 mM EDTA), and the eluted in elution buffer (1% SDS and 0.1 M NaHCO3). DNA was reverse-crosslinked by incubation at 65°C overnight. After RNase and Proteinase K treatment, DNA was purified and prepared for next-generation sequencing using the NEBNext Ultra II DNA Library Prep Kit (NEB). The ChIP-seq library were sequenced using DNBSEQ-G400 platform.

For the data processing, sequencing reads were aligned to the mouse reference genome (GRCm38.p6) using BWA-mem. Low-quality alignments (MAPQ < 10) were removed, and PCR duplicates were eliminated using Picard (v2.6.0). The binding signal on cCREs was quantified using bedtools, counting sequencing reads that overlapped with cCREs by more than 20%."

4. The color scale used in Figure 2A lacks sufficient contrast. Consider enhancing the contrast or selecting a more distinct color gradient to improve readability.

Following the reviewer's suggestion, we revised Figure 2A to use a more distinct color gradient, improving the clarity and interpretability of the data.



Revised Figure 2A.

Reviewer #3 (Remarks to the Author):

In this study, Park, Park et al., focused on investigating astrocyte developmental programs linking them with the susceptibility to neuroinflammatory diseases. As the contribution of astrocytes to various conditions, despite their important role in the central nervous system (CNS), is not yet well understood, the subject is of relevance to a broad readership. The authors performed a comprehensive study of gene regulatory programs during development of astrocytes from initial gliogenesis (embryonic day 16) to mature astrocyte stage (postnatal day 30) using a reporter mouse line (Aldh1l1-EGFP transgenic) and multi-omics, including transcriptome (RNA-seq), open chromatin (ATAC-seq) and chromosomal interactions (HiCAR).

They describe gene regulatory programs characteristic for the three distinct developmental stages (based on the stage-specific cis regulatory elements and their target genes defined using the activity-by-contact model). They identified novel transcription factors (TFs) involved in astrocyte development, and in particular, the important role of NR3C1 during early postnatal period. Deletion of NR3C1 in astrocytes resulted in exacerbated inflammatory responses in adults and enhanced symptoms of a widely used model (EAE) of autoimmune neuroinflammation in general and multiple sclerosis (MS) in specific. Interestingly, they demonstrated that the lack of NR3C1 predisposed for the epigenetic profile with an accessible chromatin at inflammatory genes during early development, which exerted its function only in adults and upon challenge, leading to activation of inflammatory pathways.

This is an interesting and well-conducted study that highlights the role of astrocytes in neuroinflammation and links molecular modulations/disturbances of developmental programs with the vulnerability to neuroinflammatory pathologies later in life.

We thank the reviewer for their supportive comments that our study provides insights into the role of astrocyte in neuroinflammation and mechanical links with developmental programs. We have addressed the concern in the point-by-point response below.

Major points:

1. Given the anti-inflammatory effects of glucocorticoids (GCs), their wide use to treat acute relapses in MS, and the documented immune functions of astrocytes, the motivation to study NR3C1in EAE/MS (lines 184-187) should be adjusted to include these aspects.

We sincerely appreciate the reviewer's valuable feedback. We have revised the manuscript to include a brief overview of NR3C1 and its putative anti-inflammatory role

in the treatment of MS in the revised main text as below.

(page 18, lines 392-396).

"Our study highlights the pathological relevance and therapeutic implication of NR3C1 encoding the GR, a transcription factor activated by glucocorticoid (GC) binding that regulates anti-inflammatory transcriptional programs (Coutinho et al. 2011). Given the widespread clinical use of GCs to manage acute MS relapses, understanding the cellular and molecular mechanisms of NR3C1 in the CNS is of critical therapeutic importance."

2. Please describe the details of control groups of mice, especially in EAE experiments (a breeding scheme would be nice). What exactly is Cre- WT? I assume that they are Cre-negative littermate wild-type controls obtained from the same breeding used to generate Cre-positive KO animals? Has Cre-positive control been tested for any of the phenotypes (given potential impacts of Cre, PMID: 29336844). Since the Aldh1l1-creERT2 strain seems to have a mixed BL6/N and J background (with some contribution from FVB/N), controls are critical, especially as only two replicates were used for multi-omics profiling.

We apologize for the insufficient explanation regarding the experimental control group. We crossed Aldh1l1-creERT2 (-/-); Nr3c1$^{f/f}$ females with Aldh1l1-creERT2 (+/-); Nr3c1$^{f/f}$ males to obtain Aldh1l1-creERT2 (+/-); Nr3c1$^{f/f}$, Aldh1l1-creERT2 (-/-); Nr3c1$^{f/f}$ pups. All pups were injected with 4-hydroxytamoxifen (4-OHT) at P0 and P1 to induce astrocyte-specific Nr3c1 knockout. At the weaning point, male mice were sacrificed, and only female mice were used for further analysis. We performed genotyping to identify the presence of the Cre gene and used Aldh1l1-creERT2 (-/-) mice as controls and Aldh1l1-creERT2 (+/-) as the experimental group. To avoid confusion, we designated Aldh1l1-creERT2 (-/-); Nr3c1$^{f/f}$ as Cre^{-} Control, which refers to the littermate wild-type controls that received 4-OHT but retained intact Nr3c1 expression. Additionally, the Aldh1l1-creERT2 mice were backcrossed with BL6/J mice for more than 10 generations to minimize genetic variability and reduce potential effects of mixed background. We also acknowledge that Cre expression itself can potentially impact phenotypes. While we did not include Cre-positive, flox-negative controls in this study, previous studies using Aldh1l1-creERT2; flox negative line in the EAE model showed no significantly increased phenotype or disease progression with tamoxifen injection alone (Sheng-Wen Chen et al., 2025). Nevertheless, we agree that this remains an important consideration. These details have been included in the revised manuscript as below.

(page 21, lines 455-458).

"Aldh1l1-creERT2 mice were backcrossed with BL6/J mice more than 10 generations to minimize

genetic variability and reduce potential effects of mixed background. To minimize variability in disease onset and severity, only female mice were used for EAE induction."

3. If I understood correctly, GSEA analyses were conducted on differentially expressed genes (DEGs), not all ranked genes for a given comparison. I would advise being more stringent with thresholds (wrt detection, fold-change and significance cutoffs), given that a heterogeneity between the individual animals is likely (due to the nature of EAE disease, random distribution of inflammation in the CNS, genetic background of animals) and since only two replicates were run. For example (Fig. 4d and e) 2,256 down- and 1,895 up-regulated DEGs (based on the unadjusted p-value < 0.05) were considered for GSEA, but despite many DEGs, the fact that TF was KO and expression measured in a pure cell type, it is surprising that very few GO terms were (barely) significant. Additionally, the rationale for GSEA on up- and down-regulated genes separately, when looking at functional consequences is unclear, as functions/pathways are most often controlled by both up- and down-regulation of the constituent genes). Analyzing DEGs separately makes sense in (later) analysis when the focus is on the role of NR3C1 as a repressor or activator, but for obtaining insights into processes regulated by KO, GSEA on combined DEGs and with more stringent selection is better suited, and would give a possibility to estimate activation state of significant GO terms (a quick analysis on combined up- and down-regulated genes with FDR < 5%, 1719 DEGs, gives many more highly significant GO terms). As QC, it would be nice to know that GRs, alone or in combination with co-factors, are among predicted upstream regulators. It would be important to double-check GSEA analysis in order to establish if the statement "Unexpectedly, the DEGs in NR3C1 cKO astrocytes did not exhibit significant enrichment for immune response pathways among either up-regulated or down-regulated genes (Fig. 4e)" truly holds given the large number of DEGs.

We thank the reviewer for the thoughtful comments and have carefully reexamined our gene set enrichment strategy. In our original analysis, we used the MSigDB Hallmark gene sets (Liberzon A et al., 2015), which are highly curated and non-redundant, designed to capture well-defined biological states and signaling events. These sets were selected for their interpretability and specificity, particularly in immune and inflammatory processes, and are widely adopted for their robustness in transcriptomic studies. Since many Hallmark signatures reflect directional activation of gene modules, we initially performed separate GSEA on up- and down-regulated genes to detect potential asymmetries in transcriptional control by NR3C1.

To address concerns regarding DEG thresholds, we applied more stringent filtering (log_2 fold change > 1 and FDR < 0.05) and re-performed GSEA under three input strategies: upregulated genes, downregulated genes, and both of the genes. Across all approaches, we found that Hallmark immune-related pathways were significantly

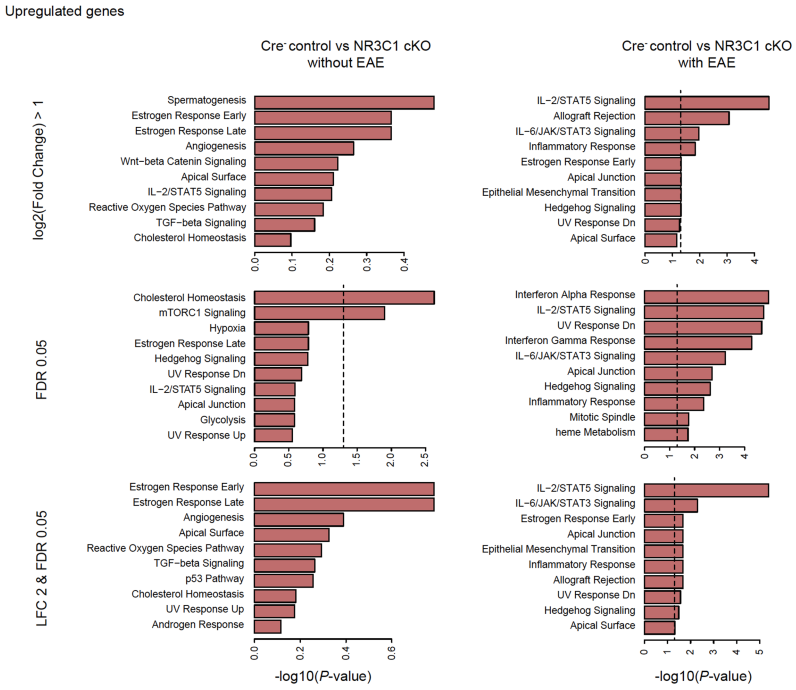
enriched only in the EAE condition, not in the naïve state (Responsive Figs. 2-4). This supports our observation that NR3C1 depletion only affects astrocytic immune-responsive genes under inflammatory condition, rather than the physiological state.

We also implemented pre-ranked GSEA using the full transcriptome. Using log_2 fold change–based ranking, we again observed strong enrichment for immune and interferon-related Hallmark gene sets specifically in the EAE condition, with no such enrichment in the naïve state (Responsive Fig. 5).

In response to the reviewer's concern about the unexpectedly weak enrichment of DEGs in the naïve state, we also performed GSEA in another database. When analyzing GO Biological Process terms, although some enriched pathways were detected in the naïve group, overall trend (weak enrichment of immune terms in DEGs in the naïve state) was similar, suggesting that transcriptional changes are present but not captured within Hallmark categories.

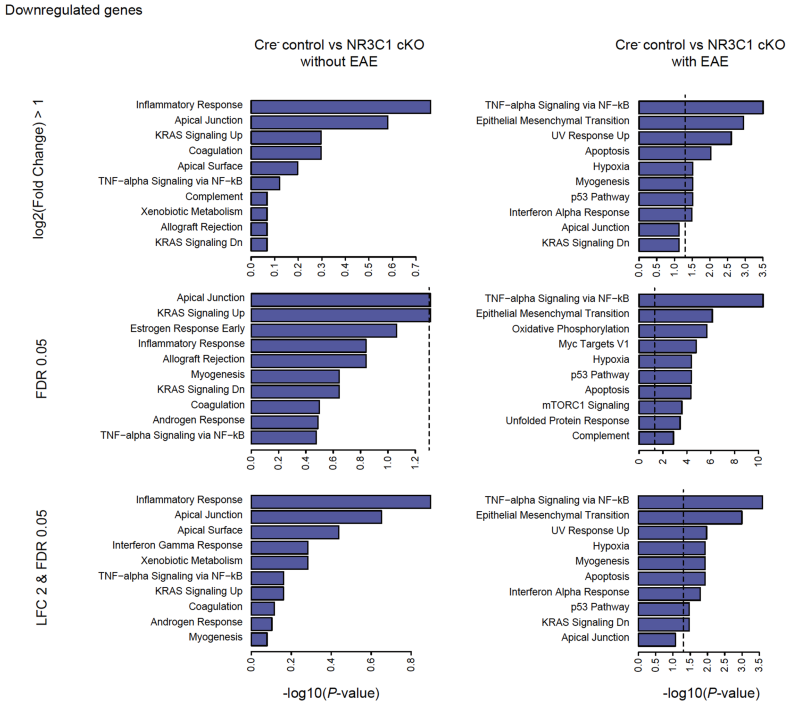
Furthermore, we found that dysregulated genes in NR3C1 cKO astrocytes were associated with genomic regions bound by NR3C1 and c-JUN, suggesting that our data reflect regulatory targets affected by NR3C1 depletion (see response to Reviewer 2 comment 1).

Together, these analyses underscore the context-dependent role of NR3C1 in shaping astrocyte immune responses and reinforce the robustness of our conclusions across analytical choices.

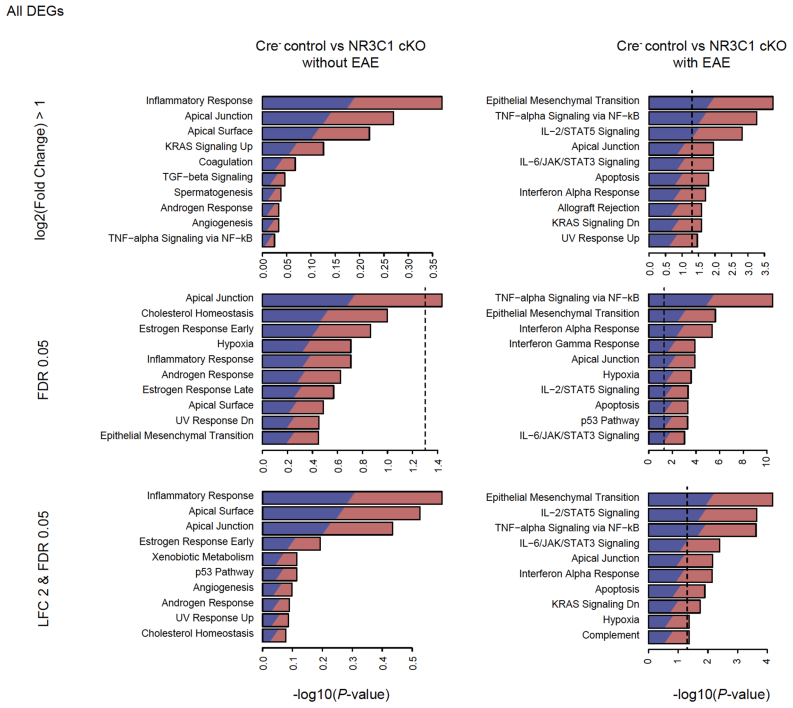


Responsive Figure 2. Top 10 enriched MSigDB Hallmark pathways of upregulated DEGs in

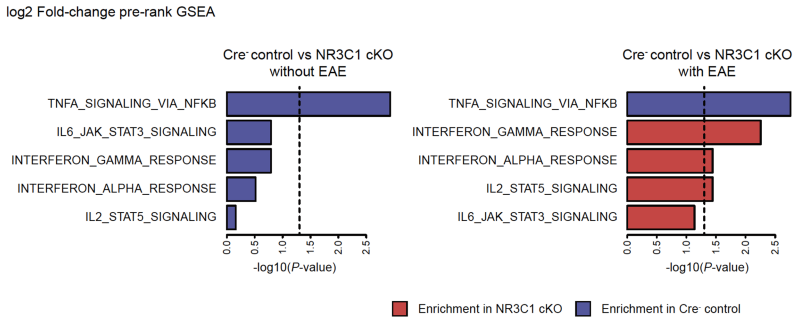
NR3C1 cKO astrocytes without EAE induction (left) and with EAE induction (right). The DEGs were defined using three different thresholds: log2 fold change > 1, FDR < 0.05, and their intersection.



Responsive Figure 3. Top 10 enriched MSigDB Hallmark pathways of downregulated DEGs in NR3C1 cKO astrocytes without EAE induction (left) and with EAE induction (right). The DEGs were defined using three different thresholds: log2 fold change > 1, FDR < 0.05, and their intersection.



Responsive Figure 4. Top 10 enriched MSigDB Hallmark pathways of overall DEGs in NR3C1 cKO astrocytes without EAE induction (left) and with EAE induction (right). The DEGs were defined using three different thresholds: log2 fold change > 1, FDR < 0.05, and their intersection.



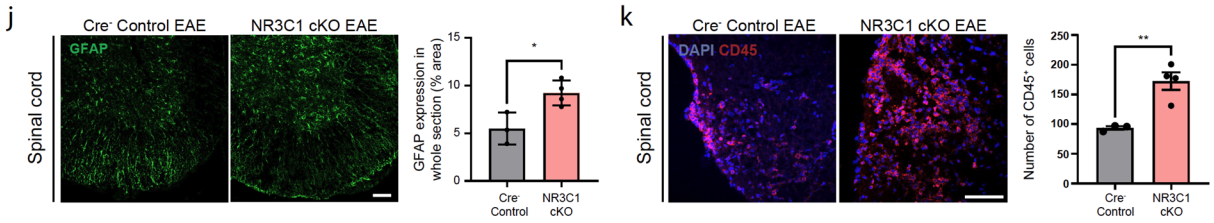
Responsive Figure 5. Rank-based enrichment analysis of MSigDB Hallmark pathways for DEGs in NR3C1 cKO astrocytes without EAE induction (left) and with EAE induction (right). The DEGs were ranked based on the level of log2 fold change to perform GSEA.

4. In MOG35-55-induced EAE on the BL6 background, the spinal cord is predominantly affected by inflammation, demyelination and axonal transection. What is the rationale for focusing on corpus callosum and cortex? Have the authors investigated any of the studied changes in spinal cord?

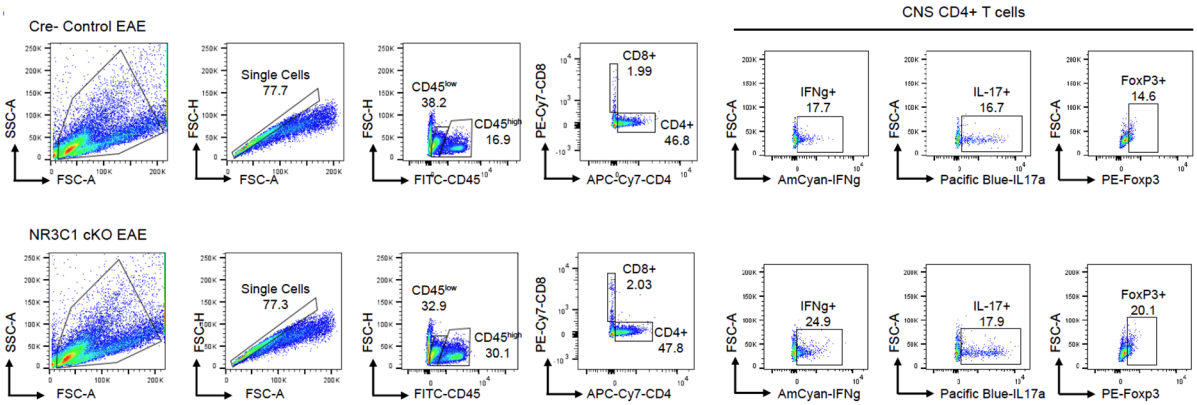
To observe the gene expression changes in NR3C1 cKO astrocytes, we focused on the cortex, where we initially identified NR3C1 as a critical developmental factor. We then further validated exaggerated immune responses in the corpus callosum, a region known to be responsive during EAE. Based on the reviewer's suggestion, we have now included analysis of the spinal cord and cerebellum and demonstrated that similar inflammatory responses can be observed in both the brain as well as spinal cord under EAE conditions (new Fig. 4j,k, new Supplementary Fig. 4b-e, see response to Reviewer 1 comment 3,4). We described these new results in the revised manuscript as below.

(pages 11-12, lines 241-247).

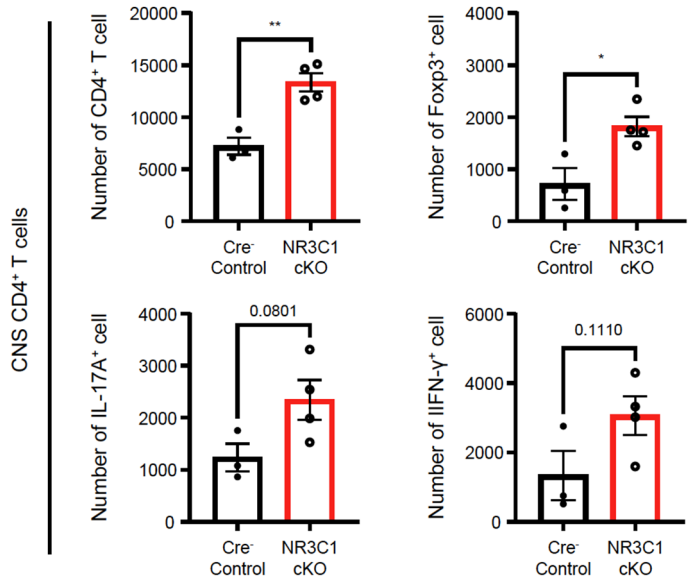
"We observed a similar increase in GFAP and IBA1 expression in the spinal cord and cerebellum of NR3C1 cKO mice following EAE induction (Fig. 4j, Supplementary Fig. 4d, e). Furthermore, NR3C1 cKO mice exhibited increased numbers of CD45+ immune cells and CD4+ T cells in the spinal cord (Fig. 4k, Supplementary Fig. 4b, c, f), indicating that NR3C1 regulates astrocyte-mediated immune responses across multiple CNS regions beyond the cortex. Taken together, these data suggest that astrocytic NR3C1 deficiency contributes to a more inflammatory immune environment."



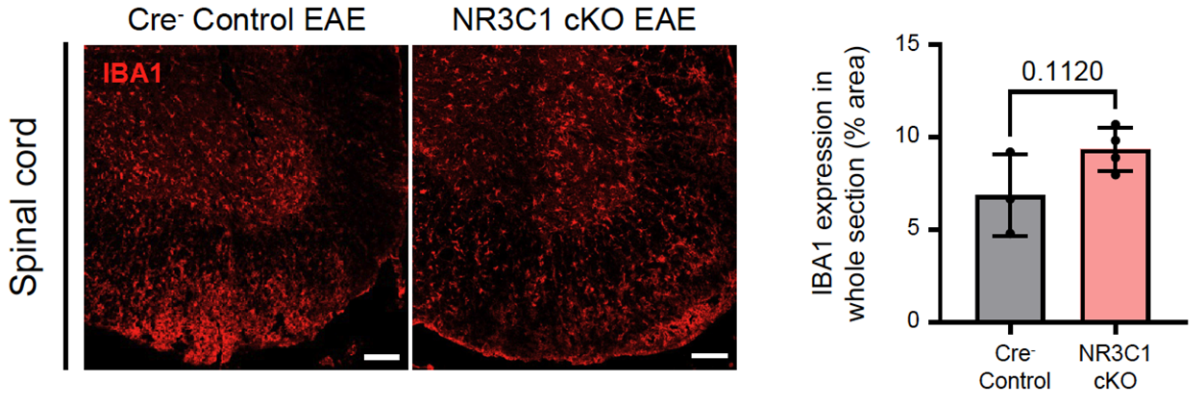
New Figure 4j, k. Representative confocal images of GFAP (j) and CD45 (k) expression in the spinal cord of Cre$^-$ control and NR3C1 cKO mice at the EAE peak (left). Bar graphs showing the area of GFAP expression (j) and the number of CD45$^+$ cells (k) in the spinal cord at the EAE peak (right). Mice used: n=3 for Cre$^-$ control and n=4 for NR3C1 cKO).



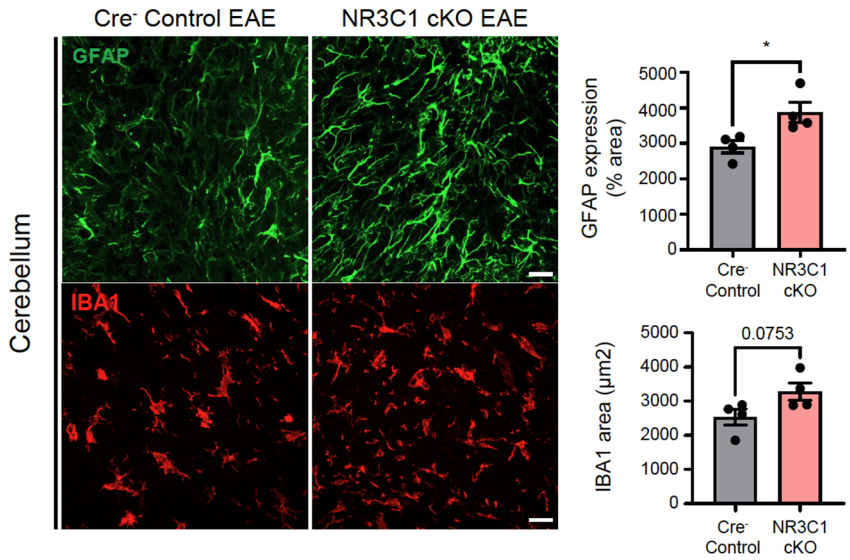
New Supplementary Figure 4b. FACS sorting schematic for CD4⁺ T cell and its subsets.



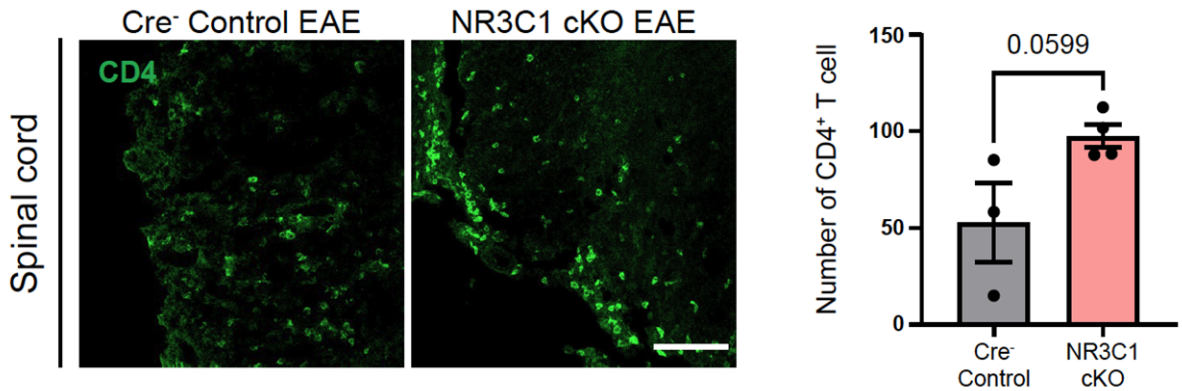
New Supplementary Figure 4c. Bar graphs showing the number of CD4+ T cell and its subsets (Foxp3+, IL-17A+, and IFN-γ+).



New Supplementary Figure 4d. Representative confocal images for IBA1 expression in the spinal cord from Cre⁻ control and NR3C1 cKO mice (left). Bar graphs showing the area of IBA1 expression (right).



New Supplementary Figure 4e. Representative confocal images for GFAP and IBA1 expression in the cerebellum from Cre⁻ control (upper) and NR3C1 cKO mice (bottom). Bar graphs showing the area of GFAP and IBA1 expression (right).



New Supplementary Figure 4f. Representative confocal images for CD4⁺ T cell infiltration in the spinal cord from Cre⁻ control and NR3C1 cKO mice (left). Bar graphs showing the number of infiltrated CD4⁺ T cell (right).

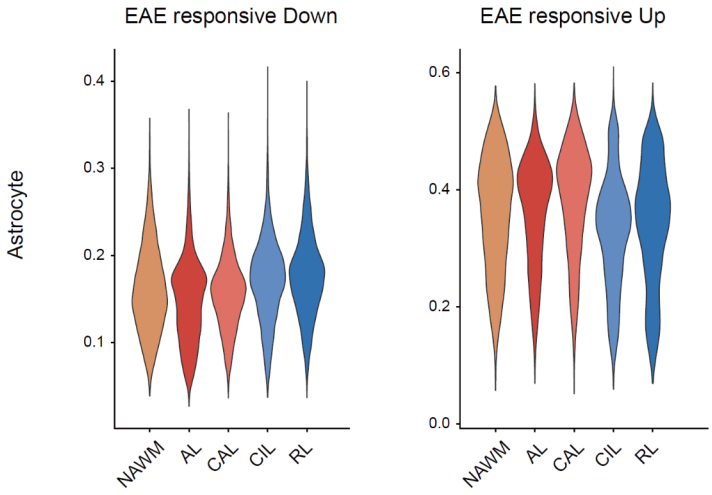
5. I could not find GSEA and the corresponding expression in MS lesions for the specific group of genes that displayed binding of NR3C1, accessible chromatin during development and get up-regulated during EAE (Fig. 6)? I might have missed it, but

this list of genes and associated functions seem the most interesting. How do they behave in MS, and if possible, present active, chronic active and inactive lesions (NAWM if available) separately? “Furthermore, the subtle reduction of NR3C1 in the corpus callosum of MS patients suggests that the role of NR3C1 in brain immune responses extends beyond the mouse EAE model”. Any chance to connect NR3C1 levels with open chromatin in astrocytes in non-inflammatory controls, NAWM, inactive, chronic inactive and active lesions in MS from a publicly available dataset? It would be very interesting to see the outcome.

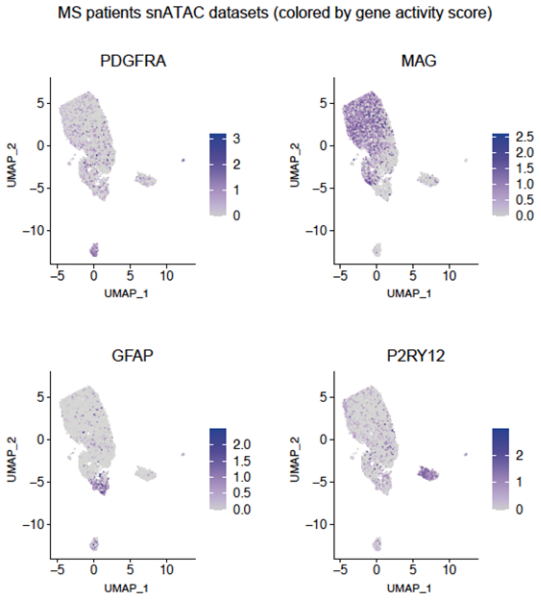
We thank the reviewer for this insightful comment highlighting the importance of genes that are developmentally primed by NR3C1 binding and chromatin accessibility, and subsequently upregulated in EAE. The GSEA and gene expression analysis for this group of genes is presented in original Figure 5, which highlights the immune responsive genes displaying NR3C1 binding and differential expression upon NR3C1 deletion during EAE. In the revised manuscript, we now provide the list of genes in new Supplementary Table 6.

To evaluate their relevance in human MS, we interrogated publicly available transcriptomic datasets of MS brain lesions (including active, chronic active, inactive lesions, and NAWM) (Macnair W et al., 2025). Notably, genes that were upregulated upon astrocyte-specific NR3C1 deletion in EAE were consistently elevated in active and chronic active MS lesions, while downregulated genes showed reduced expression, supporting a conserved regulatory role for NR3C1 in astrocyte-mediated inflammation across species (new Supplementary Fig. 5i).

We also attempted to analyze chromatin accessibility using public single-nucleus ATAC-seq datasets from MS lesions (Elkjaer et al., 2024). Unfortunately, the population of astrocyte in the datasets was insufficient to compare NR3C1-associated chromatin accessibility across lesion types (Responsive Fig. 6, WM, n=207 cells; NAWM, n=118 cells; CA, n=112 cells; RL, n=8 cells). We included the new results in the revised manuscript as below.



New Supplementary Figure 5i. Violin plots showing expression levels of DEGs at EAE peak in various brain lesions of multiple sclerosis patients (NAWM, normal-appearing white matter; AL, active lesion; CAL, chronic active lesion; CIL, chronic inactive lesion; RL, remyelinated lesion)



	WM #1	WM #21	NAWM #1	NAWM #2	NAWM #3	CA #1	CA #1	RL #1	RL #2
Astro	195	12	59	40	19	10	102	1	7
Immune	238	0	83	10	34	5	76	4	5
Oligo	3661	136	1087	632	537	437	215	91	67
OPC	142	0	46	8	38	6	40	0	0

Responsive Figure 6. UMAP plots show snATAC-seq profiles from MS patient brain samples,

colored by gene activity scores for key marker genes (top, PDGFRA for OPC, MAG for oligodendrocyte, GFAP for astrocyte, and P2RY12 for microglia). The table summarizes the number of nuclei assigned to each cell type across different lesion types (WM, white matter; NAWM, normal-appearing white matter; CA, chronic active lesion; RL, remyelinated lesion).

(page 13, lines 274-279).

"Stratified analysis of MS lesions, including normal-appearing white matter (NAWM), active lesions (AL), chronic active lesions (CAL), chronic inactive lesions (CIL), and remyelinated lesions (RL), further confirmed that genes upregulated in NR3C1 cKO astrocytes were more highly expressed in inflammatory lesion types (AL and CAL), while downregulated genes showed reduced expression in these same regions compared to less inflammatory lesions (CIL and RL) (Supplementary Fig. 5i)."

6. "Thus, our study highlights the importance of early genetic regulation and its long-term impact on health, offering potential insights for early interventions and therapeutic strategies to prevent mature-onset diseases." How does this translate to human conditions (MS in the context of NC3R1 specifically)? Is there any evidence of genetically and/or environmentally-associated changes in NC3R1 that can occur in humans and lead to vulnerability later in life? On the other hand, what happens upon treatment with GCs in MS? Can these rounds of GCs have lasting effects given the documented epigenetic imprint of GCs in peripheral immune cells (PMID: 31064978)?

Thank you for the great point! There is growing evidence that both genetic and environmental factors can influence the effects of NR3C1, contributing to long-term susceptibility to immune dysregulation. For example, early-life stress has been shown to induce hypermethylation of the NR3C1 promoter, leading to reduced expression and altered stress responses later in life (Palma-Gudiel et al., 2015). Consistently, reduced GR expression has been observed in MS patients (Bechmann et al., 2014), suggesting that such epigenetic modifications could contribute to disease vulnerability.

In terms of therapeutic aspects for NR3C1, glucocorticoid treatment is known to induce lasting epigenetic changes in peripheral immune cells. For example, Wiechmann et al. (2019) showed that dexamethasone exposure leads to rapid and transient demethylation at enhancer regions of FKBP5, resulting in increased expression. This is notable in the context of GC signaling, as Binder et al. (2008) demonstrated that elevated FKBP5 expression reduces GR sensitivity by impairing its nuclear translocation and transcriptional activity, thereby contributing to glucocorticoid resistance. Importantly, in the context of MS, Souren et al. (2019) identified stable hypomethylation at ZBTB16 in the peripheral blood of MS patients, long after high-dose methylprednisolone treatment, providing in vivo evidence that such epigenetic imprints occur in patients. These findings support the notion that GC therapy can reprogram immune cells through durable epigenetic mechanisms. Given that NR3C1 similarly governs immune regulation in astrocytes, it is plausible that repeated GC

exposure may induce comparable epigenetic modifications in astrocytes. However, further research is needed to demonstrate whether GC treatment induces epigenetic changes in astrocytes. We have included these points in the Discussion as below.

(page 19, lines 402-414).

"Beyond its diagnostic relevance, NR3C1 may be subject to long-lasting modulation following GC therapy, requiring careful consideration in treatment contexts. GC treatment is known to induce lasting epigenetic changes in immune cells. For instance, dexamethasone exposure causes transient demethylation at FKBP5 enhancer regions, upregulating its expession[42]. Elevated FKBP5, in turn, impairs GR nuclear translocation and reduces its transcriptional activity, thereby contributing to glucocorticoid resistance[43]. In MS patients, the high-dose methylprednisolone treatment triggers the persistent hypomethylation of ZBTB16 in the peripheral blood, further supporting the idea that GC-induced epigenetic imprints can be long-lasting in patients[44]. These findings collectively suggest that GC therapy can reprogram immune cells through durable epigenetic mechanisms. Given that NR3C1 similarly governs immune regulation in astrocytes, it is plausible that repeated GC exposure may induce comparable epigenetic modifications in astrocytes. However, further research is needed to demonstrate whether GC treatment induces epigenetic changes in astrocytes."

Minor points:

1. The relevance of GSEA in Fig. 3 is unclear, e.g., is the 'Multiple Sclerosis' term genes based on DEGs identified in the CNS tissue or blood? The authors should consider enrichment analysis across polygenic risk score loci, which might give more evidence for the involvement of astrocytic genes in the disease etiology.

We apologize for our insufficient description for the analysis in Fig. 3. We agree that enrichment analysis across polygenic-risk-score (PRS) loci is useful for quantifying heritability. However, there were two challenges to apply enrichment analysis across polygenic risk score loci in our analysis: 1) as our model system is mouse astrocyte it is difficult to directly map GWAS-SNPs using human specimens and 2) the limited number of putative TF bound enhancers in each astrocyte developmental stage overlapping with GWAS-SNPs, hindering the statistical power in enrichment analyses such as PRS or LDSC. Nonetheless, we attempted to perform LDSC analysis using GWAS SNPs from various brain diseases and TF-bound cCREs that were lifted over to human genomic coordinates. However, no significant heritability enrichment was observed across TFs. This lack of enrichment is likely due to the limited number of TF-bound cCREs (n = 2317 in average) and its overlaps to SNPs (n = 5 in average).

In order to overcome aforementioned limitations, we combined TF activity (enhancer openness containing TF motif × HiCAR interaction frequencies between enhancer and promoter, similar to Activity by contact model) and summed these values for each target gene. This peak-to-gene aggregation strategy consolidates the sparse TF

bound enhancer signals into a biologically meaningful gene-level score, enabling GSEA to reflect cumulative regulatory effects that are particularly relevant in the context of astrocyte development.

Moreover, the DisGeNET gene set employed in our analysis provides a valuable complementary resource, as it consolidates expertly curated, GWAS-derived, and literature-based gene–disease associations. By evaluating the gene list with scores anchored in astrocyte chromatin accessibility, our analysis can retain a cellular context. We therefore consider the gene-level approach better suited than a SNP-based test for uncovering astrocyte contributions to MS pathology. The rationale has been incorporated into the revised manuscript as below.

(page 9, lines 176–184).

"To explore the pathological associations of NR3C1 along with five astrocyte mid-stage specific TFs (FOS, EGR1, JUN, JUNB, and STAT3), we performed gene set enrichment analyses using DisGeNET26 on the predicted target genes of each TF. To identify TF target genes, we integrated chromatin accessibility data from cCREs harboring the corresponding TF motif with HiCAR-derived interaction frequencies, following an approach similar to the Activity-by-Contact (ABC) model (see Methods). Using the resulting cCRE–promoter pairs (Fig. 1c), we assigned gene-level scores by summing ABC scores of linked cCREs harboring each TF motif to consolidate the sparse TF bound enhancer signal, defining candidate genes predominantly regulated by each TF."

2. Please provide the rationale of not including Klf2, Fosb and Nr4a1 in further analysis (Fig. 3), they are also mid-stage genes that seem to exhibit exclusive perturbation impact on astrocytes.

We apologize for the insufficient description. Our single-cell transcriptome analyses in Figure 2 aimed to identify the most relevant transcription factors for astrocytes among the 55 TFs identified in Figure 2a. To achieve this, we applied two filtering criteria: TF expression (Figure 2d) and TF activity (Figure 2g). Six TFs (Jun, Fos, Junb, Stat3, Egr1, and Nr3c1) exhibited higher expression in the astrocytic lineage compared to the neuronal lineage. Among them, only Nr3c1 demonstrated lineage-specific activity in astrocytes, leading us to focus on its role in astrocyte development. We have clarified this rationale in the revised manuscript as below.

(page 8, lines 169–172)

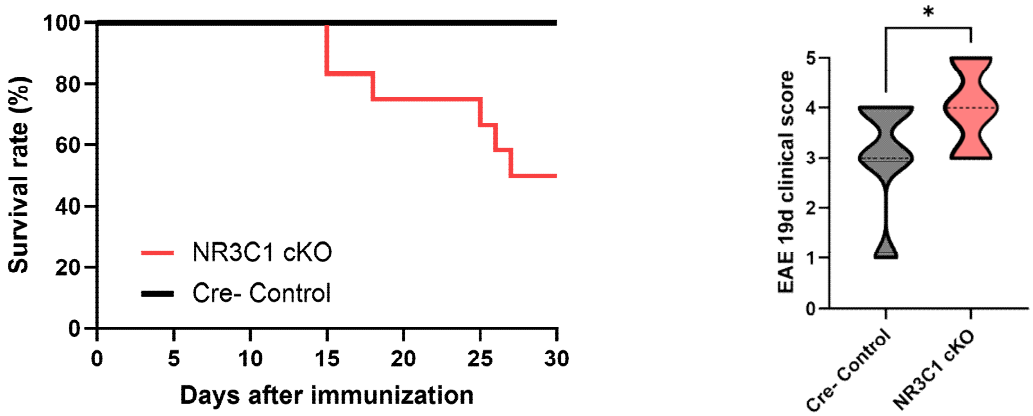
"Although several TFs that are highly expressed in astrocytes (*Jun*, *Fos*, *Egr1*, *Junb*, and *Stat3*) were predicted to influence both lineages, NR3C1 uniquely demonstrated both high expression and a lineage-specific perturbation effect in astrocytes, suggesting its critical role in early postnatal astrocyte development."

3. “Approximately half of the NR3C1 cKO mice reached a clinical score of 5, characterized by sudden death or severe paralysis.” How were these mice accounted for in the plots and statistical analysis? Please provide full statistics on the incidence and mortality.

Among the 10 EAE induced astrocyte-specific NR3C1 cKO mice, deaths began occurring from day 15, and 6 mice reached a clinical score of 5 during the 30 days of EAE clinical scoring period, characterized by sudden death. On the peak day of EAE progression, 3 out of 10 mice reached a clinical score of 5. None of their Cre- littermate controls reached a clinical score of 5. In the clinical score comparison analysis, a two-way ANOVA test was performed. All mice that reached a clinical score of 5 were considered as having a clinical score of 5 from the day they reached this score until the end of the scoring period. To provide more detailed statistics, we include survival rate graph and comparison of clinical scores on EAE peak day. (Responsive Fig. 7). We have provided additional descriptions in the revised manuscript as below.

(page 29, lines 631–633)

“All mice that reached a clinical score of 5 were considered as having a clinical score of 5 from the day they reached this score until the end of the scoring period.”



Responsive Figure 7. Survival rate graph shows over half of astrocyte-specific NR3C1 cKO mice experienced death 30 days after immunization (left). Astrocyte-specific NR3C1 cKO mice show severe disease progression (right).

4. Please provide short motivation for the use of the Aldh1l1-EGFP transgenic line, e.g., how comprehensive is the Aldh1l1 promoter. Expression is obviously stable

throughout examined stages (Suppl. Fig 1), but are there any astrocyte populations that could have been missed due to the lack of Aldh1l1 expression?

Thank you for the critical comment on our model system. We used the Aldh1l1-EGFP transgenic line because Aldh1l1 is a well-established pan-astrocytic marker with stable expression across astrocyte populations and developmental stages (Cahoy et al., 2008). This allows for comprehensive labeling of astrocytes throughout the examined time points, as shown in Supplementary Fig. 1. Notably, Aldh1l1 shows particularly robust expression in the cortical regions we analyzed, supporting the broad relevance of our findings to astrocyte development. We have clarified this point in the revised manuscript as below.

(page 4, lines 79-83).

"To investigate the dynamic gene regulation during perinatal astrocyte development, we utilized *Aldh1l1-EGFP* transgenic mouse, since ALDH1L1 is a well-established pan-astrocytic marker known for its stable expression across astrocyte populations and developmental stages. EGFP-positive astrocytes were isolated from mouse cortical tissue using fluorescence-activated cell sorting (FACS)."