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Peer Review File

The spatiotemporal transcriptional profiling of murine brain during cerebral malaria progression and after artemisinin treatment

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Chen et al. proposed a research article that constructs single-cell and spatial transcriptomic atlas of control (from public datasets), model, and artemisinin-treated groups in a mouse model of cerebral malaria. The authors attempt to elucidate the physiological and pathological mechanisms of neurological sequelae caused by malaria parasites (even after the use of antimalarial drugs). Also, the authors have explored the molecular mechanisms of cerebral malaria at the cellular level, focusing on blood-brain barrier disruption, immune infiltration, and cell-cell interactions. The data in this article are comprehensive and the research provides significant insights into the study of cerebral malaria scientifically. Besides, the research includes experiment validation (such as crucial immunofluorescence) to verify enriched key genes. The arguments of this research are well-supported, and the article follows a reasonably logical structure.

Here are a few crucial issues that may require further clarification from the authors:
1. If I am not mistaken, the authors didn’t remove batch effects of the data before cell clustering analysis. Could they provide UMAP plots assessing batch effects among samples within the same group in the supplementary figures?

2. The cell-type-specific marker genes used for cell-type and cell-subtype classification in the article should be referenced with the source literature. It is very important to respect the results of previous research. I strongly encourage the authors to present this information in a table.

3. The authors used a 1% threshold of malaria parasite gene expression reads to determine if an RBC was infected with the parasite. Could they provide the rationale for setting this threshold? Is it possible that, during late-stage infection of mouse brain tissue, some captured single cells may actually be malaria parasite cells? If this is a possibility, how would such cells be identified through data analysis methods? Additionally, why wasn't the same analysis of PbA gene expression applied to spatial transcriptomics data?

4. In the Methods section, the authors used different thresholds (15% and 30%) for mitochondrial reads in processing single-cell and spatial transcriptomic data. If samples of two omics are from the same tissue, why did the authors use two different threshold standards? Please explain.

5. In line 154, the authors referenced public ST-seq data but needed to include a specific literature citation.

6. The article contains some inconsistencies in gene nomenclature and grammar issues (e.g., PbANKA-1340100 and PbANKA_1145800 naming inconsistency, capitalization errors, and missing spaces between words). Please thoroughly review these grammar and naming issues.

7. The introduction of the spatial transcriptomic research topic in the abstract (Line 36) and results section (Line 152) were not well-organized. Consider refining the text to better introduce the topic (combined with the current development of CM research) for better follow-up.

8. Why did the author choose only days 0, 5, and 8 in the experimental design? A specific explanation may be needed.

9. While the analysis in the article is comprehensive in my opinion, there is still room for improvement in integrating single-cell and spatial group data.

Reviewer #2 (Remarks to the Author):

Chen et al have utilised single cell RNA-sequencing and spatial transcriptomics to investigate the brain response during ECM, and following artesunate treatment of the condition. As the pathways influencing ECM development are still unclear and there is a critical need for new adjunctive therapies for cerebral malaria, the study is of some interest. However, although the authors undertake a number of investigations, the biological relevance and implications of some of the results are unclear, and many of the results confirm existing knowledge. Moreover, it is unclear whether the ST analyses provide the resolution in places to significantly enhance knowledge of the spatiotemporal intracerebral responses to artesunate treatment.

Major comments:
It is unclear what day of analysis the analyses in Fig S1 E and F was performed on. This is essential to understand the extent of ECM development in mice at the time point of artesunate treatment. Relating to this, there does not appear to be a lot of BBB damage in the model group.

For ST spot-type annotation, it is unclear why the authors did not use the scRNA-seq to more thoroughly deconvolve the spots (i.e. Cell2Location) to define the relative abundance of cell types within each spot. This would provide more detailed insight on spot identity, rather than referring to spots broadly as dominant endothelial, astrocyte etc. Indeed, due to the nature of the brain architecture, there will usually be numerous cell types in a particular spot, and this will become more evident around the cerebrovasculature in mice with ECM or following treatment. There is a concern in Fig S3c that microglial cells are within such a small area in the UMAP, when realistically they will be highly prevalent throughout the brain parenchyma. In figure 4, showing the Endo_inflam spots only in specific locations is also misleading.

It is important that the authors attempted to verify the ST data using immunofluorescence in figure 2; however, at the resolution and magnification provided, it is unclear how robust the staining is. It is difficult to visualise the CD3+ T cells and some of the staining around the ventricle looks quite diffuse. This needs to be improved.

RBC removal was apparently performed on the brains during processing for scRNA-seq. As such, how many parasitized RBCs were lost from the parasite-host analysis? It's not clear how robust this analysis is, given this caveat. Moreover, what proportion of the (parasitised)RBCs in the analysis were luminal within the vasculature compared within haemorrhages? This may substantially influence what the interaction partners are for the parasitized RBCs in the brain. Currently there is debate on the nature of pRBC interaction with brain endothelial cells during ECM, so this part of the manuscript needs to be strengthened or removed.

The authors indicate that their data emphasises an important role of inflamed microglial cells in engaging with T cells to mediate experimental cerebral malaria (ECM) pathology. However, there is little evidence of T cells crossing into the brain parenchyma during ECM to engage with microglial cells. As such, the authors need to verify such interactions occur to provide relevance for the analyses. Figure 7G – the staining for CD8 does not seem robust, particularly in the ART group. IBA-1 can also be expressed on macrophages, so co-localisation of CD8 with IBA-1 does not mean CD8+ T cells are interacting with microglia. This would need to be confirmed using a vascular marker. Finally, relating to this, there is currently very little evidence that monocytes or macrophages contribute to ECM development at the onset of the syndrome: Depletion of myeloid cells prior to infection can prevent ECM, but depletion of the cells immediately prior to onset of the syndrome has little effect. As such, the biological relevance of some of the receptor-ligand interaction analyses is unclear.

Minor
In the introduction the authors conflate studies on experimental cerebral malaria and human cerebral malaria. (For example lines 74 to 77). These should be separated and studies in human CM and murine models should be clearly defined, given there is still some debate on the pathogenesis in the human and murine syndromes.

Line 247 – there is no evidence from the data in Figure 1C that the T cells and NK cells cross the

BBB and infiltrate the brain parenchyma. Indeed, from existing literature most of the cells recruited within the brain of mice with ECM remain luminal in the vasculature, with a proportion moving into the perivascular space.

Line 155 – It appears that only 2 biological replicates were used in the model and ECM groups, so it is unclear how robust the data is. Detailed histological assessments and RMCBS scores for the mice used in the scRNA-seq and ST should be provided.

Line 335 CD45RA is not expressed in mice or used to distinguish T cell populations, so this should be altered.

In figure 6D the expression of KLRD1 is rather atypical. Also, qualitative images showing only one or two CD8+ T cells is not particularly compelling to show whether the T cell response is similar or different in the model and ART groups.

The clone of the P. berghei ANKA parasites used in the study should be provided.

The materials and methods sections for some protocols could be clearer (e.g. mIHC and ELISA).

Typo line 711

Reviewer #3 (Remarks to the Author):

In this manuscript, Chen, Bai, He and coauthors have performed a single cell and spatial transcriptomic analysis on brain samples from experimental cerebral malaria models treated and untreated with artesunate. The analysis performed by the authors is impressive and overall, well done. The study is timely, given recent advances in single cell and spatial omics and provides a useful dataset that will inform other scientists and other studies. I found really interesting the comparison between CM and CM model treated with ART, as this provides information on molecular mechanisms that could drive long term sequelae in cerebral malaria patients. The paper is well written and it easy to follow. The study has some inherent limitations, overall listed in the discussions. I would recommend its publication after the reviewers clarify some major concerns on the methodology, the analysis and the interpretation. The comments are listed in the order they appear on the manuscript.

Major comments:
Abstract: The study is very informative, but given the nature of transcriptomics it mostly suggests mechanisms of pathogenesis that need to be validated in the future by the authors or other scientists. Nevertheless, the abstract gives the impression that they tested experimentally the suggested mechanisms. I would suggest to rewrite the abstract clearly stating that the study suggests mechanisms of pathogenesis.

Computational Methods:
- Did the authors apply any batch correction for the scRNAseq analysis? I couldn't find it in the manuscript.
- Model 2 is very low in RNA count and gene number compared to the other samples. This could speak for low quality of the sample. The authors must strongly consider whether this sample should be taken out of the analysis if there are significant differences between Model1/3 vs Model 2. However, this will leave the authors with a low n, and I would suggest to get a new sample.
- The authors allocate a cell identity for each spot on the spatial transcriptomics. The visium spot size is 55um and could contain up to 10 different cell types. For example, a brain capillary has a diameter from 5-10 um, in just this small region endothelial cells, pericytes, astrocytes, iRBC and multiple immune cells can be found. In my opinion, giving a cell identity for each spot is not necessary for the the manuscript and its main conclusions, and it could be misleading for many of the readers not familiar with the methodology. If the authors prefer to keep this type of analysis, at least they should acknowledge this limitation early on in the results section text, as this

technique is probably new to many readers.

Experimental Methods:
- The authors applied a red blood cell removal solution on the dissociated brain tissue, could this affect the recovery of infected red blood cells from the samples and the posterior analysis.
- Given the limitation of spot size in spatial transcriptomics, I strongly suggest a quantification of immunofluorescences shown in Figure 4C, 5D, 6D and 7G.

Results:
- Figure 1B: The number of neurons in the single cell RNAseq analysis is significantly low, taken into account the elevated number of neurons in the brain and the prevalence of neurons in the spatial analysis (Figure 2D). Is this a consequence of the methodology chosen for tissue dissociation?

- Calculations of Proportion of cells (Figure 1D, 4D, 5B, 6C). The proportion of cells types is informative, but similarly to Figure 3D I would suggest to add the number cells. This would provide additional information. For example, a massive increase in a cell population could mask relevant findings in other populations.

- Simplify Figure 3: I would suggest to simplify this figure and the text describing it. The manuscript has already a large amount of valuable information and some sections can be further extended (see comment below on neutrophils). Overall, the figure provides little information on disease pathogenesis, besides the validation that the authors found PbiRBC in the brain of CM model. Some of the ligand-receptor interactions suggested in Figure 3J have probably little relevance in disease pathogenesis. For example, to my knowledge VEGFA expression has not been described before for RBC. And if it does, it is quite likely at negligible levels compared to other cell types (leukocytes, brain parenchyma cells). Likewise, what is the biological relevance that the authors want to convey in Figure 3G showing the top Pb transcripts? Overall, this figure raises many questions and it provides little information on disease pathogenesis.

- The identification of the endothelial phenotype is very interesting and informative, could the authors allocate this phenotype to arteries, veins and, capillaries or is it widespread?

- A brief description of the function of the markers being used in Figure 4C, 5D, 6D and 7G is often missing in the text. This is important for readers outside the field. For example, a description of what is KLRD1 (Figure 6D) cannot be found.

- In figure 4G and Figure S4 E and F the authors claim that expression of leukocyte adhesion markers and antigen processing is higher in ECM models compared to BBB establishment, and that expression of BBB establishment is recovered in ART model. I disagree with this interpretation. This conclusion cannot be extracted from the graph shown in the main figures. This result is one of the main highlights of the discussion, so effort should be taken to unambiguously support the claim. A graph showing a ratio of BBB establishment/ antigen processing + leukocyte adhesion could be clearer?

- The increase in neutrophils in Figure 5B is remarkable. Given the recent relevance of neutrophils in both ECM and HCM described in Knackstedt et al (PMID: 31628160) I strongly suggest to extend the analysis.

- Why is the antigen presentation so spatially localized? I would expect it to be much widely distributed given the increases seen in the scRNAseq analysis, especially on microglia.

- Line 330-331. The increase in lymphocyte populations is remarkable and not highlighted in the text. I would suggest to add a sentence.

- Line 355-369 Breaking down the GO terms in cell type populations could be very informative. If possible. I strongly suggest to expand this analysis.

- Line 387-390 and Figure 7E. There is a lot to take in in this graph and is difficult to interpret due

to the small size, I would suggest that in this figure the authors just include the relevant ligand-receptor interactions highlighted in the text and move the others to supplemental. Besides, there is an alignment problem with the X axis labels.

- Line 443-445: The authors suggest that the OB neurons are insensitive to ART treatment, alternatively, it could be that the damage does not reverse with treatment.

Discussion:
- Early on in the discussion the authors should state more clearly the differences between experimental and human CM. For example, the second paragraph puts in context the results of the current manuscript into sequestration mechanisms in human CM. Recent reports have suggested that the accumulation of P. berguei in the brain microvasculature is mediated by mechanical trapping rather than cytoadhesion (Strangward et al., 2017 PMID: 28273147). The authors should be carefully on directly translating the pathogenic mechanisms found in the ECM into cytoadhesion mechanisms in HCM.

- Line 489: As mentioned earlier, the evidence of the link between leukocyte adhesion and BBB breakdown is not well supported by the current data (Figure 4G).

Minor.

The authors could consider to rename the experimental conditions to control, CM and ART (or CM-ART). Naming the CM as "Model" is confusing, as the other two experimental conditions are also modelling different outcomes of the disease

Line 305: Figure reference is missing.

Line 315: Only H2-D1 is shown in the graph of MVC comparison

Line 377: When the authors mention T/NK interactions it gives the impression that it could be with each other, rephrase so it is clear that the authors are focusing on interactions of T AND NK cells with other cell types.

Figure 7E: There is something wrong with the X axis of the graph.
Line 341-342: Where are the & of CD8 T cells coming from, the % listed are very high and different than in Figure 6C.
Line 492: Brain hydrostatic pressure instead of blood pressure?
Line 537-539: I would suggest removing this sentence, as the authors suggest the IFNg dynamics and kinetics are complicated, so it is necessary to introduce a mechanism in hepatocytes and P. vivax
Heatmaps of multiple figures. The color legend indicates relative expression. Relative to what? Sometimes all the conditions are shown. Sometimes the label "relative expression" is missing.
Figure 2: The color code chosen for Neuron and Oligo masks the colors for the other cell type identities, making the figure difficult to interpret.
Figure4E,6E, S4E, S5D: The identity of the spots could be highlighted by a color outline. Otherwise, we don't know what we are looking at.
Figure 4E. Why is region four described if only region 1 and 2 is shown in 4F and 4G?
Figure 8G: of which model?
Figure S1:F I would suggest to use a higher magnification. iRBC sequestration, enlarged perivascular space and vascular leakage are difficult to see in the provided images.
Figure S4A, S5B: The color code on the top of the heat maps is not outlined on the figure legend. I would suggest removing it or referencing to the color code in the main figure.
Figure S4B: Endo AP is not defined in the text. Do the authors mean Endo-Hb?

Point-by-point response to the comments

We thank the reviewers for the insightful comments and constructive suggestions that have helped us with the preparation of a revised manuscript. We have performed additional experiments to address all the concerns and comments raised by our reviewers. Here we submitted a substantially improved manuscript along with our point-by-point response.

On the following pages, the comments of all reviewers #1, #2, and #3 are presented below in purple, italicized font text point-by-point. Our response is given in black, normal font text, with the figure numbers or revised positions highlighted in red. All the authors have read the revised manuscript and agree with the contents.

Reviewer #1

Chen et al. proposed a research article that constructs single-cell and spatial transcriptomic atlas of control (from public datasets), model, and artemisinin-treated groups in a mouse model of cerebral malaria. The authors attempt to elucidate the physiological and pathological mechanisms of neurological sequelae caused by malaria parasites (even after the use of antimalarial drugs). Also, the authors have explored the molecular mechanisms of cerebral malaria at the cellular level, focusing on blood-brain barrier disruption, immune infiltration, and cell-cell interactions. The data in this article are comprehensive and the research provides significant insights into the study of cerebral malaria scientifically. Besides, the research includes experiment validation (such as crucial immunofluorescence) to verify enriched key genes. The arguments of this research are well-supported, and the article follows a reasonably logical structure. Here are a few crucial issues that may require further clarification from the authors:

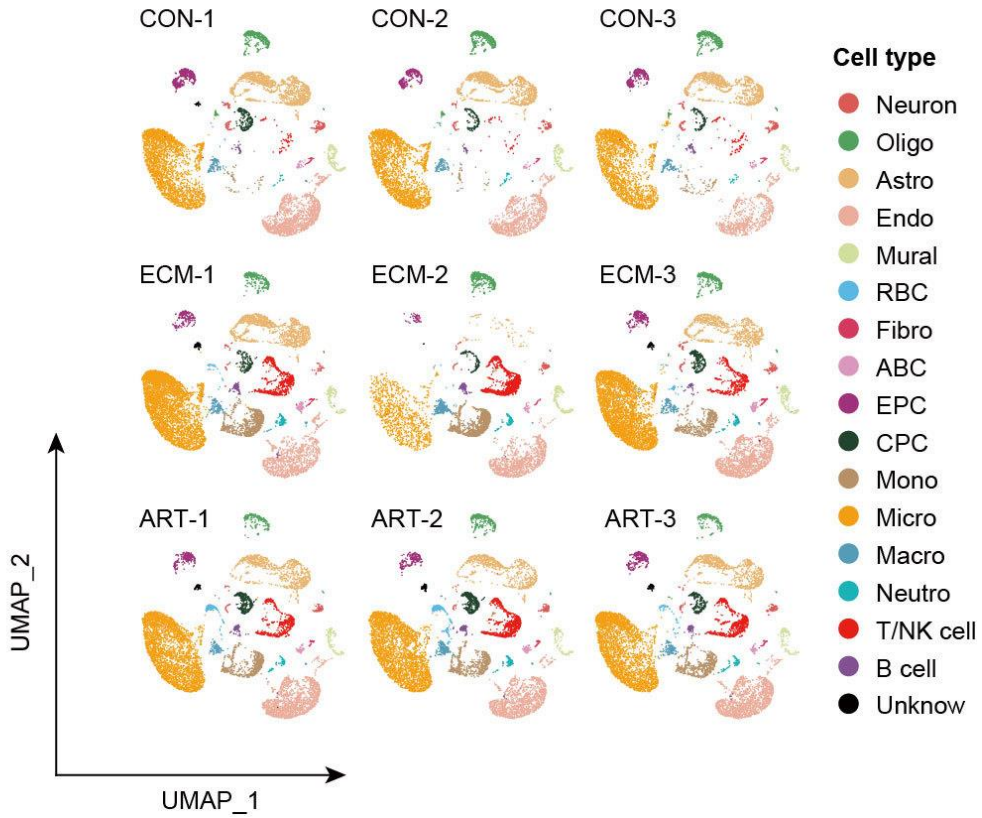
We appreciate your efforts in reviewing our manuscript and thanks for the positive and detailed feedback. According to your suggestion, we have addressed your concerns as shown below.

R1_1. If I am not mistaken, the authors didn't remove batch effects of the data before cell clustering analysis. Could they provide UMAP plots assessing batch effects among samples within the same group in the supplementary figures?

✓**Response:**

We apologize for the carelessness and omission of description in the manuscript. Actually, we have performed canonical correlation analysis to remove batch effects and dataset integration before cell clustering analysis of the scRNA-seq dataset. Here, we provide the individual UMAP plots to assess the batch effects among samples within the same group in the supplementary figures (Supplementary Fig. 2e), and we also have added a description of this part in the **Methods** (Line 789-791**)** and **Results** (Line 141-142**)** parts in the revised manuscript.

Supplmentary Fig. 2e



Supplementary Fig. 2e Splited UMAP plot shows distribution of various cell types of three biological replicates of three groups in the scRNA-seq dataset, colored by cell types. Oligo: oligodendrocyte, Astro: astrocyte, Endo: endothelial cells, Mural: Mural cell, RBC: red blood cell, Fibro: fibroblast, ABC: arachnoid barrier cells, EPC: ependymocytes, CPC: choroid plexus epithelial cells, Mono: monocyte, Micro: microglia, Macro: macrophage, Neutro: neutrophils.

R1_2. The cell-type-specific marker genes used for cell-type and cell-subtype classification in the article should be referenced with the source literature. It is very important to respect

the results of previous research. I strongly encourage the authors to present this information in a table.

✓Response:

Thanks for your valuable comment. To respect the results of previous research and to make our analysis results more reproducible, we here organized and listed the references with the source literature used for cell-type and cell-subtype classification in our article (Supplementary Table. 1).

Supplementary Table1. the reference of cell-type-specific marker genes list

Cell type	Marker	Reference	Cell subtype	Marker	Reference
Neuron	*Sox11, Nrxn3, Ntn1*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	Neuron_Pro	*Mki67*	**Hochgerner H, et al. *Nat Neurosci.* 2023 Dec;26(12):2237-2249.; Shi Y, et al. *Adv Sci (Weinh).* 2022 Mar;9(7):e2104112.**
			Neuron_IP	*Ascl1*	
			Neuron_IM	*Dcx, Tubb3*	
			Neuron_M_ex	*Rbfox3, Dlg4, Camk2a, Slc17a6, Slc17a*	
			Neuron_M_in	*Gad1, Gad2, Slc32a1*	
Oligo: oligodendrocyte	*Mbp*	**Lau SF, et al. *Proc Natl Acad Sci U S A.* 2020 Oct 13;117(41):25800-25809.**	——	——	——
Astro: astrocyte	*Adloc , Aldh1l1, Slc1a2, S100b, Aqp4, Apoe*	**Zheng K, et al. *J Cereb Blood Flow Metab.* 2022 Jan;42(1):56-73.**	Astro_C1	*Agt, Itih3, Nnat*	**Hasel P, et al. *Nat Neurosci.* 2021 Oct;24(10):1475-1487. ; Batiuk MY, et al. *Nat***
			Astro_C2	*Mfge8, Ptn*	
			Astro_C3	*Cx3cr1, Csf1r*	
			Astro_C4	*Gria1, Hopx*	
			Astro_C5	*Gfap, Meg3, Cd9*	

					Commun. **2020 Mar 5;11(1):1220.**
Endo: endothelial cells	*Cldn5*，*Kdr, Pecam1*	**Lau SF, et al. *Proc Natl Acad Sci U S A*. 2020 Oct 13;117(41):25800-25809.**	Endo_Art	*Bmx, Efnb2, Vegfc*	**Vanlandewijck M, et al. *Nature.* 2018 Feb 22;554(7693):475-480. ; Garcia FJ, et al. *Nature.* 2022 Mar;603(7903):893-899.**
			Endo_Cap	*Mfsd2a, Tfrc, Slc7a5*，*Slc16a1*	
			Endo_Ven	*Vwf, Slc38a5, Ackr1, Lrg1, Vwf, Ch25h, Vcam1, Icam1*	
			Endo_Inflam	*Ccl3, Ccl4, Ccl5*	
			Endo_Hb	*Hba-a1, Hba-a2*	
Mural: Mural cell	*Kcnj8*	**Ximerakis M, et al. *Nat Neurosci*. 2019 Oct;22(10):1696-1708.**	aSMC	*Acta2, Myl9, Myh11, Tagln*	**Vanlandewijck M, et al. *Nature.* 2018 Feb 22;554(7693):475-480. ; Garcia FJ, et al. *Nature.* 2022 Mar;603(7903):893-899.**
			vSMC	*Acta2, Abcc9, Pdgfrb, Vtn*	
			Pericyte	*Kcnj8*	
RBC: red blood cell	*Hba-a1*	**Cheng CK, et al. *J Adv Res.* 2023 Jan;43:187-203.**	hRBC (healthy RBC)	*Hba-a1*	**De Niz M, et al. *Nat Commun.* 2016 May 26;7:11659. ; Ruberto AA, et al. *Sci Rep.***
			iRBC (infected RBC)	*PBANKA_1340100* ， *PBANKA_1145800* *PBANKA_1101300*	

					2021 Feb 22;11(1):4127.
Fibro: fibroblast	*Dcn*	**Chu H, et al. *Exp Ther Med.* 2018 Sep;16(3):1729-1734.**	——	——	——
ABC: arachnoid barrier cells	*Slc47a1*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	——	——	——
EPC: ependymocytes	*Ccdc153*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	——	——	——
CPC: choroid plexus epithelial cells	*Ttr*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	——	——	——
Mono: monocyte	*Plac8*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	Mono_classical	*Ccr2, Ly6c2*	**Pombo Antunes AR, et al. *Nat Neurosci.* 2021 Apr;24(4):595-610.; Hammond MD, et al. *J Neurosci.* 2014 Mar 12;34(11):3901-9.**
			Mono_non-classical	*Ear2, Ace, Eno3*	
Micro: microglia	*Aif1*	**Kenkhuis B, et al. *Neurobiol Dis.* 2022**	Micro_Home	*Tmem119, P2ry12*	**Zhan L, et al.**
			Micro_Ap	*H2-Oa, H2-M3*	

		Jun 1;167:105684.	Micro_Inflam	*Ccl3, Ccl4*	***Elife.* 2020 Oct 15;9:e51796.**
			Micro_IEG	*Jun, Fos*	
			Micro_RNASplic	*Snrnp70, Luc7l2*	
Macro: macrophage	*Pf4*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	Macro_Home	*Mrc1, Cd163*	**Jordão MJC, et al. *Science.* 2019 Jan 25;363(6425):eaat7554.**
			Macro_Ap	*H2-Aa, H2-Ab1*	
Neutro: neutrophils	*S100a9*	**Ximerakis M, et al. *Nat Neurosci.* 2019 Oct;22(10):1696-1708.**	Neutro_Home	*Ltf, Ngp*	**Li X, et al. *J Neuroinflammation.* 2022 Apr 7;19(1):83.**
			Neutro_Activated	*Il1b*	
			Neutro_Chemo	*Ccl6, Ccl9*	
T/NK cell: Tlymphocyte and NK cell	*Cd3e*	**Guo K, et al. *Front Cell Dev Biol.* 2021 Feb 22;9:624711.**	CD4_Tn	*Tcf7, Ccr7, Lef1*	**Zheng L, et al.*Science.* 2021 Dec 17;374(6574):abe6474.**
			CD4_Tm	*S100a4, Cd40lg, Cxcr6, Cxcr3*	
			CD4_Tpro	*Mki67, Stmn1*	
			CD8_Tn	*Tcf7, Ccr7, Lef1*	
			CD8_Tem	*Fasl, Cxcr6, Cxcr3*	
			CD8_CTL	*Ccl5, Klrd1, Gzmb*	
			CD8_Tpro	*Mki67, Stmn1*	
			γdT	*Trdc*	
			NK	*Ncr1*	
B cell	*Cd79a*	**Guo K, et al. *Front Cell Dev Biol.* 2021 Feb 22;9:624711.**	——	——	——

References:

[1] Lau SF, et al. Single-nucleus transcriptome analysis reveals dysregulation of angiogenic endothelial cells and neuroprotective glia in Alzheimer's disease. ***Proc Natl Acad Sci U S A***. 2020 Oct 13;117(41):25800-25809.**PMID:32989152**

[2] Zheng K, et al. Single-cell RNA-seq reveals the transcriptional landscape in ischemic

stroke. ***J Cereb Blood Flow Metab.*** 2022 Jan;42(1):56-73. **PMID: 34496660**

[3] Cheng CK, et al. SOX4 is a novel phenotypic regulator of endothelial cells in atherosclerosis revealed by single-cell analysis. ***J Adv Res.*** 2023 Jan;43:187-203. **PMID: 36585108**

[4] Chu H, et al. Effects of transplanted adipose derived stem cells on the expressions of α-SMA and DCN in fibroblasts of hypertrophic scar tissues in rabbit ears. ***Exp Ther Med.*** 2018 Sep;16(3):1729-1734. **PMID: 30186394**

[5] Kenkhuis B, et al. Co-expression patterns of microglia markers Iba1, TMEM119 and P2RY12 in Alzheimer's disease. ***Neurobiol Dis.*** 2022 Jun 1;167:105684. **PMID: 35247551**

[6] Ximerakis M, et al. Single-cell transcriptomic profiling of the aging mouse brain. ***Nat Neurosci***. 2019 Oct;22(10):1696-1708. **PMID: 31551601**

[7] Guo K, et al. Single-Cell RNA Sequencing With Combined Use of Bulk RNA Sequencing to Reveal Cell Heterogeneity and Molecular Changes at Acute Stage of Ischemic Stroke in Mouse Cortex Penumbra Area. ***Front Cell Dev Biol.*** 2021 Feb 22;9:624711. **PMID: 33692998**

[8] Hochgerner H, et al. Neuronal types in the mouse amygdala and their transcriptional response to fear conditioning. ***Nat Neurosci.*** 2023 Dec;26(12):2237-2249. **PMID: 37884748**

[9] Shi Y, et al. Single-Nucleus RNA Sequencing Reveals that Decorin Expression in the Amygdala Regulates Perineuronal Nets Expression and Fear Conditioning Response after Traumatic Brain Injury. ***Adv Sci (Weinh).*** 2022 Mar;9(7):e2104112. **PMID: 35038242**

[10] Hasel P, et al. Neuroinflammatory astrocyte subtypes in the mouse brain. ***Nat Neurosci.*** 2021 Oct;24(10):1475-1487. **PMID: 34413515**

[11] Batiuk MY, et al. Identification of region-specific astrocyte subtypes at single cell resolution. ***Nat Commun.*** 2020 Mar 5;11(1):1220. **PMID: 32139688**

[12] Vanlandewijck M, et al. A molecular atlas of cell types and zonation in the brain vasculature. ***Nature.*** 2018 Feb 22;554(7693):475-480. **PMID: 29443965**

[13] Garcia FJ, et al. Single-cell dissection of the human brain vasculature. ***Nature.*** 2022 Mar;603(7903):893-899. **PMID: 35158371**

[14] De Niz M, et al. The machinery underlying malaria parasite virulence is conserved between rodent and human malaria parasites. ***Nat Commun.*** 2016 May 26;7:11659. **PMID: 27225796**

[15] Ruberto AA, et al. Single-cell RNA sequencing reveals developmental heterogeneity

among Plasmodium berghei sporozoites. ***Sci Rep.*** 2021 Feb 22;11(1):4127. **PMID: 33619283**

[16] Pombo Antunes AR, et al. Single-cell profiling of myeloid cells in glioblastoma across species and disease stage reveals macrophage competition and specialization. ***Nat Neurosci.*** 2021 Apr;24(4):595-610. **PMID: 33782623**

[17] Hammond MD, et al. CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage. ***J Neurosci.*** 2014 Mar 12;34(11):3901-9. **PMID: 24623768**

[18] Zhan L, et al. A MAC2-positive progenitor-like microglial population is resistant to CSF1R inhibition in adult mouse brain. ***Elife.*** 2020 Oct 15;9:e51796. **PMID: 33054973**

[19] Jordão MJC, et al. Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. ***Science.*** 2019 Jan 25;363(6425):eaat7554. **PMID: 30679343**

[20] Li X, et al. Single-cell transcriptomic analysis of the immune cell landscape in the aged mouse brain after ischemic stroke. ***J Neuroinflammation.*** 2022 Apr 7;19(1):83. **PMID: 35392936**

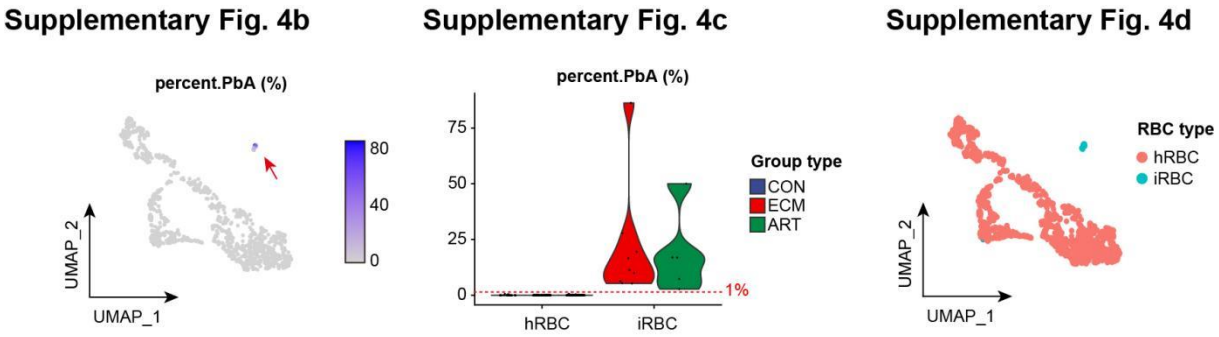
[21] Zheng L, et al. Pan-cancer single-cell landscape of tumor-infiltrating T cells. ***Science.*** 2021 Dec 17;374(6574):abe6474. **PMID: 34914499**

R1_3. The authors used a 1% threshold of malaria parasite gene expression reads to determine if an RBC was infected with the parasite. Could they provide the rationale for setting this threshold? Is it possible that, during late-stage infection of mouse brain tissue, some captured single cells may actually be malaria parasite cells? If this is a possibility, how would such cells be identified through data analysis methods? Additionally, why wasn't the same analysis of PbA gene expression applied to spatial transcriptomics data?

✓**Response:**

Thanks for your detailed review and nice question. Firstly, after extracting 933 red blood cells (RBC) population from the integrated scRNA-seq dataset, we evaluated the *Plasmodium berghei* ANKA (PbA) gene expressed percentage (percent.PbA) of RBC across the three groups (Supplementary Fig. 4b), finding that the percent.PbA in the ECM group was dramatically higher than the other two groups (Supplementary Fig. 4c). The max value of percent.PbA in hRBC of the CON group is 0.61%, which might be attributed to the homologous gene expression between the mice and PbA parasite. For example, we found that most of these reads in the CON group were mapped to *PBANKA_0700761*, a 28S ribosomal RNA of parasite.

According to the Basic Local Alignment Search Tool (BLAST) result, this gene has relatively higher identity with 28S ribosomal RNAs in mice (percent identity: 89.19%, E value:7e-126). Therefore, we used a 1% threshold of percent.PbA to determine whether an RBC was infected with the parasite, which could distinguish healthy RBA (hRBC) and infected RBC (iRBC), eventually obtaining 919 hRBC and 14 iRBC (Supplementary Fig. 4d). We have proved the rationale for setting this threshold in the revised manuscript (Line 204-208).



Supplementary Fig. 4b: Featureplot shows the expression level of percent.PbA in the RBC dataset. **Supplementary Fig. 4c**: Vlnplot shows the level of percent.PbA of hRBC (of three groups) and iRBC (of ECM and ART groups), the horizontal dash line in red indicates the percentage cutoff (1%). **Supplementary Fig. 4d**: UMAP plot shows the annotation result of hRBC and iRBC, colored by RBC types.

Secondly, during the entire intra-erythrocytic developmental cycle, *plasmodium* parasite mainly parasitically resides in the RBC of the host, and cannot exist in the host cells freely (**Hafalla JC, et al. *Immunol Rev*. 2011;240(1):297-316.**). According to recent single *plasmodium* transcriptomics studies, most of them identified *plasmodium* parasites via analyzing the infected red blood cells with parasites in *in vitro* culture models **(Poran, A, et al. *Nature.* 2017;551(7678):95-99.**). The direct identification of malaria parasites or sporozoites requires complicated pre-processing such as flow sorting, magnetic bead sorting, or density gradient (**Howick VM, et al. *Science*. 2019; 365(6455):eaaw2619.; Ruberto AA, et al. *Sci Rep.* 2021 Feb 22;11(1):4127.**), which was not performed in our study. Here, we mainly use this pipeline to indirectly profile the gene expression characteristics of *plasmodium* by analyzing infected RBC. However, we did not establish a long-term *in vivo* or *in vitro* model in the current study, thus we cannot rule out the possibility of direct detection of malaria parasites via such an analysis process. We acknowledge that this a meaningful issue, it is beyond the scope of the current study and worthy of further research.

Lastly, we actually tried to apply the analytical process to our ST-seq dataset to directly locate the *plasmodium* infected-relevant regions. However, our Spaceranger's quantitative results indicated the absence of infected RBC among all spots. This result could be attributed to the limitation of the composition of sequencing reads within spots, the sensitivity and coverage of sequencing reads, and the sagittal sections of the brain used for ST-seq experiment. Therefore, we did not mention the results about applying this pipeline to the ST-seq dataset.

References:

1. Hafalla JC, et al. Cell biology and immunology of malaria. ***Immunol Rev***. 2011;240(1):297-316. **PMID: 21349101.**

2. Poran, A, et al. Single-cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. ***Nature***. 2017;551(7678):95-99**. PMID: 29094698.**

3. Howick VM, et al. The Malaria Cell Atlas: Single parasite transcriptomes across the complete Plasmodium life cycle. ***Science***. 2019; 365(6455):eaaw2619**. PMID: 31439762.**

4. Ruberto AA, et al. Single-cell RNA sequencing reveals developmental heterogeneity among Plasmodium berghei sporozoites. ***Sci Rep.*** 2021 Feb 22;11(1):4127. **PMID: 33619283.**

R1_4. In the Methods section, the authors used different thresholds (15% and 30%) for mitochondrial reads in processing single-cell and spatial transcriptomic data. If samples of two omics are from the same tissue, why did the authors use two different threshold standards? Please explain.

✓**Response:**

Thanks for your detailed reviews of our research. Previous studies have reported that the up-regulation of RNA levels of mitochondrial genes was correlated with cell death or injury. Hence, the cells that expressed a higher proportion of mitochondrial genome UMIs should be considered as low cellular quality cells and be filtered in the further analysis (**Galluzzi L, et al. *Nat Rev Mol Cell Biol*. 2012;13(12):780-788.; Ilicic T, et al. *Genome Biol*. 2016;17:29.**). However, this parameter (the cutoff of the proportion of mitochondrial genome UMIs) is highly dependent on the tissue type, technical characteristics and the biological questions to be investigated (**AlJanahi AA, et al. *Mol Ther Methods Clin Dev*. 2018;10:189-196.**).

In our single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics sequencing (ST-

seq) datasets, even though the samples are from the same brain tissue, the experimental process and the generation of two omics datasets differ according to the manufacturer's protocol. For example, tissue dissociation and cell suspensions made many neuron cells vulnerable and subsequently unable to pass the standards of quality control (QC). Thus, we adopted a stricter QC threshold (15% mitochondrial reads) for the scRNA-seq dataset, to obtain a high-quality cellular transcriptomics dataset. Moreover, we want to retain sufficient number of spots for subsequent studies, that we needed to set a looser threshold (30% mitochondrial reads) in the ST-seq dataset.

In addition, there are several studies employing both scRNA-seq and ST-seq methods, that also used different QC thresholds in UMIs, mitochondrial reads precent etc. (**Hasel, Philip et al. *Nature neuroscience.* 2021: 1475-1487.; Lee, Sangderk et al. *Cell reports* 2023: 112196.**). Therefore, it is reasonable that we adopt different QC cutoffs of mitochondrial read percent for the two omics datasets.

References:

1. Galluzzi L, et al. Mitochondria: master regulators of danger signalling. ***Nat Rev Mol Cell Biol.*** 2012;13(12):780-788. **PMID: 23175281**

2. Ilicic T, et al. Classification of low quality cells from single-cell RNA-seq data. ***Genome Biol.*** 2016;17:29. **PMID: 26887813**

3. AlJanahi AA, et al. An Introduction to the Analysis of Single-Cell RNA-Sequencing Data. ***Mol Ther Methods Clin Dev.*** 2018;10:189-196. **PMID: 30094294**

4. Hasel, Philip et al. Neuroinflammatory astrocyte subtypes in the mouse brain. ***Nature neuroscience.*** 2021: 1475-1487. **PMID: 34413515**

5. Lee, Sangderk et al. APOE modulates microglial immunometabolism in response to age, amyloid pathology, and inflammatory challenge. ***Cell reports*** 2023: 112196. **PMID: 36871219**

R1_5. In line 154, the authors referenced public ST-seq data but needed to include a specific literature citation.

✓**Response:**

Thanks for your valuable comment. In this study, we have retrieved and integrated four public ST-seq datasets of healthy mouse brain tissue as Control group for further comparison, and we have added a specific literature citation/website

(https://www.10xgenomics.com/cn/resources/datasets/) in our revised manuscript as per your recommendations.

Samples	Slice	Links
Mouse Brain Serial Section 1	Sagittal-Anterior	https://www.10xgenomics.com/resources/datasets/mouse-brain-serial-section-1-sagittal-anterior-1-standard-1-1-0
Mouse Brain Serial Section 1	Sagittal-Posterior	https://www.10xgenomics.com/cn/resources/datasets/mouse-brain-serial-section-1-sagittal-posterior-1-standard-1-1-0
Mouse Brain Serial Section 2	Sagittal-Anterior	https://www.10xgenomics.com/cn/resources/datasets/mouse-brain-serial-section-2-sagittal-anterior-1-standard-1-1-0
Mouse Brain Serial Section 2	Sagittal-Posterior	https://www.10xgenomics.com/cn/resources/datasets/mouse-brain-serial-section-2-sagittal-posterior-1-standard-1-1-0

R1_6. The article contains some inconsistencies in gene nomenclature and grammar issues (e.g., PbANKA-1340100 and PbANKA_1145800 naming inconsistency, capitalization errors, and missing spaces between words). Please thoroughly review these grammar and naming issues.

✓**Response:**

Thanks for your detailed investigation and valuable suggestions, we have thoroughly reviewed the grammar and naming issues including naming inconsistency, capitalization errors, and missing spaces between words, and carefully corrected these mistakes and updated the figures and manuscript.

R1_7. The introduction of the spatial transcriptomic research topic in the abstract (Line 36) and results section (Line 152) were not well-organized. Consider refining the text to better introduce the topic (combined with the current development of CM research) for better follow-up.

✓**Response:**

Thanks for your great suggestion on improving the accessibility of our manuscript. To this point, we have rewritten the description of the spatial transcriptomic research topic in the **Abstract** (Line 7-10) and **Results** section (Line 146-148). In combination with the current development of CM research, we highlight the power and advantages of spatial transcriptomic technology, so as to better introduce the topic in our study.

R1_8. Why did the author choose only days 0, 5, and 8 in the experimental design? A specific explanation may be needed.

✓Response:

1. Thank you for point this out. As the schematic plot shows (Fig. 1a), we injected PbA-infected red blood cells into healthy mice on day 0 (d0), and evaluated body weight, malaria infection, survival condition, and scores of the rapid murine coma and behavior scale (RMCBS), as indicatives of ECM. Based on the results of our preliminary experiments, the mice in the ECM group began to manifest the syndrome of ECM on 5 days (d5) after PbA infection, with significantly lower RBMCS. ECM mice exhibited a spike in infection and death on d5, and a sharp increase in death on 8 days (d8), as previously reported (**Carroll RW, et al. *PLoS One*. 2010;5(10): e13124.; Capuccini B, et al. *Sci Rep*. 2016, 19; 6:39258.**). According to malaria treatment guidelines, artesunate (ART) injection can be used for the treatment of malaria for three days in most cases (**Hess KM, et al. *Ann Pharmacother.* 2010;44(7-8):1250-1258.**), thus we treated the mice in the ECM group with ART on d5. After the administration of ART for 3 days, most of these ECM conditions were alleviated, and the mice from all groups were anesthetized and sacrificed on d8. This experimental design is thus essential to understand the extent of ECM development in mice at the time point of artesunate treatment, and we have added a specific explanation in the **Results** part in the revised manuscript.

Fig. 1a

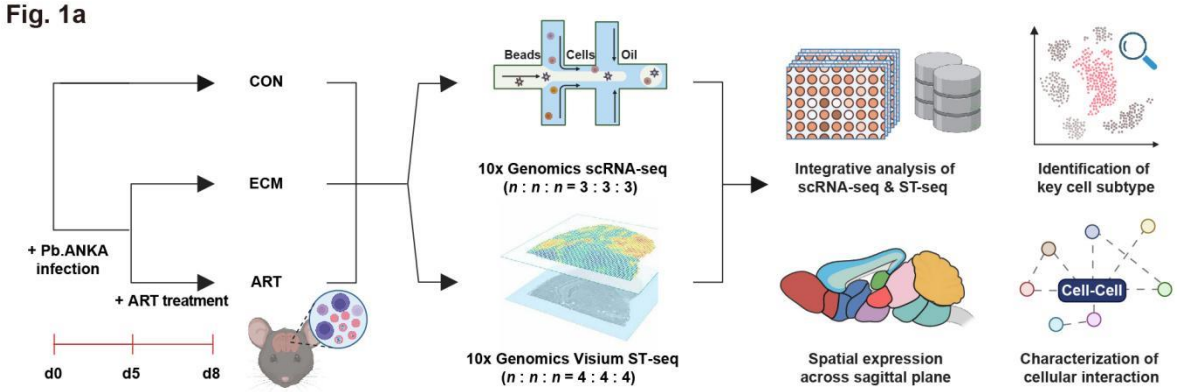


Fig. 1a Scheme depicts the experimental process and analytical workflow of murine brain tissues from CON, ECM and ART groups on d8 via integrating scRNA-seq and ST-seq technologies (created with BioRender.com).

References:

1. Carroll RW, et al. A rapid murine coma and behavior scale for quantitative assessment of murine cerebral malaria. ***PLoS One***. 2010;5(10): e13124. **PMID:** 20957049

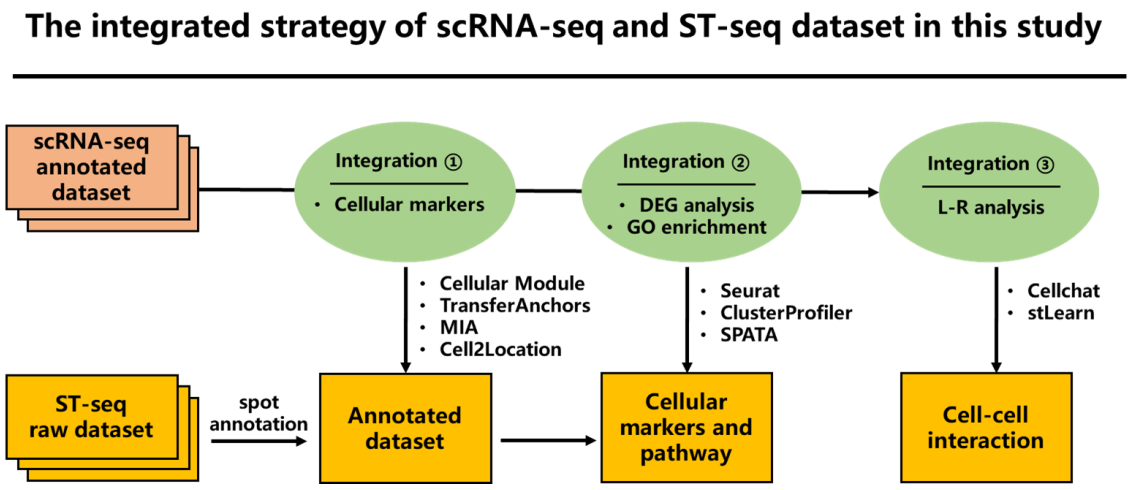
2. Capuccini B, et al. Transcriptomic profiling of microglia reveals signatures of cell activation and immune response, during experimental cerebral malaria. ***Sci Rep.*** 2016, 19; 6:39258. **PMID: 27991544**

3. Hess KM, et al. Intravenous artesunate for the treatment of severe malaria. ***Ann Pharmacother.*** 2010;44(7-8):1250-1258. **PMID :20551300**

R1_9. While the analysis in the article is comprehensive in my opinion, there is still room for improvement in integrating single-cell and spatial group data.

✓**Response:**

Thanks for your kind suggestions. We appreciate your appraisal of our analysis and acknowledge that there is still room for improvement in the integration of the two datasets. We have thus adopted additional analysis methods and highlighted our integrated strategy in the **Methods** and **Results** parts in our revised manuscript. To make it as clear as possible, we here made a diagram to demonstrate the integration strategy of the scRNA-seq and ST-seq datasets in this study. As shown in the following figure, our analysis has three main integrative sections.



① the integration of scRNA-seq and ST-seq dataset for spot annotation:

Based on the well annotated scRNA-seq dataset, our analysis has applied four multi-omics integration methods: (i) Cellular module score, (ii) Transfer anchor, (iii) Multimodal intersection analysis (MIA) and (iv) Cell2Loaction algorithm, to evaluate the accuracy and concordance of our spot types' annotation in the ST-seq dataset labeled by SingleR package. Among these, the integrating methods (i) and (ii) have been employed in previous studies, and iii and iv were added as other reviewers suggested.

② the integration of scRNA-seq and ST-seq datasets for DEG and pathways analysis:

We employed Seurat and ClusterProfiler packages to investigate the cellular transcriptome alteration and enriched process of various cell types during ECM development and ART treatment, identifying the representative DEGs and pathways. Using SPATA method, we further explored the spatial intensity of these DEG and pathways at different regions and spot types.

③ the integration of scRNA-seq and ST-seq datasets for ligand-receptor (L-R) pairs analysis:

We used the CellChat R package to calculate the L-R interaction scores among different cell/spot types in the scRNA-seq and ST-seq datasets, to identify the overlapping L-R pairs within the scRNA-seq and ST-seq datasets. We also used stLearn algorithm to evaluate and validate the spatial distribution of specific L-R pairs identified above.

Reviewer #2

Chen et al have utilised single cell RNA-sequencing and spatial transcriptomics to investigate the brain response during ECM, and following artesunate treatment of the condition. As the pathways influencing ECM development are still unclear and there is a critical need for new adjunctive therapies for cerebral malaria, the study is of some interest. However, although the authors undertake a number of investigations, the biological relevance and implications of some of the results are unclear, and many of the results confirm existing knowledge. Moreover, it is unclear whether the ST analyses provide the resolution in places to significantly enhance knowledge of the spatiotemporal intracerebral responses to artesunate

treatment.

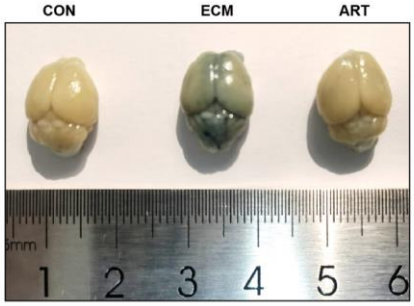
We greatly appreciate your professional review work on our article. The reviewer has given an accurate summary of our work and brought forward constructive questions. According to your excellent suggestions, we have made extensive corrections to our previous draft. The detailed corrections are listed below.

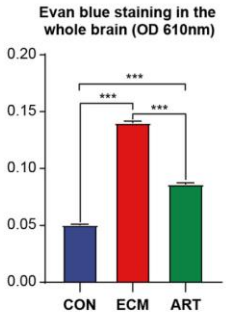
R2_1. It is unclear what day of analysis the analyses in Fig S1 E and F was performed on. This is essential to understand the extent of ECM development in mice at the time point of artesunate treatment. Relating to this, there does not appear to be a lot of BBB damage in the model group.

✓**Response:**

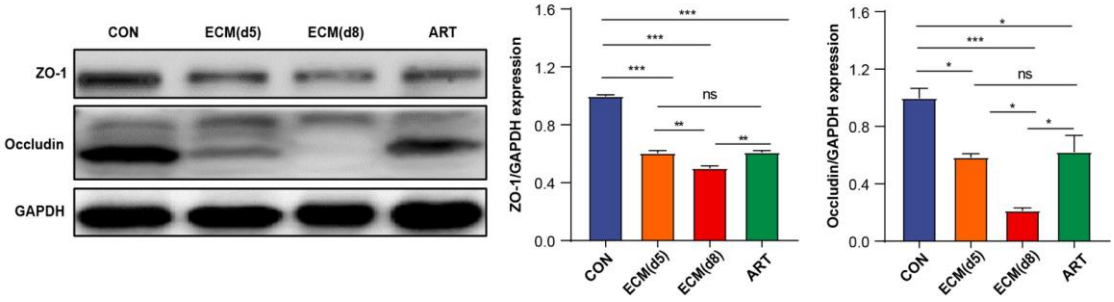
We appreciate your kind reminder, and acknowledge that the days setting is essential to understand the extent of ECM progression in mice at the time point of artesunate treatment. Firstly, the analysis in Supplementary Fig. 1e (Evans blue staining) and Supplementary Fig. 1g (H&E staining) was performed on 8 days (d8). Secondly, we used Evans blue dye to evaluate vascular permeability in murine brains, and our results indicate that the Evans blue dye leaked in large amounts in the ECM group compared with the CON and ART groups on d8, in agreement with previous studies (**Ahishali B, et al. *Methods Mol Biol.* 2021;2367:87-103.; Kuehlwein JM, , et al. *Immunology*. 2020;159(2):193-204.; Xu G, van Bruggen R, Gualtieri CO, et al. *Cell Rep*. 2020;32(12):108170.**). Lastly, we also evaluated the expression level of tight junction proteins such as ZO-1 and Occludin in the CON, ECM(d5), ECM(d8) and ART groups. We found that the expression of these two proteins significantly decreased in the ECM(d5) and ECM(d8) groups compared with that in the CON group. However, the BBB injury of the ART group were partly alleviated when compared with the ECM(d8) group, but not restored to healthy baseline (CON group) (Supplementary Fig. 1f). These results indicate that the BBB damage occurs in the brain tissues of the ECM(d8) group, which can be used as a follow-up study.

Supplementary Fig. 1e





Supplementary Fig. 1f



Supplementary Fig. 1e Evans blue staining of whole murine brain tissues (left panel) and the quantitative statistics (right panel) in three groups (n = 6 samples per group). **Supplementary Fig. 1f** The protein expression of ZO-1 and Occludin by western blot (left) and quantitative statistics (right panel) corresponding to groups in CON, ECM (d5), ECM (d8), and ART groups (n = 3 samples per group).

References:

1. Ahishali B, et al. Evaluation of Blood-Brain Barrier Integrity Using Vascular Permeability Markers: Evans Blue, Sodium Fluorescein, Albumin-Alexa Fluor Conjugates, and Horseradish Peroxidase. ***Methods Mol Biol.*** 2021;2367:87-103. **PMID**: **32785841**

2. Kuehlwein JM, , et al. Protection of Batf3-deficient mice from experimental cerebral malaria correlates with impaired cytotoxic T-cell responses and immune regulation. ***Immunology.*** 2020;159(2):193-204. **PMID**: **31631339**

3. Xu G, van Bruggen R, Gualtieri CO, et al. Bisphosphoglycerate Mutase Deficiency Protects against Cerebral Malaria and Severe Malaria-Induced Anemia. ***Cell Rep.*** 2020;32(12):108170. **PMID**: **32966787**

R2_2. For ST spot-type annotation, it is unclear why the authors did not use the scRNA-seq to more thoroughly deconvolve the spots (i.e. Cell2Location) to define the relative abundance of cell types within each spot. This would provide more detailed insight on spot identity, rather

than referring to spots broadly as dominant endothelial, astrocyte etc. Indeed, due to the nature of the brain architecture, there will usually be numerous cell types in a particular spot, and this will become more evident around the cerebrovasculature in mice with ECM or following treatment. There is a concern in Fig S3c that microglial cells are within such a small area in the UMAP, when realistically they will be highly prevalent throughout the brain parenchyma. In figure 4, showing the Endo_inflam spots only in specific locations is also misleading.

✓**Response:**

Thanks for your valuable comments and suggestions. To profile the transcriptional alteration of various cell types identified in the scRNA-seq dataset in the spatial context, we here assigned the dominant identities such as Endo, Immune and Neuron etc. to the spots in the integrated ST-seq dataset. Based on the current resolution of Visium method, the cerebrovascular architecture of ECM murine brain might contain numerous cells within a particular spot, and we have acknowledged these limitations in the ST-seq dataset, and modified our description of the identities of these spots as "main spot type" in the revised manuscript to avoid ambiguity. As you suggested, we have applied several methods to evaluate the accuracy and provide more detailed insight on spot identities.

In addition to the cellular module score and TransferAnchors integrated methods, we performed two other methods including multimodal intersection analysis (MIA) (**Moncada R, et al. *Nat Biotechnol.* 2020 Dec;38(12):1476.**) and cell2location (**Kleshchevnikov V, et al. *Nat Biotechnol.* 2022 May;40(5):661-671.)** to evaluate the relative abundance of cell types within each spot in the ST-seq datasets. We observed that Neuron, Oligo and CPC spots have high specificity of the corresponding cellular types in the MIA results, in agreement with our previous analysis (Fig. 2b). In the deconvoluted results by cell2location, we observed the co-location of serval cell types within the same spots at some regions, while the predominant identity of each spot was basically consistent with the annotated spot types, such as Neuron, Oligo, Immune and Endo spots in the ECM_1_P slide (Fig. 2c). Among them, immune cells (involving Macro, Micro, Mono and T/NK cells) were distributed diffusely in some regions of the brain parenchyma, and significantly enriched at the Immune spots, surrounded by Endo spots.

As for the key subtypes such as Micro_Inflam and Endo_Inflam, we calculated the module scores based on the cluster-specific genes of various spots in the ECM_1_P of ST-seq dataset. We found that the Endo_Inflam subtype mainly distributed at the Endo spots, while Micro_Inflam has elevated module scores throughout the brain parenchyma, in agreement with their actual prevalence in the brain tissue (Fig. 3d). Therefore, we have adjusted the description about the spatial distribution of Micro_Inflam (being more prevalent throughout the brain tissue rather than confined within such a small area). Despite of this, Micro_Inflam in Immune spots exhibited higher scores that non-Immune spots, supporting our spots' annotation. Furthermore, we also performed cell2location on the key subtypes including Endo_Inflam, Micro_Inflam and CD8_CTL to further explore their spatial distribution, demonstrating the co-localization of these three subtypes within the Immune-Endo spots (Fig. 6g). Taken together, we have validated the annotation of main spot types via additional methods for integrating the scRNA-seq and ST-seq datasets, observing high consistency among these results.

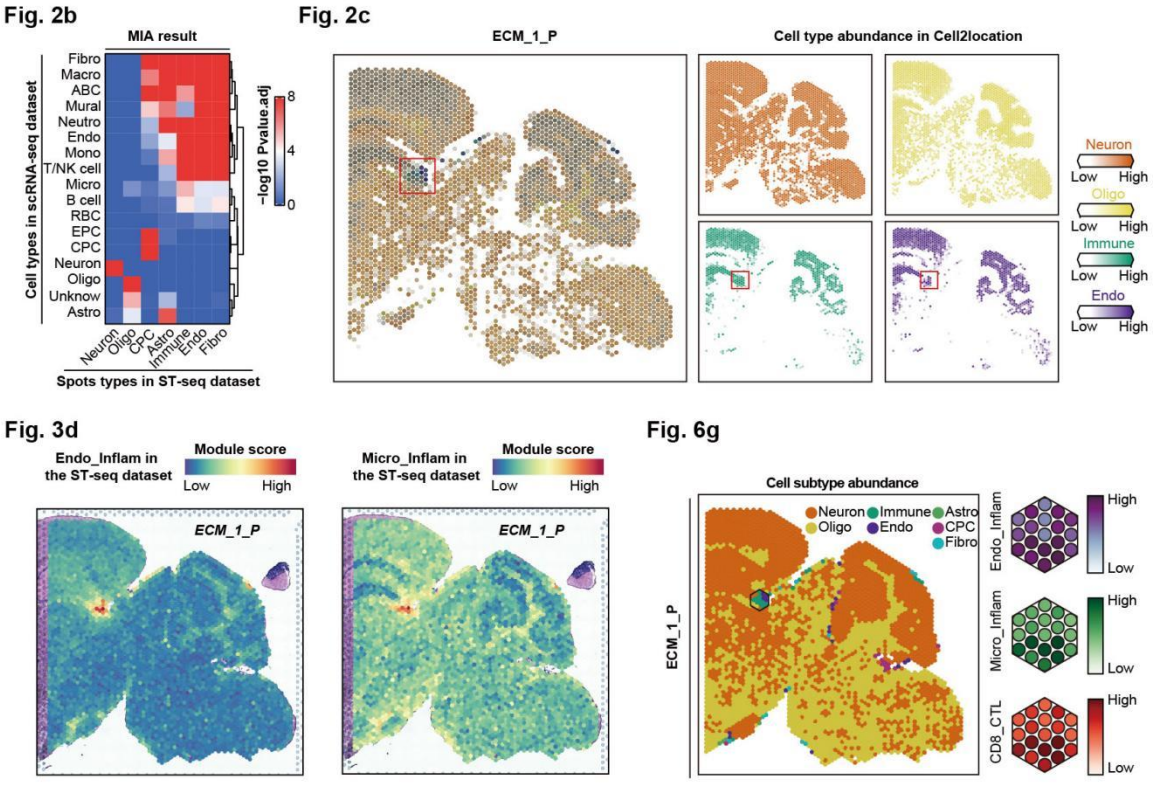


Fig. 2b The heatmap indicates the correlation significance of cell types in scRNA-seq and spot types in ST-seq dataset using MIA method. **Fig. 2c** The visualization indicates the estimated cell abundances (color intensity) of key cell types in ECM_1_P slide using cell2location method. **Fig. 3d** Module scores distribution of Endo_Inflam and Micro_Inflam subtypes in the ECM_1_P slide in the ST-seq dataset. **Fig. 6g** The visualization indicates the estimated cell

abundances (color intensity) of key cell types (Endo_Inflam, Micro_Inflam and CD8_CTL) in ECM_1_P slide using cell2location method.

Reference:

1. Moncada R, et al. Integrating microarray-based spatial transcriptomics and single-cell RNA-seq reveals tissue architecture in pancreatic ductal adenocarcinomas. ***Nat Biotechnol***. 2020 Dec;38(12):1476. **PMID: 31932730**.

2. Kleshchevnikov V, et al. Cell2location maps fine-grained cell types in spatial transcriptomics. ***Nat Biotechnol.*** 2022 May;40(5):661-671. **PMID: 35027729.**

R2_3. It is important that the authors attempted to verify the ST data using immunofluorescence in figure 2; however, at the resolution and magnification provided, it is unclear how robust the staining is. It is difficult to visualise the CD3+ T cells and some of the staining around the ventricle looks quite diffuse. This needs to be improved.

✓**Response:**

Thanks for your valuable comment**.** We have optimized our immunofluorescence results, to visualize the T cell (marked by $CD3^+$) and Myeloid cell (marked by $CD68^+$) more clearly (Fig 2f). We also observed similar distribution of T cell and Myeloid cell at the local regions, indicating the robustness of the immunofluorescence staining in our study.

Fig. 2f

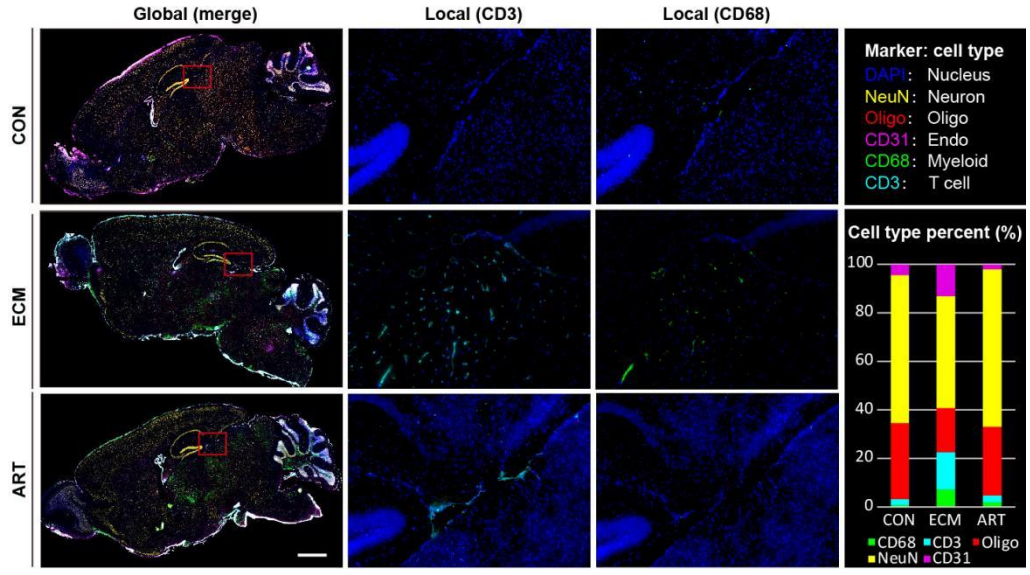


Fig .2f Six-plex staining panels show the cellular components and distribution of the whole brain tissues (left panel), CD3 and CD68 markers (middle panel), and cellular proportions (right panel) of each group, colored by cell type markers.

R2_4. RBC removal was apparently performed on the brains during processing for scRNA-seq. As such, how many parasitized RBCs were lost from the parasite-host analysis? It's not clear how robust this analysis is, given this caveat. Moreover, what proportion of the (parasitised)RBCs in the analysis were luminal within the vasculature compared within haemorrhages? This may substantially influence what the interaction partners are for the parasitized RBCs in the brain. Currently there is debate on the nature of pRBC interaction with brain endothelial cells during ECM, so this part of the manuscript needs to be strengthened or removed.

✓**Response:**

Thanks for your valuable comment. We conducted the RBC removal process in the scRNA-seq workflow for each sample among all three groups. Thus, it is inevitable that both parasitized and healthy RBC would be lost during the brain tissue dissociation. Therefore, the quantitative analysis of these RBC would be difficult. Using this pipeline, we have identified 919 hRBC and 14 iRBC in total, and most of the iRBC were from the ECM group. Taking this into account, we have removed the cellular number and proportion analysis of RBC in the **Results** part. We have also acknowledged that cautions should be taken regarding that the number and proportion of iRBC might not represent the parasitemia level among the three groups.

Despite the limitation, we found that the identification of iRBC agreed well with the expression patterns of representative markers in RBCs (*Mm10_Hba-a1*) and PbA parasites (*PBANKA_1340100*, encodes L-lactate dehydrogenase, LDH). We also identified the expression of two orthologues PfEMP-1 genes, including *PBANKA_1145800* (encodes membrane associated histidine-rich protein 1a, MAHRP1a) and *PBANKA_1101300* (encodes skeleton-binding protein 1, SBP1) in iRBC with parasites (**Ruberto AA, et al. *Sci Rep.* 2021 Feb 22;11(1):4127. ; De Niz M, et al. *Nat Commun.* 2016 May 26;7:11659.**) (Supplementary Fig. 4f). Compared to hRBC, we found the enhanced expression of some specific T-cell epitopes genes bound by MHC class-I in iRBC, including *PBANKA_1034400, PBANKA_1112400, PBANKA_1360100*, and *PBANKA_1145800*, which might together contribute to the occurrence of ECM (**Lin J, et al. *BMC Microbiol.* 2023 Sep 21;23(1):264.**) (Supplementary Fig. 4g).

Therefore, these results indicate consistency with previous studies and the robustness of this analysis pipeline.

Strangward et al. have reported that >90% of intracerebral iRBC during ECM were intracapillary, rather than within hemorrhages (**Strangward P, et al. *PLoS Pathog.* 2017;13(3):e1006267.**). Currently, we are not able to determine the proportion of iRBC locating at different locations (luminal within the vasculature compared within hemorrhages), due to the lack of representative and reliable locational markers in the scRNA-seq dataset. In addition, since we didn't identify the distribution of RBC in our ST-seq dataset, which attributes to the limitation of the composition of sequencing reads within spots, the sensitivity and coverage of sequencing reads, etc. Thus, we cannot determine the regional types of iRBC in the spatial context.

We acknowledge that this substantially limits the identification of interaction partners of the iRBC in the brain, and that it is insufficient to uncover the nature of iRBC interaction with brain endothelial cells during ECM based on our datasets. Thus, we have removed the cellular integration analysis in the Results part of the revised manuscript, as indicated by the reviewer.

Reference:

1. Ruberto AA, et al. Single-cell RNA sequencing reveals developmental heterogeneity among Plasmodium berghei sporozoites. ***Sci Rep.*** 2021 Feb 22;11(1):4127. **PMID: 33619283**

2. De Niz M, et al. The machinery underlying malaria parasite virulence is conserved between rodent and human malaria parasites. ***Nat Commun.*** 2016 May 26;7:11659. **PMID: 27225796**

3. Lin J, et al. Identification of disease-related genes in Plasmodium berghei by network module analysis. ***BMC Microbiol.*** 2023 Sep 21;23(1):264. **PMID: 37735351**

4.Strangward P, et al. A quantitative brain map of experimental cerebral malaria pathology. ***PLoS Pathog.*** 2017;13(3):e1006267. **PMID: 28273147**

R2_5. The authors indicate that their data emphasises an important role of inflamed microglial cells in engaging with T cells to mediate experimental cerebral malaria (ECM) pathology. However, there is little evidence of T cells crossing into the brain parenchyma during ECM to engage with microglial cells. As such, the authors need to verify such interactions occur to provide relevance for the analyses. Figure 7G – the staining for CD8 does not seem robust, particularly in the ART group. IBA-1 can also be expressed on

macrophages, so co-localisation of CD8 with IBA-1 does not mean CD8+ T cells are interacting with microglia. This would need to be confirmed using a vascular marker. Finally, relating to this, there is currently very little evidence that monocytes or macrophages contribute to ECM development at the onset of the syndrome: Depletion of myeloid cells prior to infection can prevent ECM, but depletion of the cells immediately prior to onset of the syndrome has little effect. As such, the biological relevance of some of the receptor-ligand interaction analyses is unclear.

✓**Response:**

We appreciate your valuable comments. Here, we have performed additional mIHC and IF experiments to verify the occurrence of these interactions and provide support for our analyses, with improved robustness of CD8 staining in each group.

To further elaborate, mIHC results showed that Endothelial cells (marked by CD31) were colocalized with Myeloid cells (marked by CD68) and T cells (marked by CD3), indicating that these cells exhibit a high probability of communication (Fig. 6h). Particularly, some CD3+ cells overlapped with CD68+ cells, but not with CD31+ cells, indicating that these cellular interactions might occur outside the cerebral microvessels. In the IF results, we used CD8a (the marker of CD8 T cells), IBA1 (the marker of microglia cells), and CD31 antibodies (the vascular marker) to confirm the engagement of microglial cells with T cells in mediating ECM pathology (Fig. 6i). The staining results were consistent with the mIHC assay, in that CD8a markers were mainly co-located with CD31. However, we also found that there were some co-localizations of CD8a with IBA1 but not with CD31, indicating that a portion of $CD8^+$ T cells may interact with microglia cell in brain parenchyma.

The up-regulation of antigen presenting molecules (MHC-I) would suggest a role in migration, antigen-presentation, and activation of $CD8^+$ T cells, which are crucial players in the pathogenesis of ECM **(Hansen DS. *PLoS Pathog*. 2012;8(12):e1003045.)**. In fact, $CX3CR1^+$ cells (inflammatory monocytes/macrophage, microglia, and dendritic cells) with activated MHC-I, have been observed close to form stable interactions with T cells in mice infected with PbA parasite **(Shaw TN, et al. *PLoS Pathog*. 2015 Nov 12;11(11):e1005210.)**. Meanwhile, Capuccini et al. have reported the activation of antigen presentation of microglia during ECM using gene expression microarrays, which supports our finding (**Capuccini B et al. *Sci Rep.* 2016;6:39258.**).

Considering that activated endothelial cells can cross-present malaria antigen to $CD8^+$ T cells (**Howland SW, et al. *PLoS Pathog*. 2015;11(6):e1004963.)**, therefore, interaction between Microglia and $CD8^+$ T cell might not be the priming event for the latter's activation, as that is thought to take place already on endothelial cells (before T cell entering the brain parenchyma). It is possible that following interaction with microglia, a secondary activation and amplification of the response of $CD8^+$ T cell contributes to additional brain injuries. Collectively, we have updated these results and discussed the role of microglia and its interaction with T cells in the revised manuscript.

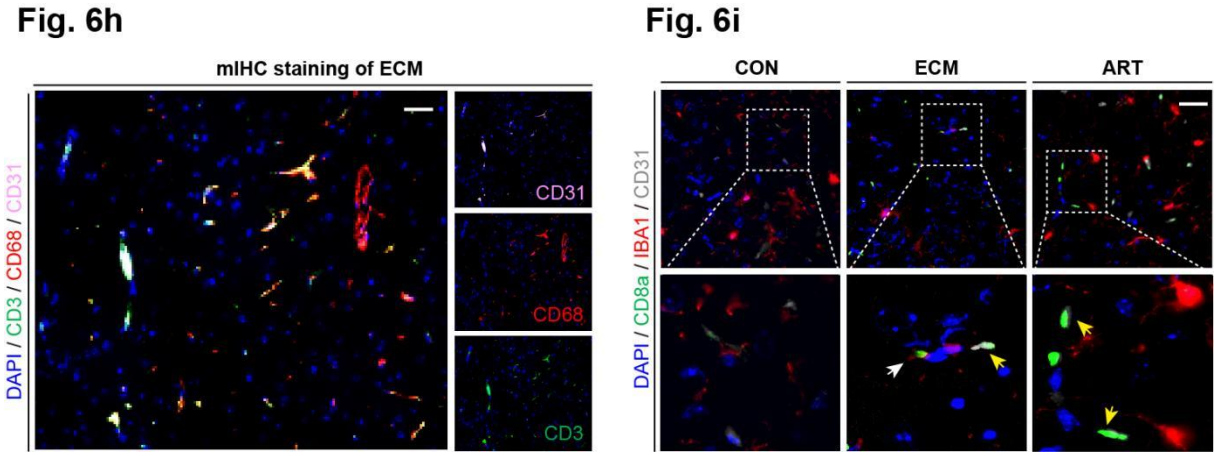


Fig. 6h The representative mIHC staining of ECM group (CD3, green; CD68: red; CD31: pink; DAPI: blue), scale bar = 20 μm. **Fig. 6i** Immunofluorescence staining of co-location of Endothelial cells, $CD8^+$ T cells and Microglia (CD8a, green; IBA1, red; CD31: grey; DAPI: blue) among three groups (n = 3 samples per group), the interactions were highlight in white arrow (outside the microvessels) or yellow arrows (within the microvessels), scale bar = 20 μm.

References:

1. Hansen DS. Inflammatory responses associated with the induction of cerebral malaria: lessons from experimental murine models. ***PLoS Pathog*.** 2012;8(12):e1003045. **PMID: 23300435**

2. Shaw TN, et al. Perivascular Arrest of CD8+ T Cells Is a Signature of Experimental Cerebral Malaria. ***PLoS Pathog***. 2015 Nov 12;11(11):e1005210. **PMID: 26562533.**

3**.** Capuccini B, et al. Transcriptomic profiling of microglia reveals signatures of cell activation and immune response, during experimental cerebral malaria. ***Sci Rep.*** 2016;6:39258. **PMID: 27991544**

4. Howland SW, et al. Activated Brain Endothelial Cells Cross-Present Malaria Antigen. ***PLoS Pathog.*** 2015;11(6):e1004963. **PMID: 26046849**

R2_6. In the introduction the authors conflate studies on experimental cerebral malaria and human cerebral malaria. (For example lines 74 to 77). These should be separated and studies in human CM and murine models should be clearly defined, given there is still some debate on the pathogenesis in the human and murine syndromes.

✓**Response:**

Thanks for your kind suggestions. Although there are numerous similarities of cerebral syndromes caused by *Plasmodium* parasites between experimental cerebral malaria (ECM) and human cerebral malaria (HCM) (**de Souza JB, Riley EM. *Microbes Infect.* 2002 Mar;4(3):291-300.**), there remain some debates on the pathogenesis in the human and murine syndromes during cerebral malaria (**Strangward P, et al. *PLoS Pathog.* 2017 Mar 8;13(3):e1006267.**). We do acknowledge that we should have clearly defined and separated the HCM and ECM studies in the introduction. Thus, we have made corrections in the introduction part of our revised manuscript (Line 44-51). Thank you again for your kind reminder.

References:

1. de Souza JB, Riley EM. Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. ***Microbes Infect.*** 2002 Mar;4(3):291-300. **PMID: 11909739**.

2. Strangward P, et al. A quantitative brain map of experimental cerebral malaria pathology. ***PLoS Pathog.*** 2017 Mar 8;13(3):e1006267. **PMID: 28273147**.

R2_7. Line 247 – there is no evidence from the data in Figure 1C that the T cells and NK cells cross the BBB and infiltrate the brain parenchyma. Indeed, from existing literature most of the cells recruited within the brain of mice with ECM remain luminal in the vasculature, with a proportion moving into the perivascular space.

✓**Response:**

We greatly acknowledge your kind advice. Intracerebral T cell accumulation during PbA infections have been observed in mice with ECM, and CD8+ T cells are known to play a critical role in during ECM development **(Belnoue E et al. *J Immunol.* 2002;169(11):6369–75.)**.

However, according to a report by Strangward et al., T cells are predominantly located luminal or abluminal to the cerebral microvasculature, or accumulated in larger-calibre vessels during ECM. There is no evidence of extravasation of T cells into the brain parenchyma, with few T cells observed within the parenchyma even when accompanied with intracerebral hemorrhage **(Strangward P, et al. *PLoS Pathog.* 2017 Mar 8;13(3):e1006267.)**. Thus, we have made corrections in the **Results** part (Fig. 1c) in our revised manuscript (Line 141-144). Thank you for your kind reminder.

References:

1. Belnoue E et al. On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. ***J Immunol.*** 2002;169(11):6369–75. **PMID: 12444144**

2. Strangward P et al. A quantitative brain map of experimental cerebral malaria pathology. ***PLoS Pathog.*** 2017;13(3):e1006267. **PMID: 28273147**

R2_8. Line 155 – It appears that only 2 biological replicates were used in the model and ECM groups, so it is unclear how robust the data is. Detailed histological assessments and RMCBS scores for the mice used in the scRNA-seq and ST should be provided.

✓Response:

Thanks for your valuable comment. We had listed the detailed RMCBS scores over 8 days in the CON, ECM and ART groups. As expected, our results are similar to a previously reported work (**Carroll RW, et al. *PLoS One*. 2010;5(10):e13124.)**. We hope this process will address your concern about the biological duplication.

Group type	NO.	Coordinate		Exploratory behavior	Power		Reflexes and self-preservation				Health behavior	Total points	scRNA-seq	ST-seq
		Gait	Balance	Motor function	Posture	Body strength	Touch escape	Auricle reaction	Foot pinch pressure	Oppress	Gloss			
CON	1	2	2	2	2	2	2	2	2	2	2	20	Yes	No
	2	2	2	2	2	2	2	2	2	2	2	20	Yes	No
	3	2	2	2	2	2	2	2	2	1	2	19	Yes	No
ECM	1	1	0	1	1	1	0	1	1	0	1	7	Yes	No
	2	1	1	0	1	1	1	1	1	0	1	8	Yes	No
	3	1	1	0	1	2	0	1	1	0	1	8	Yes	No
	4	1	0	1	1	1	0	1	1	0	1	7	No	Yes
	5	1	1	0	1	1	1	1	1	0	1	8	No	Yes
ART	1	1	2	1	1	1	1	1	2	1	1	12	Yes	No
	2	2	1	2	1	1	1	1	1	2	1	13	Yes	No
	3	1	1	1	0	1	2	2	1	2	1	12	Yes	No
	4	2	1	2	1	1	1	1	1	2	1	13	No	Yes
	5	1	1	1	0	1	2	2	1	2	1	12	No	Yes

References:

1. Carroll RW, et al. A rapid murine coma and behavior scale for quantitative assessment of murine cerebral malaria. ***PLoS One***. 2010;5(10):e13124. **PMID: 20957049.**

R2_9. Line 335 CD45RA is not expressed in mice or used to distinguish T cell populations, so this should be altered.

Response:

Thank you for pointing this out. In our previous study, we found that the Cd8_TEMRA subtype highly expressed the proinflammatory cytokines and cytotoxic genes such as *Fasl*, *Klrd1, Ccl5, Gzma, Gzmb* and *Gzmk*, which is similar to the expressed characteristics of $CD8^+$ TemRA cells in previous studies (**Li W, et al. *Exp Hematol Oncol.* 2023 May 8;12(1):44.; Gate D, et al. *Nature*. 2020 Jan;577(7790):399-404.)**. However, we have neglected the species-specific expression of CD45RA and we apologize for our carelessness. Thus, we have changed the name of this cellular population to Cd8_CTL cells based on the expression of these effector markers. Thanks again for your reminder and your kind suggestions.

References:

1. Li W, et al. Identification of potential resistance mechanisms and therapeutic targets for the relapse of BCMA CAR-T therapy in relapsed/refractory multiple myeloma through single-cell sequencing. ***Exp Hematol Oncol.*** 2023 May 8;12(1):44. **PMID: 37158921.**

2. Gate D, et al. Clonally expanded CD8 T cells patrol the cerebrospinal fluid in Alzheimer's disease. ***Nature***. 2020 Jan;577(7790):399-404. **PMID: 31915375.**

R2_10. In figure 6D the expression of KLRD1 is rather atypical. Also, qualitative images showing only one or two CD8+ T cells is not particularly compelling to show whether the T cell response is similar or different in the model and ART groups.

✓**Response:**

We appreciate your comment and agree with your opinion. Here, we optimized the concentration of antibody KLRD1, re-performed the immunofluorescence staining experiment, and displayed the results in the proper scale to improve the representativeness of Fig. 5d. In addition, in order to reflect the response pattern of the CD8_CTL subgroup, we profiled the significant pseudotime DEGs and GO enrichment analysis of each cluster gene set along the trajectory (Fig. 5f). The increased expression level of Stat3 (**Liu M, et al. *PLoS One*.**

2012;7(3):e34280.) and Gzmk (**Mogilenko DA, et al. *Immunity.* 2021 Jan 12;54(1):99-115.e12.**) was further validated by WB assay (Fig. 5g). These results indicate that CD8_CTL was recruited and activated upon PbA infection in the ECM group, and partially alleviated after ART treatment.

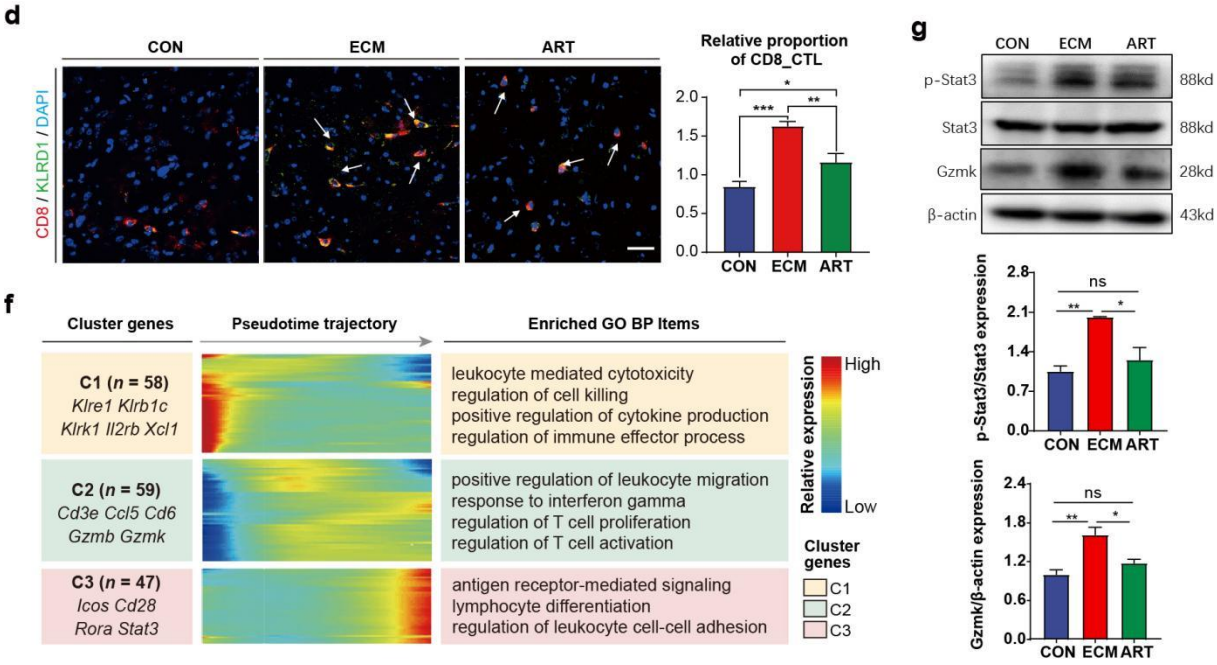


Fig. 5d Immunofluorescence staining of CD8_CTL markers (CD8, red; KLRD1: green; DAPI: blue) among three groups (n = 3 samples per group), scale bar = 25 μm. ***：$p < 0.001$, **：$p < 0.01$, *：$p < 0.05$. **Fig. 5f** Pseudotemporal expression pattern analysis shows the representative DEGs along with the inferred trajectory and enriched biological processes terms. **Fig. 5g** Protein expression levels of p-Stat3/Stat3 and Gzmk among three groups (n = 3). **□p□ 0.01, *□p□ 0.05, ns: no significance

References:

1. Liu M, et al. Heme mediated STAT3 activation in severe malaria. ***PLoS One***. 2012;7(3):e34280. **PMID: 22479586.**

2. Mogilenko DA, et al. Comprehensive Profiling of an Aging Immune System Reveals Clonal GZMK+ CD8+ T Cells as Conserved Hallmark of Inflammaging. ***Immunity.*** 2021 Jan 12;54(1):99-115.e12. **PMID: 33271118.**

R2_11. The clone of the P. berghei ANKA parasites used in the study should be provided.

✓**Response:**

Thank you for pointing this out. We have identified the clone of the *Plasmodium berghei* ANKA (PbA) parasite via examining the DNA sequence of the nuclear Internal Transcribed Spacer 2 (ITS2). The ITS2 sequence of the malaria parasite used in this study is Gene-PBANKA_0521221. The detailed sequence information can be found at https://plasmodb.org/plasmo/app/record/gene/PBANKA_0521221.We have uploaded the DNA sequence dataset and provided the clone of the P. berghei ANKA parasites in the Methods section of the revised manuscript.

R2_12. The materials and methods sections for some protocols could be clearer (e.g. mIHC and ELISA).

✓**Response:**

Thanks for your kind suggestion. We have updated the protocols of the mIHC and ELISA experiments with more details in the Method sections to make it clearer (Line 669-685, 707-725).

mIHC:

The antigenic binding sites were visualized using the Opal 5-Color Manual IHC Kit (PerkinElmer) according to the manufacturer's protocol. First, the paraffin sections of the brain were baked, dewaxed, underwent antigenic repair, incubated with H2O2 solution to remove endogenous peroxide, and blocked with BSA solution for 10min. Then, after removal of the blocking buffer, the slices underwent multiplex fluorescence staining for a panel of cellular markers (CD3, 1:50; CD68, 1:50, Oligo,1:500, NeuN, 1:20000 and CD31, 1:500) for 1 hour at room temperature. After cleaning the slides for three times with TBST, the secondary antibody was added and incubated at room temperature for 10min. After cleaning the slides with TBS, Opal dye diluent (dilution ratio 1:100) was added and incubation was performed at room temperature for 10min. The primary and secondary antibodies were removed by microwave treatment, in a similar process to antigen repair. Importantly, the procedures for each marker were completed one by one: repeated step closure, primary antibody incubation, secondary antibody incubation, Opal dye display, and microwave treatment until all indicators were labeled. At the end, DAPI working liquid was added, incubated at room temperature for 5 min, and the slides were cleaned by TBST. The slides were then sealed with fluorescent anti-

quenching tablets. Finally, automatic quantitative pathological imaging system (TissueGnostics, TissueFAXS Spectra S) was used for spectral scanning and analysis of slices.

ELISA:

The ELISA kit in this experiment adopts the Sandwich-ELISA principle (Elabscience, E-EL-M0089/E-EL-M2615c, China). The plates were coated with anti-mouse NSE and UCHL1 antibodies. Mouse INSE and UCHL1 in plasma samples or standards would bind to the coated antibodies during the experiment, and the free components would be washed away. Biotinylated anti-mouse NSE and UCHL1 antibodies and horseradish peroxidase-labeled avidin were added sequentially. Anti-mouse NSE and UCHL1 antibodies bind to mouse NSE and UCHL1 bound to coated antibodies, and biotin binds specifically to avidin to form immune complexes, and free components are washed away. The color-developing substrate (TMB) was added, the TMB appeared blue when catalyzed by horseradish peroxidase, and turned yellow when the stop solution was added. The OD values were measured at 450 nm wavelength with a microplate reader, and the concentrations of NSE and UCHL1 were positively proportional to the OD 450 values. The concentrations of NSE and UCHL1 in the samples were calculated by drawing standard curves. Specific operations were as follows: Add 100μL standard or plasma sample to the wells and incubate for 90 min at 37 °C; Discard the liquid, immediately add 100μL Biotinylated Detection Ab working solution to each well. Incubate for 60 min at 37 °C; Aspirate and wash the plate for 3 times; Add 100μL HRP conjugate working solution; Incubate for 30 min at 37 °C. Aspirate and wash the plate for 5 times; Add 90μL Substrate Reagent. Incubate for 15 min at 37 °C; Add 50μL Stop Solution; Read the plate at 450nm immediately; Calculation of the results.

R2_13. Typo line 711

✓**Response:**

We apologize for the careless mistake in Line 711, and we have deleted the redundant text in this part (Line 778). Thank you for your kind reminder.

Reviewer #3

In this manuscript, Chen, Bai, He and coauthors have performed a single cell and spatial transcriptomic analysis on brain samples from experimental cerebral malaria models treated

and untreated with artesunate. The analysis performed by the authors is impressive and overall, well done. The study is timely, given recent advances in single cell and spatial omics and provides a useful dataset that will inform other scientists and other studies. I found really interesting the comparison between CM and CM model treated with ART, as this provides information on molecular mechanisms that could drive long term sequelae in cerebral malaria patients. The paper is well written and it easy to follow. The study has some inherent limitations, overall listed in the discussions. I would recommend its publication after the reviewers clarify some major concerns on the methodology, the analysis and the interpretation. The comments are listed in the order they appear on the manuscript.

We appreciate your efforts in reviewing our manuscript and thank you for your positive and detailed feedback. We fully agree with your kind comments and understand your concerns about the probe selectivity and specificity in the current manuscript. Based on your suggestions, we have addressed your concerns as shown below.

R3_1. Abstract: The study is very informative, but given the nature of transcriptomics it mostly suggests mechanisms of pathogenesis that need to be validated in the future by the authors or other scientists. Nevertheless, the abstract gives the impression that they tested experimentally the suggested mechanisms. I would suggest to rewrite the abstract clearly stating that the study suggests mechanisms of pathogenesis.

✓**Response:**

Thank you for pointing this out. By integrating scRNA-seq and ST-seq methods, we have strived to provide a valuable resource for understanding malaria parasite-host interaction mechanisms and shed light on the adjuvant therapies development. Due the nature of transcriptomics, we acknowledge that most of our suggested mechanisms of pathogenesis need to be validated in the future by the authors or other scientists. Therefore, we have rewritten the **Abstract** clearly stating that the study suggests mechanisms of pathogenesis.

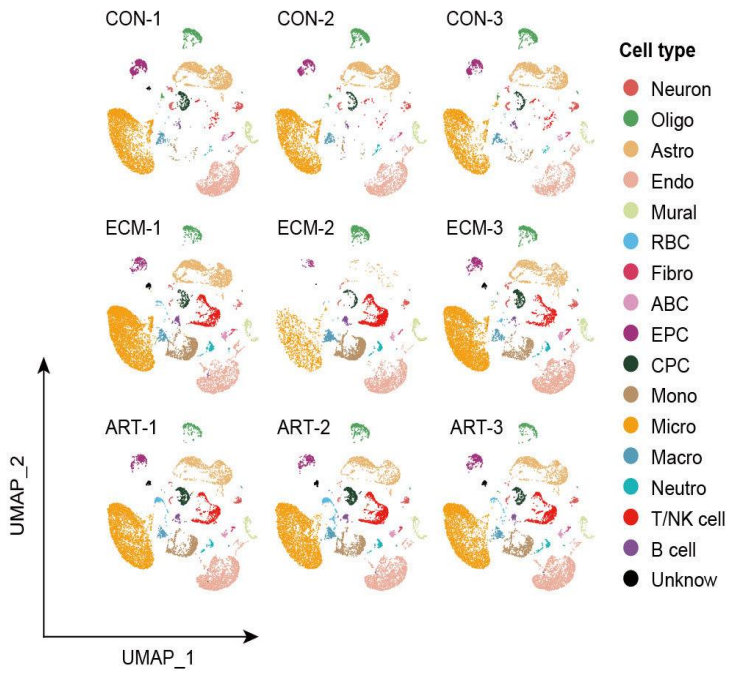
Computational Methods:

R3_2. Did the authors apply any batch correction for the scRNAseq analysis? I couldn't find it in the manuscript.

✓**Response:**

We apologize for the carelessness and omission of description in the manuscript. Actually, we have performed canonical correlation analysis to remove batch effects and dataset integration before cell clustering analysis of scRNA-seq dataset. Here, we provide the spited UMAP plots to assess the batch effects among samples within the same group in the supplementary figures (Supplementary Fig. 2e), and we also have added a description of this part in the **Methods** and **Results** parts in the revised manuscript.

Supplmentary Fig. 2e



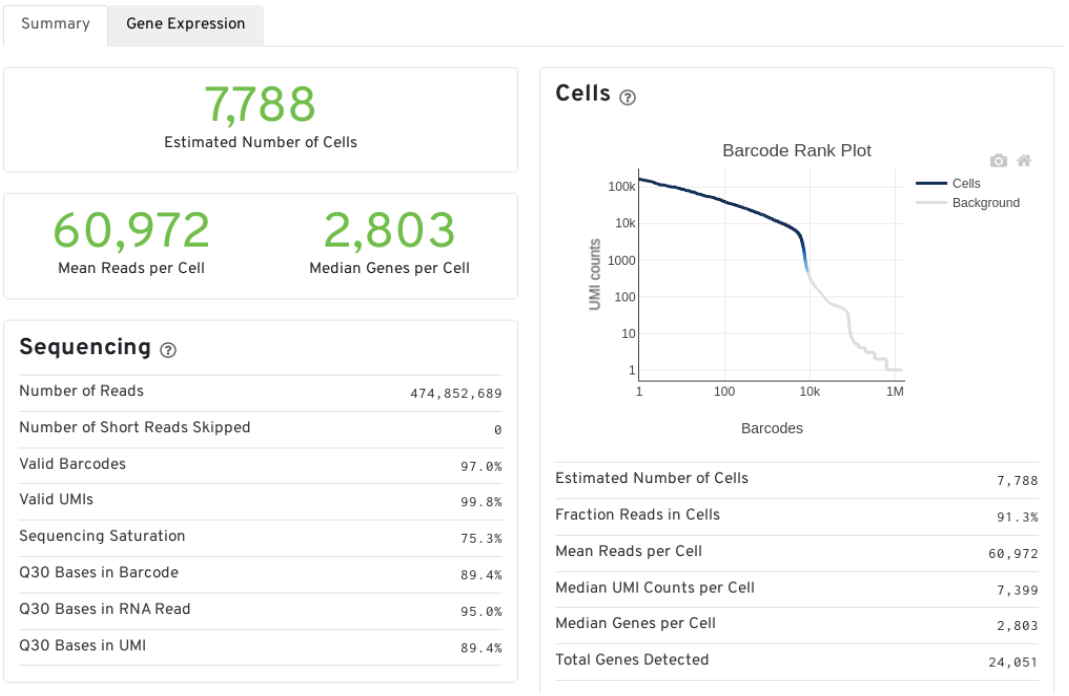
Supplementary Fig. 2e Splited UMAP plot shows distribution of various cell types of three biological replicates of three groups in the scRNA-seq dataset, colored by cell types. Neuron, Oligo: oligodendrocyte, Astro: astrocyte, Endo: endothelial cells, Mural: Mural cell, RBC: red blood cell, Fibro: fibroblast, ABC: arachnoid barrier cells, EPC: ependymocytes, CPC: choroid plexus epithelial cells, Mono: monocyte, Micro: microglia, Macro: macrophage, Neutro: neutrophils.

R3_3. Model 2 is very low in RNA count and gene number compared to the other samples. This could speak for low quality of the sample. The authors must strongly consider whether this sample should be taken out of the analysis if there are significant differences between Model1/3 vs Model 2. However, this will leave the authors with a low n, and I would suggest

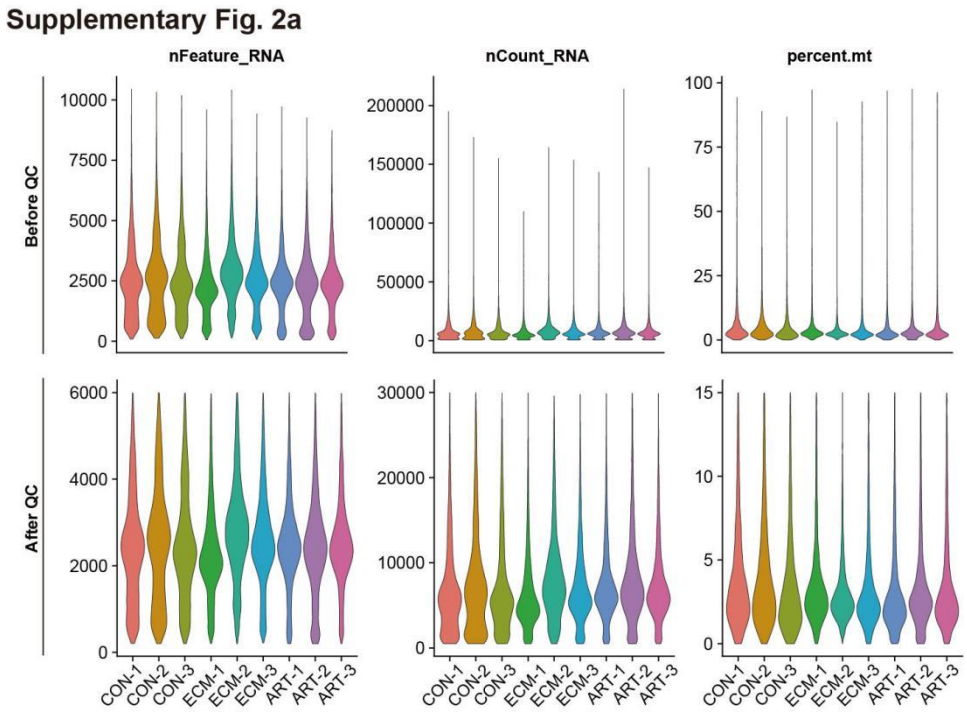
to get a new sample.

✓**Response:**

Thank you for your kind suggestion, we have supplemented additional and necessary experiments to support our results and to update our scRNA-seq results extensively. Using another cohort of experimental cerebral malaria (ECM) murine models that employs the PbA infection in C57BL/6J mice, we evaluated body weight (average value = 18.2 g□, plasmodium infection rate (average value = 24.4 %□, and RMCBS (average value = 7□to make the conditions comparable with the previous cohort. As previously described in the Methods part, the brain tissue of new ECM samples was subject to 10x Genomics scRNA-seq workflow. As demonstrated in the quality metrics produced by CellRanger, we have acquired a new sample with 7,788 cells, 60,972 mean reads/cell and 2,803 median genes/cell.



As shown in Supplementary Fig. 2a, the data quality of this new sample was comparable with other two samples in the ECM group, making the quality of the scRNA-seq dataset good enough for the subsequent analysis. More importantly, we have replaced the old low-quality sample with the new sample in the ECM group, and performed the scRNA-seq analysis part again. We found that the cellular identities and their proportion of most cell types were similar to the analyzed results in the old integrated scRNA-seq dataset, indicating the robustness of our results (Fig. 1d).



Supplementary Fig 2a Violin plots show the relative level of feature numbers, UMI counts, and mitochondria gene percentage in each sample before (top panel) and after (bottom panel) quality control in the scRNA-seq dataset.

R3_4. The authors allocate a cell identity for each spot on the spatial transcriptomics. The visium spot size is 55um and could contain up to 10 different cell types. For example, a brain capillary has a diameter from 5-10 um, in just this small region endothelial cells, pericytes, astrocytes, iRBC and multiple immune cells can be found. In my opinion, giving a cell identity for each spot is not necessary for the the manuscript and its main conclusions, and it could be misleading for many of the readers not familiar with the methodology. If the authors prefer to keep this type of analysis, at least they should acknowledge this limitation early on in the results section text, as this technique is probably new to many readers.

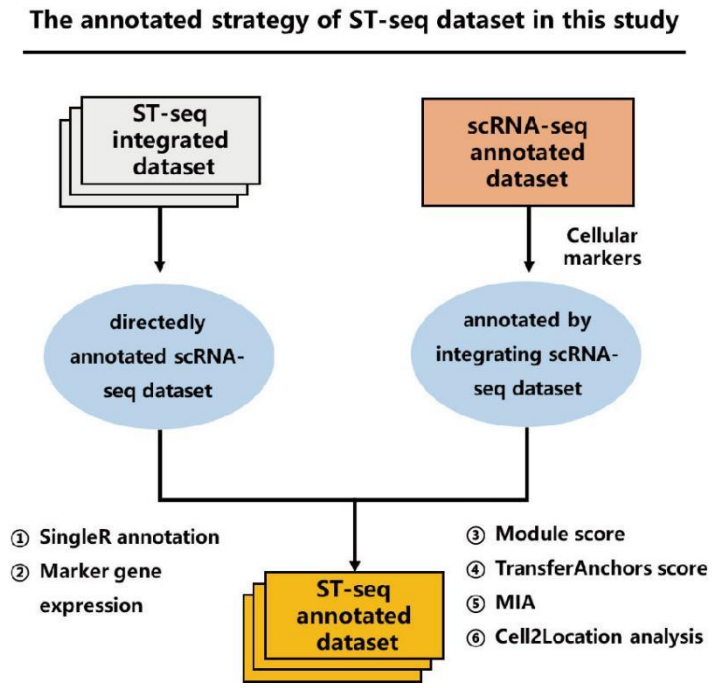
✓Response:

Thanks for your valuable comments and suggestions. To profile the transcriptional alteration of various cell types identified in the scRNA-seq dataset at the spatial context, we here assigned the dominant identities such as Endo, Immune and Neuron etc. to the spots in the integrated ST-seq dataset. Thus, it is necessary to ascribe a cell identity for each spot in the ST-seq dataset. Based on the current resolution of Visium method, the cerebrovascular architecture of ECM

murine brain might contain numerous cells within a particular spot. Here, we have acknowledged these limitations in the ST-seq dataset and modified our description of the identities of these spots as "main spot type" in the revised manuscript to avoid ambiguity to many readers.

As demonstrated in Supplementary Fig. 3c, we have adopted several annotation and deconvolution approaches to improve the reliability the spots' annotation in ST-seq dataset, including direct annotation methods: □SingleR annotation, □Marker gene expression, and integrating methods: □ Module score of cell type-specific markers, □TransferAnchors score, □ Multimodal intersection analysis (MIA) (**Moncada R, et al.** ***Nat Biotechnol.*** **2020 Dec;38(12):1476.**), and □ cell2location (**Kleshchevnikov V, et al.** ***Nat Biotechnol.*** **2022 May;40(5):661-671.**) analysis. Taken together, we have validated the annotation of main spot types via various methods for integrating scRNA-seq and ST-seq dataset, observing high consistency among these annotation methods.

Supplementary Fig. 3c



Supplementary Fig. 3c Scheme depicts the annotated strategy for spot types in the ST-seq dataset, direct annotation methods: ①SingleR annotation, ②Marker gene expression, and integrating methods: ③ Module score of cell type-specific markers, ④TransferAnchors score, ⑤ Multimodal intersection analysis (MIA) and ⑥ cell2location analysis.

Reference:

1. Moncada R, et al. Integrating microarray-based spatial transcriptomics and single-cell RNA-seq reveals tissue architecture in pancreatic ductal adenocarcinomas. ***Nat Biotechnol***. 2020 Dec;38(12):1476. **PMID: 31932730**.

2. Kleshchevnikov V, et al. Cell2location maps fine-grained cell types in spatial transcriptomics. ***Nat Biotechnol.*** 2022 May;40(5):661-671. **PMID: 35027729.**

Experimental Methods:

R3_5. The authors applied a red blood cell removal solution on the dissociated brain tissue, could this affect the recovery of infected red blood cells from the samples and the posterior analysis.

✓**Response:**

Thanks for your valuable comments. According to 10x Genomics' scRNA-seq experimental protocol, we conducted the process of RBC removal for each sample among the three groups. It is therefore inevitable that both infected and healthy RBC would be lost during the experiment, and quantitative analysis of these RBC would be difficult. Taking this into account, we have acknowledged the limitation that the use of red blood cell lysate would certainly affect subsequent studies such as quantitative analysis, and we have removed the cellular number and proportion analysis of RBC in the **Results** part. We have also indicated that cautions should be taken regarding the fact that the number and proportion of iRBC might not represent the parasitemia level among three groups.

Despite the limitation, we found that the identification of iRBC agreed well with the expression patterns of representative markers in RBC (*Mm10_Hba-a1*) and PbA parasite (*PBANKA_1340100*, encodes L-lactate dehydrogenase, LDH). We also identified the expression of two orthologues of PfEMP-1 genes, including *PBANKA_1145800* (encodes membrane associated histidine-rich protein 1a, MAHRP1a) and *PBANKA_1101300* (encodes skeleton-binding protein 1, SBP1) in iRBC with PbA parasites (**Ruberto AA, et al. *Sci Rep*. 2021;11(1):4127.; De Niz M, et al. *Nat Commun*. 2016;7:11659.**) (Supplementary Fig. 4f). Compared to hRBC, we found the enhanced expression of some specific T-cell epitopes genes bound by MHC class-I in iRBC, including H2-Db restricted (*PBANKA_1034400,*

PBANKA_1112400) and H2-Kb restricted (*PBANKA_1360100, PBANKA_1145800*), which might together contribute to the occurrence of ECM (**Lin J, et al. *BMC Microbiol.* 2023;23(1):264.**) (Supplementary Fig. 4g). Therefore, these results indicate concordance with previous studies and the robustness of our analysis pipeline.

References:

1. Ruberto AA, et al. Single-cell RNA sequencing reveals developmental heterogeneity among Plasmodium berghei sporozoites. ***Sci Rep.*** 2021;11(1):4127. **PMID: 33619283**

2. De Niz M, et al. The machinery underlying malaria parasite virulence is conserved between rodent and human malaria parasites. ***Nat Commun.*** 2016;7:11659. **PMID: 27225796**

3. Lin J, et al. Identification of disease-related genes in Plasmodium berghei by network module analysis. ***BMC Microbiol.*** 2023;23(1):264. **PMID: 37735351**

R3_6. Given the limitation of spot size in spatial transcriptomics, I strongly suggest a quantification of immunofluorescences shown in Figure 4C, 5D, 6D and 7G.

✓**Response:**

We appreciate your comment. We had added up the quantification analysis of immunofluorescences results of the **Fig. 3c, 4c, 5d** in the revised manuscript. Besides, **Fig. 7g** is a qualitative result indicating the co-colocalization of different cell types, which might not be suitable for quantification.

Fig. 3c

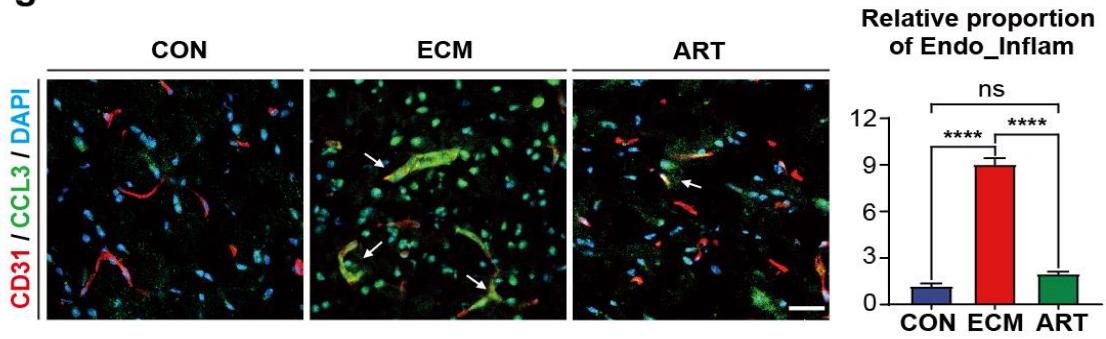


Fig. 4c

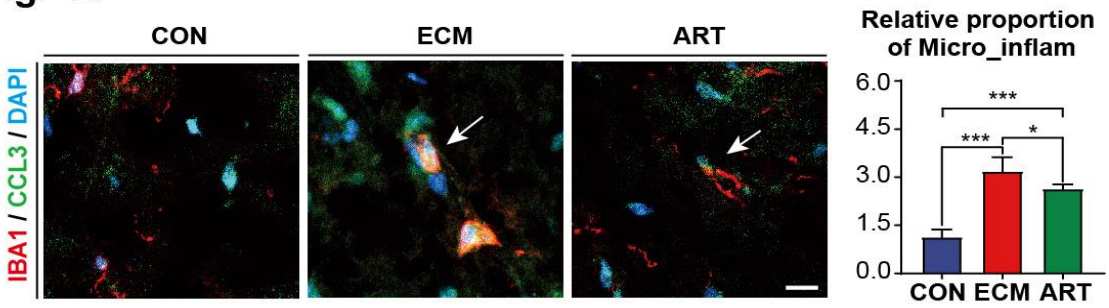


Fig. 5d

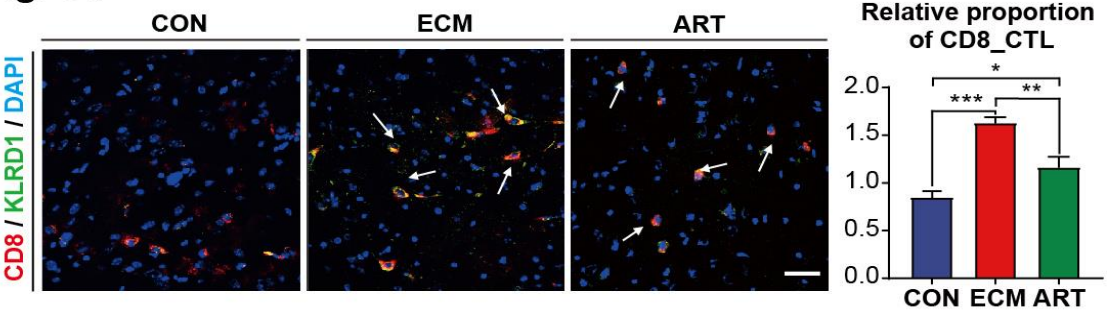


Fig. 3c Immunofluorescence staining of Endo_Inflam markers (left panel, CD31, red; CCL3: green; DAPI: blue) and the statistical analysis (right panel) among three groups (n = 3 samples per group), scale bar = 25 μm. ****： $p < 0.0001$, ns: no significance. **Fig. 4c** Immunofluorescence staining of Micro_Inflam markers (IBA1, red; CCL3: green; DAPI: blue) among three groups (n = 3 samples per group), scale bar = 10 μm. ***： $p < 0.001$, *： $p < 0.05$. **Fig. 5d** Immunofluorescence staining of CD8_CTL markers (CD8, red; KLRD1: green; DAPI: blue) among three groups (n = 3 samples per group), scale bar = 25 μm. ***： $p < 0.001$, **： $p < 0.01$, *： $p < 0.05$.

Results:

R3_7. Figure 1B: The number of neurons in the single cell RNAseq analysis is significantly low, taken into account the elevated number of neurons in the brain and the prevalence of neurons in the spatial analysis (Figure 2D). Is this a consequence of the methodology chosen for tissue dissociation?

✓**Response:**

Thanks for your valuable question. Indeed, brain tissue mainly consists of neuron and glial cells, while the proportion of neuron cells in most of our scRNA-seq datasets is much fewer than expected. The main reasons were: (1) neuron cells can easily cause plugging, due to their special protruding structure resulting in a very large size (up to 100μm or more); (2) neuron cells are relatively fragile and easily damaged during the brain tissue dissociation (**Tasic B, et al. *Nature*. 2018 Nov;563(7729):72-78.; Armand EJ, et al. *Neuron*. 2021 Jan 6;109(1):11-26. 3)**. Thus, we obtained lower numbers of neuron cells in the scRNA-seq dataset when compared with that in the ST-seq dataset (without tissue dissociation step).

To confirm this, we additionally performed single-nucleus RNA sequencing (snRNA-seq) on one ECM sample, allowing us to profile the transcriptomic information from isolated nucleus (**Koenitzer JR, et al. *Am J Respir Cell Mol Biol*. 2020 Dec;63(6):739-747.; Denisenko E, et al. *Genome Biol*. 2020 Jun 2;21(1):130.)**. As the following Figure shows, neuron cells accounted for 77.8% (1567 neurons of 2014 nucleus) in the scRNA-seq dataset, with an underrepresentation of lymphocytes within brain tissue. As you mentioned, our result indicates that the difference of cellular proportion between scRNA-seq and ST-seq datasets can be mainly attributed to our chosen methodology. In this study, as we mainly focused on the comprehensive changes within the brain microenvironment, we chose to employ scRNA-seq technology on the whole brain tissues.

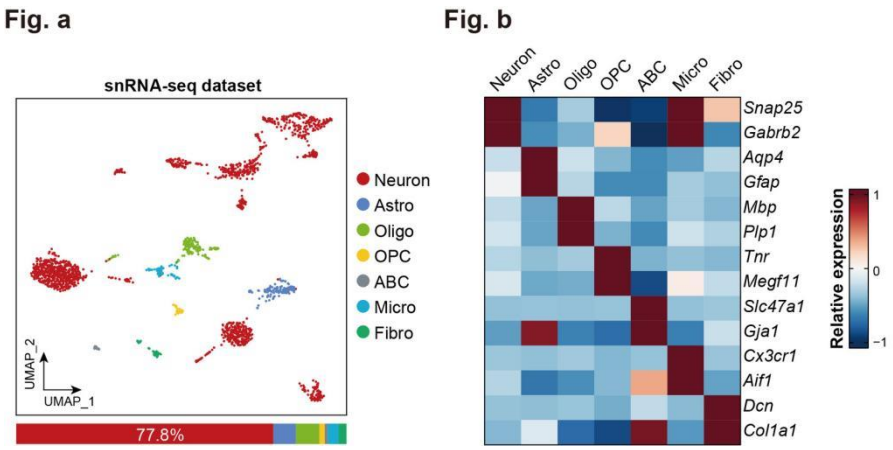


Fig. The snRNA-seq dataset of a brain sample of ECM mice.

Fig. a. UMAP plot (top) of 8 cell types of 2,014 single nuclei from BM sample. Bar plot (bottom) shows the relative proportions of each cell type. Astro: astrocyte, Oligo: oligodendrocyte, OPC: oligodendrocyte progenitor cell, ABC: arachnoid barrier cells, Micro: microglia, Fibro: fibroblast. **Fig. b.** Heatmap shows the relative expression level of the cluster-specific markers in each cell type in the snRNA-seq dataset.

References:

1. Tasic B, et al. Shared and distinct transcriptomic cell types across neocortical areas. ***Nature***. 2018 Nov;563(7729):72-78. **PMID: 30382198**.
2. Armand EJ, et al. Single-Cell Sequencing of Brain Cell Transcriptomes and Epigenomes. ***Neuron***. 2021 Jan 6;109(1):11-26. **PMID: 33412093**.
3. Koenitzer JR, et al. Reduced Dissociation Bias and Improved Rare Cell-Type Detection Compared with Single-Cell RNA Sequencing. ***Am J Respir Cell Mol Biol***. 2020 Dec;63(6):739-747. **PMID: 32804550**.
4. Denisenko E, et al. Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows. ***Genome Biol***. 2020 Jun 2;21(1):130. **PMID: 32487174**.

R3_8. Calculations of Proportion of cells (Figure 1D, 4D, 5B, 6C). The proportion of cells types is informative, but similarly to Figure 3D I would suggest to add the number cells. This would provide additional information. For example, a massive increase in a cell population could mask relevant findings in other populations.

✓**Response:**

Thanks for your suggestions. We have taken into consideration that relevant populational changes in other (sub) celltypes might be masked by a massive increase in a specific (sub) celltype, and that the absolute counts can offer more precise insights into the composition of cell populations as you mentioned. Therefore, we have added the numbers of each cell type in Figure 1d, 3b, 4b, and 5c, and we have also summarized the cellular number and proportion information in Supplementary Table2 of the revised manuscript.

Fig. 3b

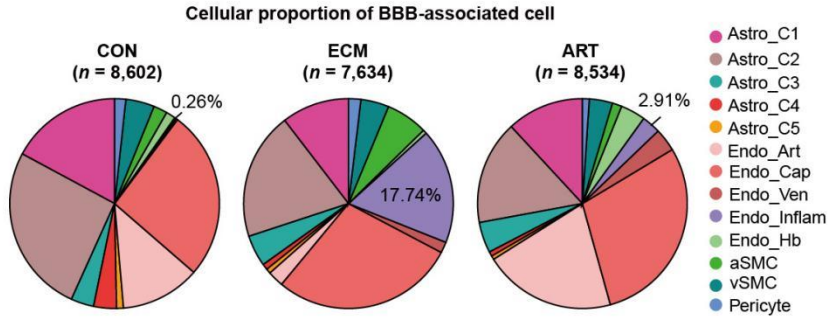


Fig. 4b

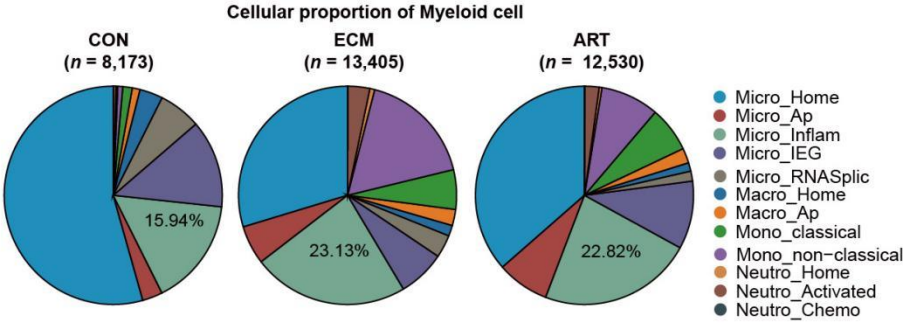


Fig. 5c

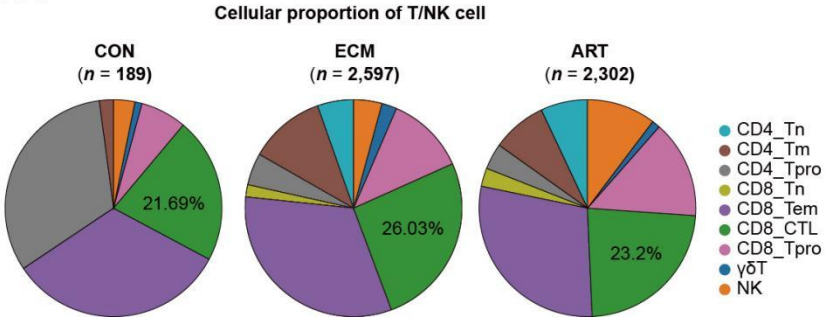


Fig. 3b Pie chart depicts the relative proportion of BBB-associated subtypes among three groups, colored by cellular subtypes. **Fig. 4b** Pie chart depicts the relative proportion of myeloid subtypes among three groups, colored by cellular subtypes. **Fig. 5c** Pie chart depicts the relative proportion of lymphocyte subtypes among three group types, colored by cellular subtypes.

R3_9. Simplify Figure 3: I would suggest to simplify this figure and the text describing it. The manuscript has already a large amount of valuable information and some sections can be further extended (see comment below on neutrophils). Overall, the figure provides little information on disease pathogenesis, besides the validation that the authors found PbiRBC in the brain of CM model. Some of the ligand-receptor interactions suggested in Figure 3J have probably little relevance in disease pathogenesis. For example, to my knowledge VEGFA expression has not been described before for RBC. And if it does, it is quite likely at negligible levels compared to other cell types (leukocytes, brain parenchyma cells). Likewise, what is the biological relevance that the authors want to convey in Figure 3G showing the top Pb transcripts? Overall, this figure raises many questions and it provides little information on disease pathogenesis.

✓**Response:**

Thanks for your nice suggestions. In this part, we developed a parasite-host strategy to identify infected RBC (iRBC) and healthy RBC (hRBC), and profile the transcriptional changes between iRBC and hRBC. Here, we identified several PbA-genes including two orthologues PfEMP-1 genes (*PBANKA_1145800, PBANKA_1101300*, which are important for PbA infection) and MHC-I genes (*PBANKA_1034400*, *PBANKA_1112400*, *PBANKA_1360100*, *PBANKA_1145800*) in iRBC with PbA parasites, which might together contribute to the occurrence of ECM. Therefore, our result demonstrates high concordance with previous studies and highlights the robustness of this analysis pipeline. However, we acknowledged that this analysis pipeline still has some limitations, and we have simplified this analysis part and placed this section into the Supplemental Figures as you suggested.

Moreover, as demonstrated in the following **Fig. a**, we have evaluated the expression levels of the ligand and receptor genes among different cell types, finding that these genes such as *Vegfa*, *Nrp1* were mainly expressed in other cell types rather than RBC, which is in agreement with your opinions. Therefore, we have removed ligand-receptor interactions in this part, which has probably little relevance in disease pathogenesis.

In the differentially expressed genes (DEGs) results, all of the PbA- genes in iRBC were considered as DEGs compared to hRBC, which caused difficulties in determining the significant genes in iRBC. Therefore, we evaluated the various PbA- genes' abundance among

the iRBC, and further identified the top10 PbA- expressed genes as well as the biological processes that these genes might involve, such as heat shock protein, ribosomal protein, and plasmodium exported protein. The results obtained here enriched our knowledge about the transcriptional reprograms of infected erythrocytes, and provide candidate parasite genes for further ECM investigation.

Fig. a

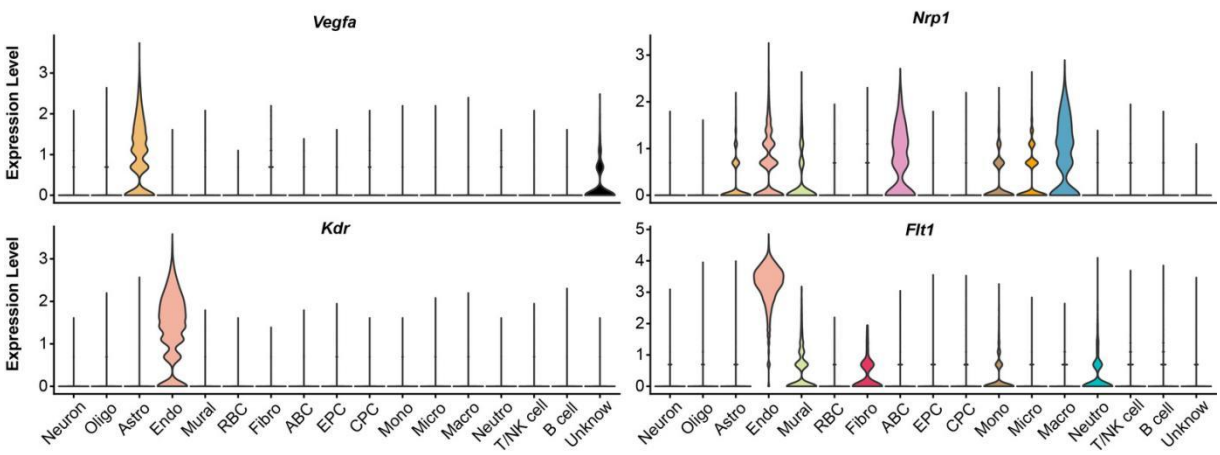


Fig. a The Violin plots show the expression level of *Vegfa*, *Nrp1*, *Kdr*, and *Flt1* among various cell types in the scRNA-seq dataset.

R3_10. The identification of the endothelial phenotype is very interesting and informative, could the authors allocate this phenotype to arteries, veins and, capillaries or is it widespread?

✓**Response:**

Thanks for your kind advice. Along with the arteriovenous axis, we have identified 3 endothelial subtypes: arteriole endothelial (Endo_Art), venule endothelial (Endo_Ven) and capillary endothelial, as well as 2 other subtypes with high chemokines (Endo_Inflam) and hemoglobin (Endo_Hb) expression (Supplementary Fig. 4a).

R3_11. A brief description of the function of the markers being used in Figure 4C, 5D, 6D and 7G is often missing in the text. This is important for readers outside the field. For example, a description of what is KLRD1 (Figure 6D) cannot be found.

✓**Response:**

Thanks for your kind suggestion. We have already added functional descriptions for each

section in the revised manuscript, as the following table shows.

Subtype	Locations	marker	*description*	Line
Endo_Inflam	Figure 3c	CD31, CCL3	endothelial marker CD31 and inflammatory factor CCL3	251-253
Micro_Inflam	Figure 4c	IBA1, CCL3	IBA1 (encoded by microglial marker *Aif1*) and CCL3 (encoded by inflammatory marker *Ccl3*)	321-325
CD8_CTL	Figure 5d	CD8, KLRD1	CD8 and killer cell lectin-like receptor subfamily D member 1(KLRD1)	376-377
CCI	Figure 6i	Cd8, IBA1	$CD8^+$ T and microglia cells (marked by CD8 and IBA1, respectively)	421-423

R3_12. In figure 4G and Figure S4 E and F the authors claim that expression of leukocyte adhesion markers and antigen processing is higher in ECM models compared to BBB establishment, and that expression of BBB establishment is recovered in ART model. I disagree with this interpretation. This conclusion cannot be extracted from the graph shown in the main figures. This result is one of the main highlights of the discussion, so effort should be taken to unambiguously support the claim. A graph showing a ratio of BBB establishment / antigen processing + leukocyte adhesion could be clearer?

✓**Response:**

Thanks for your valuable comment. To evaluate the BBB integrity among the three groups, we measured the expression of tight junction proteins including ZO-1 and Occludin at each time point using Western-blot assay. Compared with day0, protein expression in the ECM group decreased significantly on ECM(d5) and ECM(d8). ART could partly alleviate the decreased expression of these protein to be closer to the level on day5 but not reaching the baseline level. Therefore, we adjusted our conclusion that "the expression of BBB establishment is recovered in ART group"

To better demonstrate the relationship among BBB establishment, antigen processing and leukocyte adhesion, we here calculated the module scores of relevant pathways among Endo subtypes in our scRNA-seq dataset, in which Endo_Inflam showed higher antigen processing

and presentation, leukocyte adhesion to vascular endothelial cell, and the lowest establishment of blood brain barrier (Fig. 3f). As for Endo_Inflam subtype, we found that the establishment of blood brain barrier module scores was negatively correlated with antigen processing and presentation (r = -0.26), as well as leukocyte adhesion to vascular endothelial cell module scores (r = -0.16) (Fig. 3g). Thus, scRNA-seq dataset indicates the critical role of the Endo_Inflam subtype during ECM, and the connection of BBB disruption with increased antigen-presentation and leukocyte adhesion.

Supplementary Fig. 1f

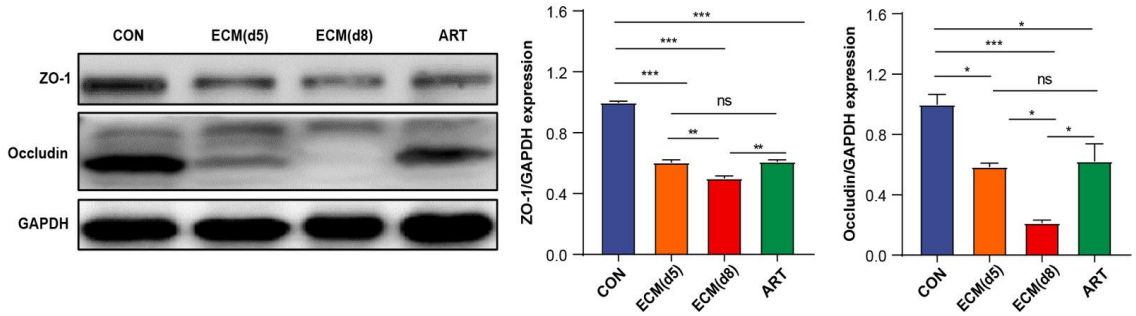


Fig. 3f

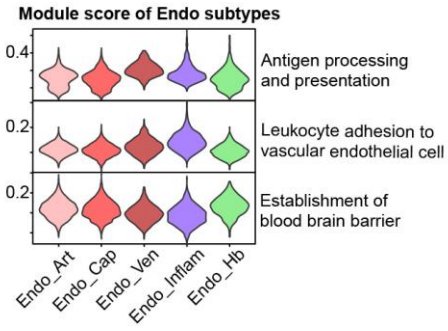
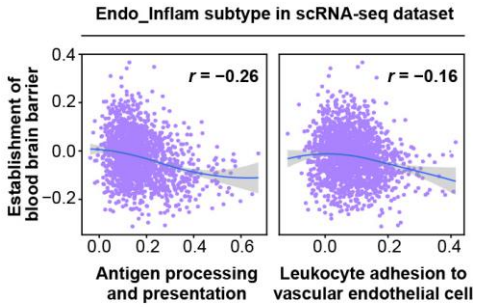


Fig. 3g



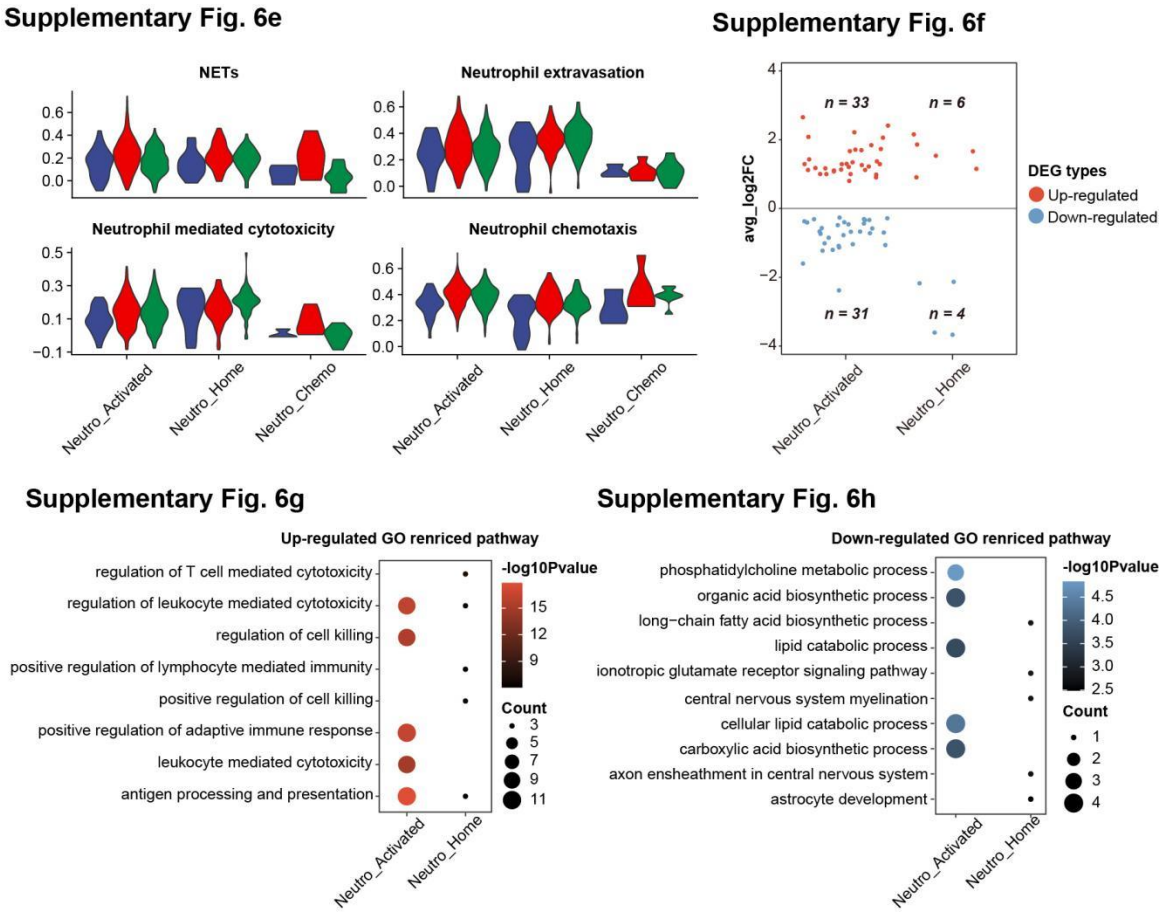
Supplementary Fig. 1f the protein expression of ZO-1 and Occludin by western blot (left) and quantitative statistics (right panel) corresponding to groups in CON, ECM(d5), ECM(d8), and ART groups (n = 3). **Fig. 3f** Vlnplot shows the module scores of antigen processing and presentation, leukocyte adhesion to vascular endothelial cell, and establishment of blood brain barrier among Endo subtypes in the scRNA-seq dataset, colored by Endo subtypes. **Fig. 3g** The scatter plots show the module scores' correlation coefficient (Pearson's r) between establishment of blood brain barrier and antigen processing and presentation (left panel), and leukocyte adhesion to vascular endothelial cell (right panel) of Endo_Inflam subtype in the scRNA-seq dataset, respectively.

R3_13. The increase in neutrophils in Figure 5B is remarkable. Given the recent relevance

of neutrophils in both ECM and HCM described in Knackstedt et al (PMID: 31628160), I strongly suggest to extend the analysis.

✓Response:

Thanks for your nice suggestions. We observed the increased proportion of activated neutrophils (Neutro_Activated) in the ECM group (Fig. 4b). Considering the important role of neutrophil during ECM progression, we subset Neutro subtypes and calculated the module scores of several pathways, including neutrophil extracellular traps (NETs), neutrophil extravasation, neutrophil mediated cytotoxicity. We found that most of these pathways was enhanced among three subtypes in ECM group compared with the other two group types (Supplementary Fig. 6e). Furthermore, we performed DEGs analysis of ECM versus CON group (MVC) among Neutro subtypes to explore the transcriptional programs upon PbA infection (Supplementary Fig. 6f). The up-regulated DEGs were enriched in cell killing, antigen processing and presentation, and down-regulated DEGs associated with lipid catabolic process and carboxylic acid biosynthetic process, emphasizing the activation characteristics during ECM (Supplementary Fig. 6g, h).



Supplementary Fig. 6e Violin plots show the module scores of neutrophil related biological processes across three Neutro subtypes. **Supplementary Fig. 6f** The up- and down-regulated DEGs between ECM and CON groups in the Neutro subtypes. **Supplementary Fig. 6g-h** Dotplots show the enriched biological processes of up- regulated (**Supplementary Fig. 6g**) and down-regulated (**Supplementary Fig. 6h**) DEGs.

R3_14. Why is the antigen presentation so spatially localized? I would expect it to be much widely distributed given the increases seen in the scRNAseq analysis, especially on microglia.

✓**Response:**

Thanks for your valuable comments. In cell-cell communications analysis, the enhanced antigen presentation activities are mainly attributed to the interaction from Micro, Macro, Mono, and Endo cells to T/NK cell in the scRNA-seq dataset. According to our spots annotation result, these cells were labeled with "Immune" and "Endo" spots, and these spots were spatially localized at a small region rather than widely distributed across the brain sagittal plane in the ST-seq dataset. Specifically, we investigated the spatial expression pattern of antigen presentation-associated genes such as *H2-K1*, and *H2-D1* in the ECM_1 sample (as the following figures show), finding that these genes were highly co-localized with the distribution of "Immune" and "Endo" spots (Fig. 2a). Thus, the spatially localized antigen presentation might be caused by the distribution of identified Immune/Endo spots, especially on microglia.

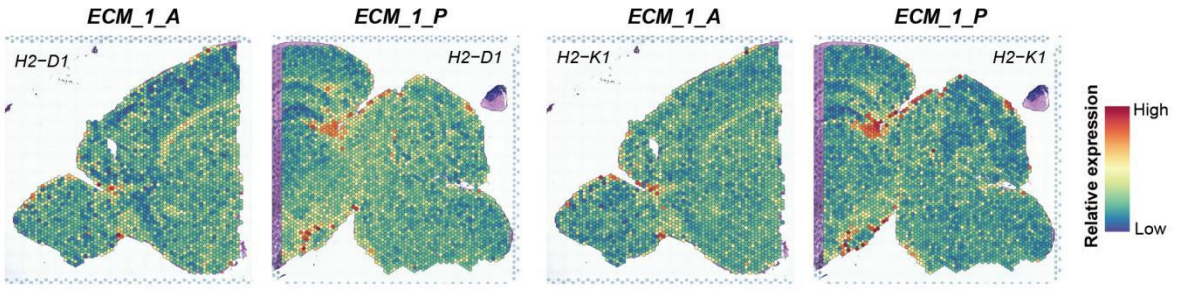


Figure. The relative expression levels of antigen presentation-associated genes (*H2-D1* and *H2-K1*) across the brain tissue in ECM_1_A and ECM_1_P of ST-seq dataset.

R3_15. Line 330-331. The increase in lymphocyte populations is remarkable and not highlighted in the text. I would suggest to add a sentence.

✓**Response:**

Thanks for your kind reminding. We have underlined the dramatic increase of lymphocyte populations in the revised manuscript (Line 360-362).

R3_16. Line 355-369 Breaking down the GO terms in cell type populations could be very informative. If possible. I strongly suggest to expand this analysis.

✓**Response:**

Thanks for your kind reminder. We have supplemented the relevant description in Line 382-388 as follows: "Cluster 1 genes (*n* = 58) predictably participated in leukocyte mediated cytotoxicity and cell killing, highly expressing *Klre1*, *Klrk1* and *Xcl1*. Cluster 2 genes (*n* = 59) were mainly associated with leukocyte migration and T cell activation, represented by *Ccl5*, *Gzmb* and *Gzmk*. *Gzmb* from the infiltrating $CD8^+$ T cells plays a critical role in the effector phase during ECM infection. Cluster 3 genes (*n* = 47) participated in antigen receptor-mediated signaling and lymphocyte differentiation (highly expressing *Icos*, *Cd28* and *Stat3*). Stat3 is a signaling molecule that involved in ECM pathogenesis (**Liu M, et al. *PLoS One*. 2012;7(3):e34280.**), while Gzmk is a cytotoxic molecule which enhanced inflammatory functions (**Mogilenko DA, et al. *Immunity*. 2021 Jan 12;54(1):99-115.e12.**). Furthermore, we next verified the elevated expression levels of Stat3 and Gzmk protein in ECM group (Fig. 5g), as well as the enhanced score of T cell migration as well as T cell mediated cytotoxicity of Immune spots in the ECM group, which was partly alleviated after ART treatment in the ST-seq dataset (Fig. 5h).

Reference:

1. Liu M, Amodu AS, et al. ***PLoS One.*** 2012;7(3):e34280. **PMID: 22479586.**

2. Mogilenko DA, et al. ***Immunity.*** 2021 Jan 12;54(1):99-115.e12. **PMID: 33271118.**

R3_17. Line 387-390 and Figure 7E. There is a lot to take in in this graph and is difficult to interpret due to the small size, I would suggest that in this figure the authors just include the relevant ligand-receptor interactions highlighted in the text and move the others to supplemental. Besides, there is an alignment problem with the X axis labels.

✓**Response:**

Thanks for your suggestions. We have simplified the content in this figure, and emphasized the

critical ligand-receptor interactions such as MHC-1 relevant pairs in this analysis part. Besides, the X axis labels represent the cellular interactions from the sender cell to the receiver cell types (e. g. Endo → T/NK cell). We have re-aligned the X axis labels and added black grid lines to represent the different groups (Fig. 6d).

Fig. 6d

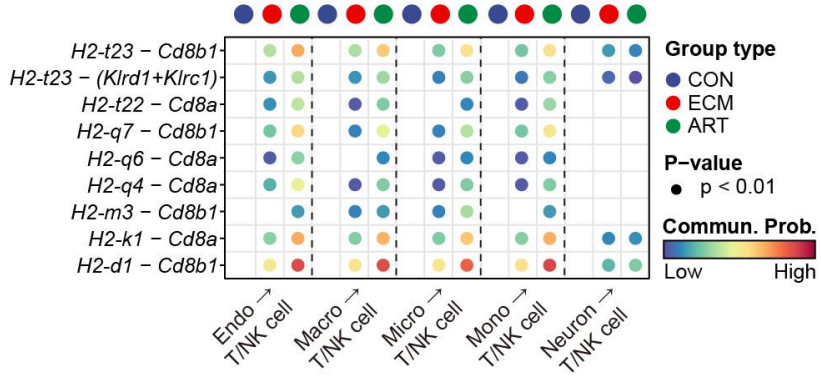


Fig. 6d Bubble plots indicate the significant MHC-I relevant ligand-receptor pairs among various sender-receiver cell types in the CON, ECM and ART groups.

R3_18. Line 443-445: The authors suggest that the OB neurons are insensitive to ART treatment, alternatively, it could be that the damage does not reverse with treatment.

✓**Response:**

Thank you for pointing this out. The insensitivity of Neuron spot in OB region to ART treatment indicates that their injury might not reverse by treatment. Thus, we have adjusted the description: "the Neuron spots in the OB region may be more responsive to PbA infection and their injury state might not reverse by ART treatment.", and added it to the revised manuscript (Line 451-452).

Discussion:

R3_19. Early on in the discussion the authors should state more clearly the differences between experimental and human CM. For example, the second paragraph puts in context the results of the current manuscript into sequestration mechanisms in human CM. Recent reports have suggested that the accumulation of P. berguei in the brain microvasculature is mediated by mechanical trapping rather than cytoadhesion (Strangward et al., 2017 PMID: 28273147). The authors should be carefully on directly translating the pathogenic

mechanisms found in the ECM into cytoadhesion mechanisms in HCM.

✓**Response:**

Thanks for your great suggestion on improving the accessibility of our manuscript. To this point, we have stated more clearly the differences between experimental and human CM in the **Introduction** part (Line 63-68) and **Discussion** part (Line 510-512, 600-605), so as to better introduce the mechanisms in our study.

R3_20. Line 489: As mentioned earlier, the evidence of the link between leukocyte adhesion and BBB breakdown is not well supported by the current data (Figure 4G).

✓**Response:**

Thanks for your nice suggestion. To address this issue, we calculated the module scores of relevant pathways among Endo subtypes in our scRNA-seq dataset, in which Endo_Inflam showed higher antigen processing and presentation, leukocyte adhesion to vascular endothelial cell, and the lowest establishment of blood brain barrier (Fig. 3f). As for Endo_Inflam subtype, we found that the establishment of blood brain barrier module scores was negatively correlated with antigen processing and presentation (r = -0.26), as well as leukocyte adhesion to vascular endothelial cell module scores (r = -0.16) (Fig. 3g). Moreover, according to the ST-seq dataset, we found the Endo spots with higher enriched scores of antigen processing, and presentation and leukocyte adhesion to vascular endothelial cell, have lower scores for the establishment of the endothelial blood-brain barrier (Fig. 3i, 3j, and Supplementary Fig. 5e, 5f) as mentioned in the response to R3_12. Therefore, our datasets have revealed the connection of BBB disruption with increased antigen-presentation and leukocyte adhesion.

Fig. 3f

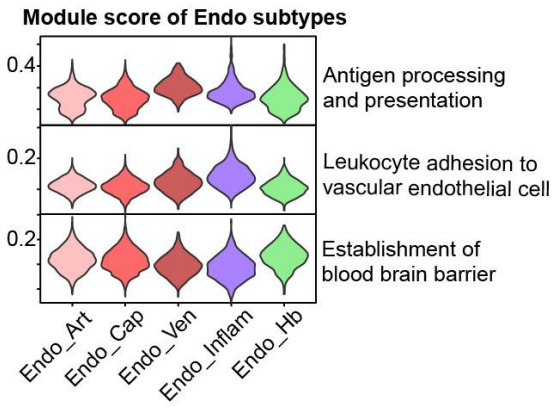


Fig. 3g

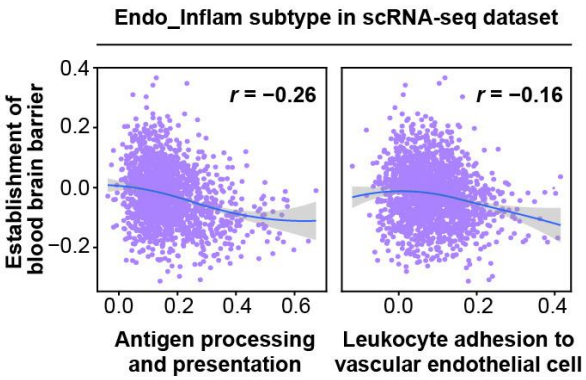


Fig. 3f Vlnplot shows the module scores of antigen processing and presentation, leukocyte adhesion to vascular endothelial cell, and establishment of blood brain barrier among Endo subtypes in the scRNA-seq dataset, colored by Endo subtypes. **Fig. 3g** The scatter plots show the module scores' correlation coefficient (Pearson's r) between establishment of blood brain barrier and antigen processing and presentation (left panel), and leukocyte adhesion to vascular endothelial cell (right panel) of Endo_Inflam subtype in the scRNA-seq dataset, respectively.

R3_21. The authors could consider to rename the experimental conditions to control, CM and ART (or CM-ART). Naming the CM as "Model" is confusing, as the other two experimental conditions are also modelling different outcomes of the disease.

✓**Response:**

Thanks for your nice suggestion. We do acknowledge that naming the CM as "Model" is confusing. To make the group identities clearer, we have renamed the experimental conditions to Con(control), ECM (experimental cerebral malaria), and ART (ART treatment) groups.

R3_22. Line 305: Figure reference is missing.

✓**Response:**

Thanks for your reminder. We have added the figure reference (Supplementary Fig. 6c) in Line 327.

R3_23. Line 315: Only H2-D1 is shown in the graph of MVC comparison

✓**Response:**

Thanks for your kind reminder. We have updated Fig. 4d, showing the top 5 up-regulated DEGs such as *H2-D1*, *H2-K1*, and *B2m* in the MVC and AVC comparisons.

Fig. 4d

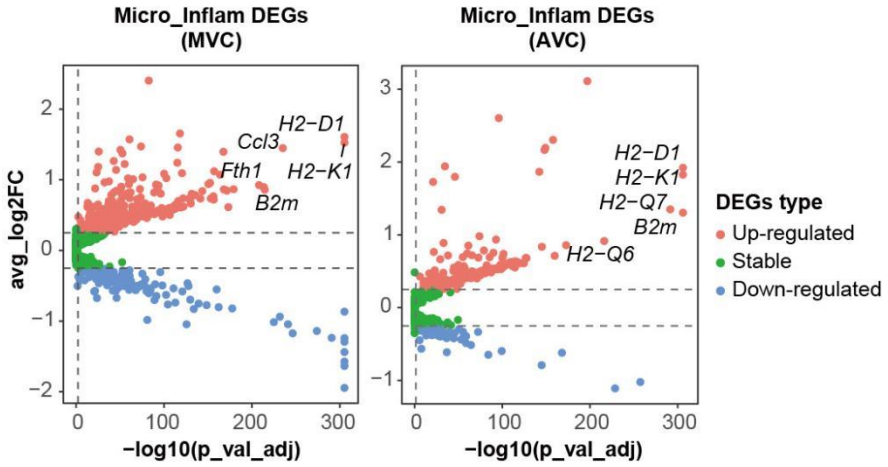


Fig. 4d Volcano plots show the DEGs patterns of Micro_Inflam cells in ECM versus CON (MVC) as well as ART versus CON (AVC), labeled by top 5 up-regulated (red) genes.

R3_24. Line 377: When the authors mention T/NK interactions it gives the impression that it could be with each other, rephrase so it is clear that the authors are focusing on interactions of T AND NK cells with other cell types.

✓**Response:**

Thanks for your kind suggestions. T lymphocyte or NK cell was identified as T/NK cell in Fig 1b. To avoid ambiguity, we have adjusted the statement as follows: "As shown in Fig. 6a, the interaction numbers of T/NK cell with other cell types in the ECM and ART groups were larger than that in the CON group in the scRNA-seq dataset." (Line 399-400).

R3_25. Figure 7E: There is something wrong with the X axis of the graph.

✓**Response:**

Thanks for your kind reminder. We have re-aligned the X axis labels and added black grid lines to represent the different groups (Fig. 6d).

Fig. 6d

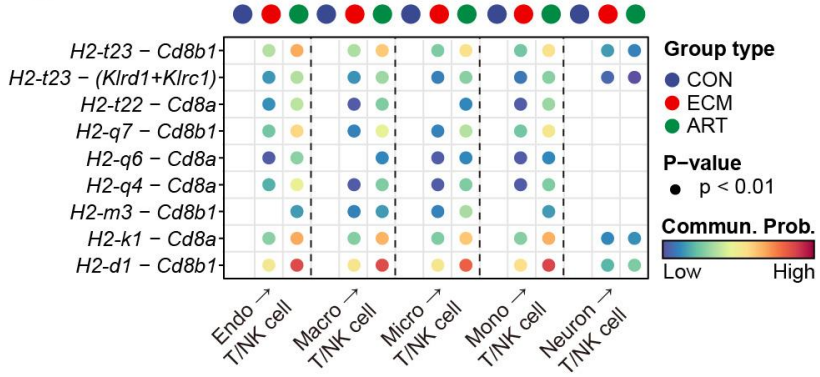


Fig. 6d Bubble plots indicate the significant MHC-I relevant ligand-receptor pairs among various sender-receiver cell types in the CON, ECM and ART groups.

R3_26. Line 341-342: Where are the & of CD8 T cells coming from, the % listed are very high and different than in Figure 6C.

✓**Response:**

Thanks for your valuable comment. We calculated the total CD8 T cell compositions among different sample types, which is the sum of CD8 T cell subtypes, including CD8_Tn, CD8_Tem, CD8_CTL and CD8_Tpro subtypes in Fig. 5c. We have also adjusted the description of this in the revised manuscript (Line 369-370).

R3_27. Line 492: Brain hydrostatic pressure instead of blood pressure?

✓**Response:**

Thanks for your kind advice. We have replaced blood pressure with brain hydrostatic pressure in the revised manuscript (Line 527).

R3_28. Line 537-539: I would suggest removing this sentence, as the authors suggest the IFNg dynamics and kinetics are complicated, so it is necessary to introduce a mechanism in hepatocytes and P. vivax.

✓**Response:**

Thanks for your kind suggestions. Considering that the pathological mechanism in hepatocytes might greatly different from that in the ECM, which is beyond the scope of this study, we have removed the content about the up-regulation of IFN-γ pathway in hepatocytes during

Plasmodium vivax infection.

R3_29. Heatmaps of multiple figures. The color legend indicates relative expression. Relative to what? Sometimes all the conditions are shown. Sometimes the label "relative expression" is missing.

✓Response:

Thanks for your pointing this out. The "relative expression" was used because we scaled gene expression levels or module scores in different cell types or group types, to facilitate the comparisons across different group or conditions. For example, in Fig. 5b, the expression of *Ncr1* is higher in NK cells relative to the other T cells. We apologize for the missing label and have added this information in the revised manuscript.

R3_30. Figure 2: The color code chosen for Neuron and Oligo masks the colors for the other cell type identities, making the figure difficult to interpret.

✓Response:

Thanks for your nice suggestions. We have changed the color panel to highlight the spatial localization of other spot types in the ST-seq dataset (Fig. 2a and Supplementary Fig. 3f).

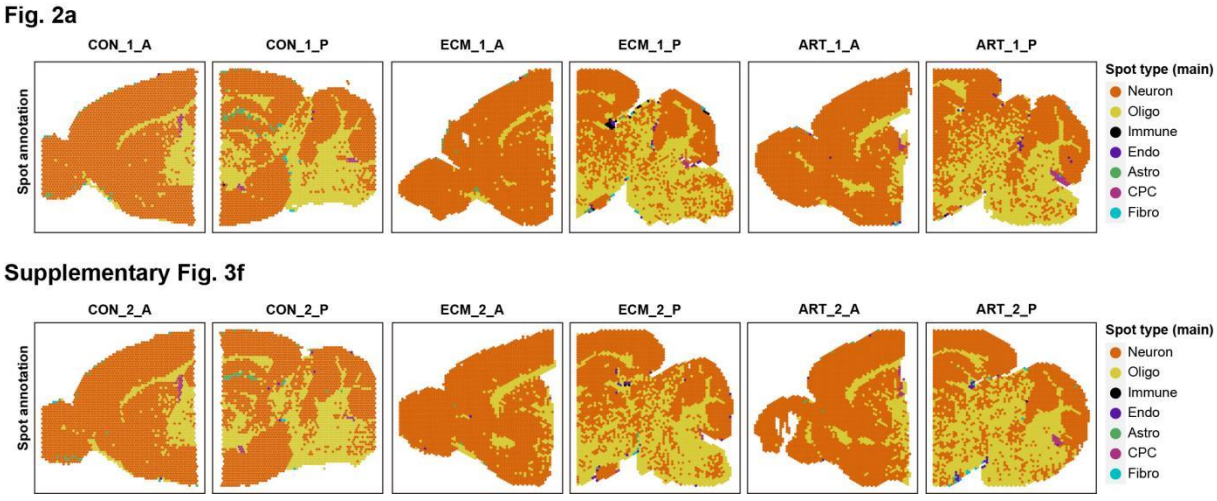


Fig. 2a the spatial landscape of CON_1_A and P, ECM_1_A and _P, ART_1_A and _P samples. **Supplementary Fig. 3f** the spatial landscape of CON_2_A and P, ECM_2_A and _P, ART_2_A and _P samples. Neuron, neuron cell; Oligo, oligodendrocyte; Astro, astrocyte; CPC, choroid plexus epithelial cells; Endo, endothelial cells; Fibro, fibroblast; Immune, immune cells

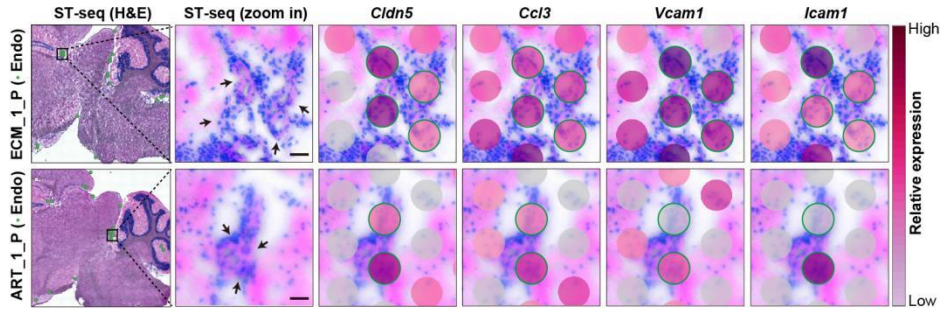
(including Macro, Micro, Mono and T/NK cells).

R3_31. Figure4E,6E, S4E, S5D: The identity of the spots could be highlighted by a color outline. Otherwise, we don't know what we are looking at.

✓**Response:**

Thanks for your nice suggestion. Using a color outline to indicate the cellular identities, we have highlighted these spots in Fig. 3e, Supplementary Fig. 5d and 6d.

Fig. 3e



Supplementary Fig. 6d

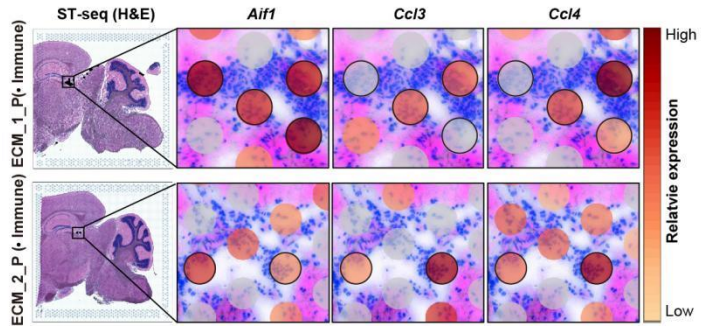


Fig. 3e The slides highlight the spatial distribution of Endo spots (column1, colored by green), the zoom in H&E staining shows the tubular structure of the blood vessels (column2, indicated by arrows), and dotplots show the spatial expression level of Cldn5, Ccl3, Vcam1, and Icam1 (column3-6) of Endo spots (outline colored by green) from ECM_1_P (top panel) and ART_1_P (bottom panel) in the ST-seq dataset, respectively. **Supplementary Fig. 6d** The slides highlight the spatial distribution of Immune spots (column1, colored by black), and dotplots show the spatial expression level of Aif1, Ccl3, and Ccl4 of Immune spots (outline colored by black) from ECM_2_P (top) and ECM_2_P (bottom panel) in the ST-seq dataset, respectively.

R3_32. Figure 4E. Why is region four described if only region 1 and 2 is shown in 4F and

4G?

✓**Response:**

Thanks for pointing this out. According to the main spatial distribution of Endo spots, we divided the locations of Endo spots within the sagittal plane into 4 main regions (zone1-4, mostly in the posterior sections) (Fig. 3h). In the ECM group, we set up a trajectory direction in ECM_1_P from zone1 to zone2; and in ECM_2_P from zone1 to zone3). In the ART group, we set up a trajectory direction in ART_1_P from zone1 to zone2; ART_2_P from zone1 to zone4) (Fig. 3i, and Supplementary Fig. 5e).

Fig. 3h

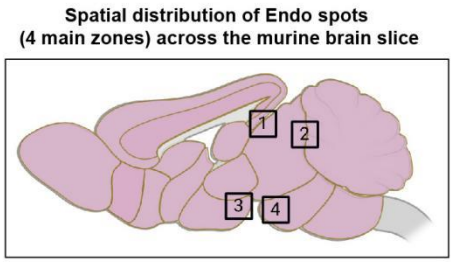
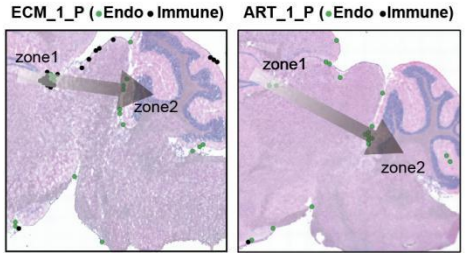


Fig. 3i



Supplementary Fig. 5e

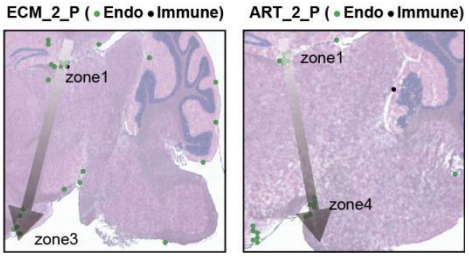


Fig. 3h The schematic diagram indicates the main distributed location (zone1-4) of Endo spots in the ST-seq dataset. **Fig.3i** The direction of the trajectory from zone1 to zone2 in the ECM_1_P and ART_1_P slides, respectively. **Supplementary Fig. 5e** The direction of the trajectory from zone 1 to zone 3 of endothelial cells in ECM_2_P slide (left), accompanied by the gene set scores along the specific trajectory (right).

R3_33. Figure 8G: of which model?

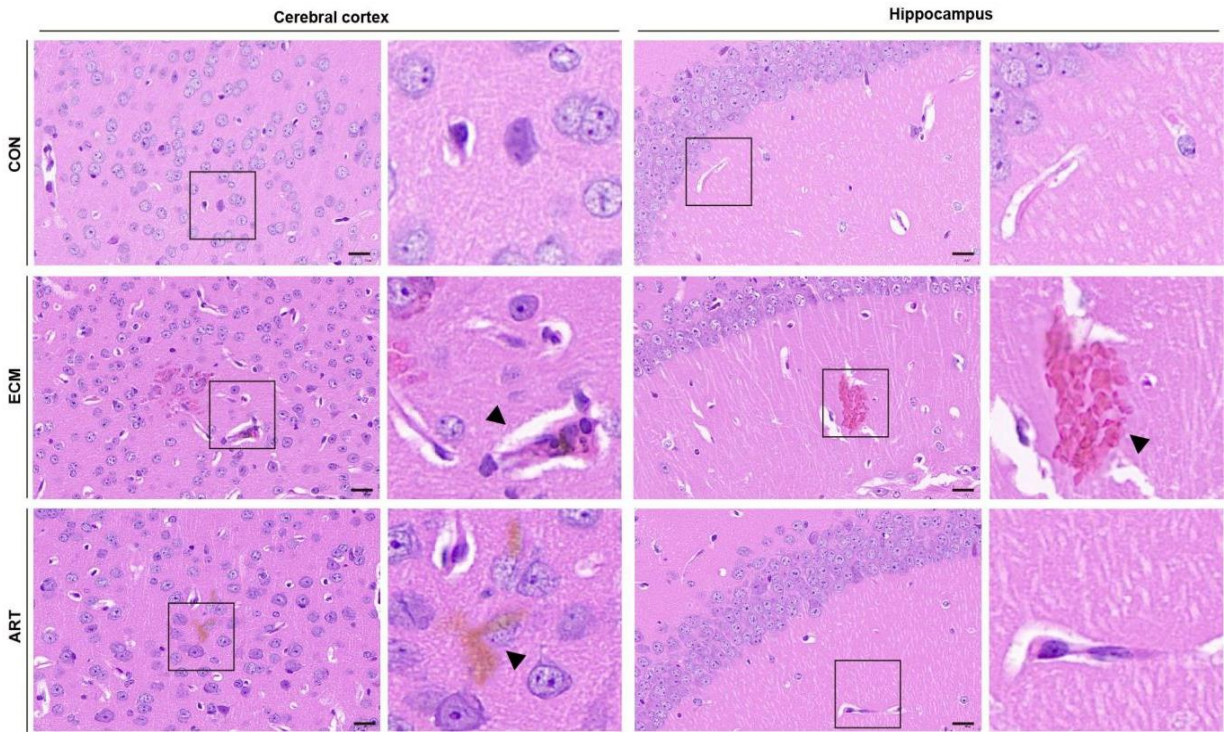
✓**Response:**

Thank you for pointing this out. The result of Fig. 7g is based on the Neuron spots of the ECM model group, we have added a label in the revised manuscript.

R3_34. Figure S1: F I would suggest to use a higher magnification. iRBC sequestration, enlarged perivascular space and vascular leakage are difficult to see in the provided images.

✓**Response:**

Thanks for your suggestions. We have taken your suggestion and provided new images with higher magnification in Supplementary Fig. 1f, to better visualize the RBC sequestration, enlarged perivascular space and vascular leakage.



Supplementary Fig. 1g H&E staining results show the cerebral cortex (left panel) and hippocampus (right panel) region of brain tissues in CON (top), ECM (middle) and ART (bottom) groups, scale bar = 25 μm.

R3_35. Figure S4A, S5B: The color code on the top of the heat maps is not outlined on the figure legend. I would suggest removing it or referencing to the color code in the main figure.

✓**Response:**

Thanks for your kind suggestions. We have removed the color code on the top of the corresponding heatmap figures such as Fig. 6b and Supplementary Fig. 5a.

R3_36. Figure S4B: Endo AP is not defined in the text. Do the authors mean Endo-Hb?

✓**Response:**

We apologize for this careless mistake. We have changed Endo_AP to Endo_Hb, thanks again for your kind reminder.

In conclusion, according to your comments, we have revised the original manuscript in detail. At the same time, we have thoroughly revised the manuscript in order to express our ideas and describe our results more clearly. We believe the manuscript has been greatly improved. Once again, thank you for the kind advice.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns with high-quality results. I am now willing to recommend publishing his work after a slight revision.

1. Check every sentence of the main text and description of results or conclusions. I can still find mistakes with a glance.

2. Reference. That is just my advice, adding more diverse articles and avoiding multiple citations to specific teams is not a bad idea, perhaps readers need a more comprehensive perspective to understand the background, conclusion, and discussion.

Reviewer #1 (Remarks on code availability):

I didn't run the code. But I have some suggestions that will not affect whether this article is considered for publication. There should be a comprehensive Readme file to introduce the tool, a Tutorial file to illustrate the installation steps and applications, and an Example file to show the process of the case study.

Reviewer #2 (Remarks to the Author):

The authors have responded to the majority of the reviewers' comments and have improved the manuscript. Overall, the scRNA-seq and ST analyses appear to have been done well and the approaches are accurately defined. I only have a couple of remaining comments.

R2.3 and R2.5 comments: The authors have attempted to address these comments but the staining of CD3 in Figure 2f still looks diffuse and it is difficult to confirm if the staining is robust. In figure 6h, CD68 staining appears strange in the main image (highlighting a vessel). In Figure 6i, it is difficult to identify the vessels and Iba1 and CD68 are not sufficient to discriminate microglia. Overall, there are still some reservations on the IF staining in the manuscript.

Whilst the authors do acknowledge some of the relevant literature, they don't take into account reports where microglia cells are depleted and where this does not significantly affect the development of ECM. As such, the relative importance of some of the inferred interactions between T cells and microglia in development of ECM is still unclear, and there is little experimental evidence that suggest that monocytes and microglia, at the time point of ECM development, contribute to disease pathogenesis as outlined in lines 530-552. Without mechanistic testing, this paragraph should be tempered.

Line 7-10 within abstract could be rewritten for clarity

Reviewer #3 (Remarks to the Author):

Overall, I believe that Chen, Bai, He, et al., have effectively addressed the majority of my concerns. I would like to emphasize the significant effort invested in reperforming the single-cell RNA sequencing (scRNAseq) analysis on a new extracellular matrix (ECM) sample and thoroughly reanalyzing the resulting data. Additionally, I commend the authors for their responsiveness to the reviewer's feedback, as evidenced by their incorporation of most suggestions, including expanding certain analyses and condensing or removing others deemed less pertinent to the narrative. The manuscript has undergone notable enhancements since its initial submission. However, there remain some minor suggestions for improvement. I strongly urge the authors to rectify any

inaccuracies in relation to the previous literature.
Minor:
New paragraph in intro: Lines 44-51. The distinction between ECM and (human) CM is still not clear. I would suggest the following modification. "Furthermore, the host's innate and adaptive immune responses in experimental cerebral malaria (ECM) are activated under the induction of the parasite biomass, accompanied by the recruitment of various immune cells such as T cell and monocytes and the release of cytokines. Alternatively, remove the sentence that refers to ECM as until now, the intro only covers disease in humans.
Line 66: remove iRBC sequestration. The sequestration levels in ECM are much lower than in humans.
Fig S1: Describe what are the arrows pointing in panel G
Line 142: "These results suggest" instead of "indicate"
Line 180: The result of the endo and immune cell distribution in panel 2C is interesting and barely described in the text
Fig 2: The resolution of panel 2f is not good. I would suggest picking better images.
Line 203: I would not qualify the percent of PbA in ethe ECM group is dramatically higher given that only 14iRBC were identified.
Line 215-218: Rephrase for accuracy. PfEMP1 does not have orthologous in P. berghei. The transcripts identified are orthologous of transcripts/proteins that quite likely contribute to trafficking of proteins expressed at the red blood cell surface.
Fig 4 and 6 and 7 and other supplementary. MVC and AVC are not clear as they do not correspond with the new term associated with the ECM condition, I would suggest to spell the comparisons or find a new acronym.
Line 235 and line 298: Spell Endo-inflam and micro_inflam on the title
Line 334 to 343. I acknowledge the authors extending their analysis on neutrophils. I would suggest to move the description of the results at the end of the section, as now it is breaking the flow on microglia associated changes.
Line 382-388 and Fig5e and f. Would it be possible that the authors depict C1, C2 and C3 in the pseudotime in 5e? What are the groups being compared in 5E in the DEG analysis?
Line 428: replace "indicating" by "suggesting"
Line 433-435: some brain regions are not spelled (HC and MB)
Figure 7e and suppl 7b. Some font colors are difficult to read when printed (CTX-2, MB, HY)
Lines 464-473. This is not necessary, but I would suggest to integrate this paragraph with the section below as they both focus on IFNg response in neurons.
Line 490-492: This sentence is confusing "We also found the persisted activation of the IFN-γ response in the ART group even after the expression of IFN-γ decreased after ART treatment (Supplementary Fig. 8f)." I don't see persistence of the IFNg activation on the western blot
Line 511 and 521: spell endothelial; spell inflammatory
Line 515-519. The reference cited in this paragraph is kind of outdated. Since 2013 there have ben multiple evidences that binding to EPCR is quite likely very relevant for cerebral malaria pathogenesis. This interaction does not occur on humans. I would suggest the authors to frame their results in the relevant literature and to clearly state when they are referring to ECM or (human) CM.
530: remove one of the two "depletion"
Line 535 and 536: spell neutrophil
Line 569-584: Caution should be taken when drawing conclusions on pathogenic transcriptomic signatures in neurons. As the authors acknowledge in the point by point response to reviewers "Thanks for your valuable question. Indeed, brain tissue mainly consists of neuron and glial cells, while the proportion of neuron cells in most of our scRNA-seq datasets is much fewer than expected. The main reasons were: (1) neuron cells can easily cause plugging, due to their special protruding structure resulting in a very large size (up to 100μm or more); (2) neuron cells are relatively fragile and easily damaged during the brain tissue dissociation (Tasic B, et al. Nature. 2018 Nov;563(7729):72-78.; Armand EJ, et al. Neuron. 2021 Jan 6;109(1):11-26. 3). Thus, we obtained lower numbers of neuron cells in the scRNA-seq dataset when compared with that in the ST-seq dataset (without tissue dissociation step). I would suggest to acknowledge the limitation on this paragraph or in the paragraph below (600-613)
Line 569: "Previous research has suggested that brain swelling and cerebral herniation are the primary causes of neuron cell death in ECM, which is similar to observations in HCM patients (85)." I would suggest to rephrase this sentence, a reference is missing on the first part and the

reference given for HCM is a seminal paper that described for the first time the association between brain swelling and cerebral hernation in fatal cerebral malaria, but the manuscript did not explore a relationship with neuron cell death.
Line 575: Spell the neuron subtypes

Point-by-point response to the comments

We thank the reviewers for the insightful comments and constructive suggestions that have helped us with the preparation of a revised manuscript. We have performed additional experiments to address all the concerns and comments raised by our reviewers. Here we submitted a substantially improved manuscript along with our point-by-point response.

On the following pages, the comments of all reviewers #1, #2, and #3 are presented below in purple, italicized font text point-by-point. Our response is given in black, normal font text, with the figure numbers or revised positions highlighted in red. All the authors have read the revised manuscript and agree with the contents.

Reviewer #1

The authors have addressed my concerns with high-quality results. I am now willing to recommend publishing his work after a slight revision.:

We appreciate your efforts in reviewing our manuscript and thanks for the positive and detailed feedback. According to your suggestion, we have addressed your concerns as shown below.

R1_1. Check every sentence of the main text and description of results or conclusions. I can still find mistakes with a glance.

✓ **Response:**

We apologize for the carelessness and omission of description in the manuscript. We have carefully checked every sentence of the main text and description of results or conclusions, to avoid mistakes in text and figures and improve the quality of our manuscript.

R1_2. Reference. That is just my advice, adding more diverse articles and avoiding multiple citations to specific teams is not a bad idea, perhaps readers need a more comprehensive perspective to understand the background, conclusion, and discussion.

✓ **Response:**

Thanks for your valuable comment. We have added additional references to provide a more comprehensive perspective to our study in the **Introduction**, **Conclusion**, and **Discussion parts** of the revised manuscript. For instance, we have added up several references in the **Introduction part** of our revised manuscript:

"Additionally, the spatial transcriptomic sequencing (ST-seq) method makes it possible to measure the topographic organization of gene expression（**Ortiz C, et al.** ***Annu Rev Neurosci,*** **Joglekar A, et al.** ***Nat Commun***). The integration of spatial information has facilitated the exploration of regional gene expression patterns and intercellular communication networks uncovered by scRNA-seq datasets (**Cheng J, et al.** ***Adv Sci (Weinh)***). These technologies have enhanced our understanding of brain biology (**Maynard KR, et al.** ***Nat Neurosci.***), organogenesis (**Lohoff T, et al.** ***Nat Biotechnol***), development (**Braun E, et al.** ***Science***), neurological diseases such as Alzheimer's Disease (**Chen WT, et al.** ***Cell***), and neuroinflammatory diseases (**Hasel P, et al.** ***Nat Neurosci***), with important implications for ECM research." (Line 75-81)

References:

[1] Ortiz C, et al. Spatial Transcriptomics: Molecular Maps of the Mammalian Brain. ***Annu Rev Neurosci***. 2021 Jul 8;44:547-562.

[2] Joglekar A, et al. A spatially resolved brain region- and cell type-specific isoform atlas of the postnatal mouse brain. ***Nat Commun***. 2021 Jan 19;12(1):463.

[3] Cheng J, et al. Multiplexing Methods for Simultaneous Large-Scale Transcriptomic Profiling of Samples at Single-Cell Resolution. ***Adv Sci (Weinh)***. 2021 Sep;8(17):e2101229.

[4] Maynard KR, et al. Transcriptome-scale spatial gene expression in the human dorsolateral 1041 prefrontal cortex. ***Nat Neurosci***. 2021 Mar;24(3):425-436.

[5] Lohoff T, et al. Integration of spatial and single-cell transcriptomic data elucidates mouse organogenesis. ***Nat Biotechnol***. 2022 Jan;40(1):74-85.

[6] Braun E, et al. Comprehensive cell atlas of the first-trimester developing human brain. ***Science***. 2023 Oct 13;382(6667):eadf1226.

[7] Chen WT, et al. Spatial Transcriptomics and In Situ Sequencing to Study Alzheimer's Disease. ***Cell***. 2020 Aug 20;182(4):976-991.

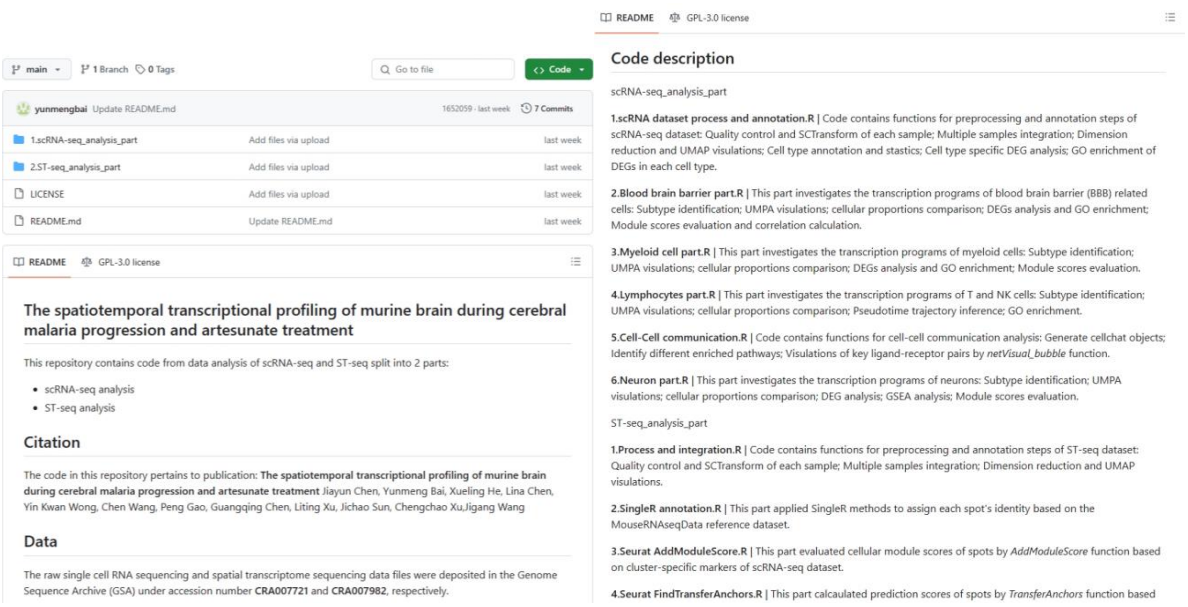
[8] Hasel P, et al. Neuroinflammatory astrocyte subtypes in the mouse brain. ***Nat Neurosci***. 2021 Oct;24(10):1475-1487.

R1_3. I didn't run the code. But I have some suggestions that will not affect whether this article is considered for publication. There should be a comprehensive Readme file to introduce the tool, a Tutorial file to illustrate the installation steps and applications, and an Example file to show the process of the case study.

✓ **Response:**

Thanks for your detailed review and nice suggestion. Following the format of previous literature

(**Vanrobaeys, Y., et al.** ***Nat Commun,*** **Handler, K., et al.** ***Nat Commun***), we have added the README.md file to github to explain the main functions of code in each step (available at: https://github.com/yunmengbai/CM_scRNA-ST-seq-code) .



References:

[1] Vanrobaeys, Y., *et al.* Mapping the spatial transcriptomic signature of the hippocampus during memory consolidation. ***Nat Commun*** **14**, 2023 Sep 29;14(1):6100.

[2] Handler, K., *et al.* Fragment-sequencing unveils local tissue microenvironments at single-cell resolution. ***Nat Commun*** **14**,2023 Nov 27;14(1):7775.

Reviewer #2

The authors have responded to the majority of the reviewers' comments and have improved the manuscript.

Overall, the scRNA-seq and ST analyses appear to have been done well and the approaches are accurately defined. I only have a couple of remaining comments.

We greatly appreciate your professional review work on our article. The reviewer has given an accurate summary of our work and brought forward constructive questions. According to your excellent suggestions, we have made extensive corrections to our previous draft. The detailed corrections are listed below.

R2_1. The authors have attempted to address these comments but the staining of CD3 in Figure 2f still looks diffuse and it is difficult to confirm if the staining is robust. In figure 6h, CD68 staining appears strange in the main image (highlighting a vessel). In Figure 6i, it is difficult to identify the vessels and Iba1 and CD68 are not sufficient to discriminate microglia. Overall, there are still some reservations on the IF staining in the manuscript.

✓ **Response:**

Thanks for your valuable comment**.** We have optimized our immunofluorescence results, to visualize the T cell and myeloid cell more clearly (**Fig 2f**). We can observe the cell body of T cell by CD8 staining, and consistent with previous publications, ECM condition clearly increase the T cell number in brain parenchyma, collectively indicating the robustness of the immunofluorescence staining of CD8 in our study. In **Fig. 6h**, we have replaced the mIHC result especially the CD68 staining. In **Fig. 6i**, we have performed additional IF staining experiments to improve the resolution and robustness of results in the revised manuscript.

Fig. 2f

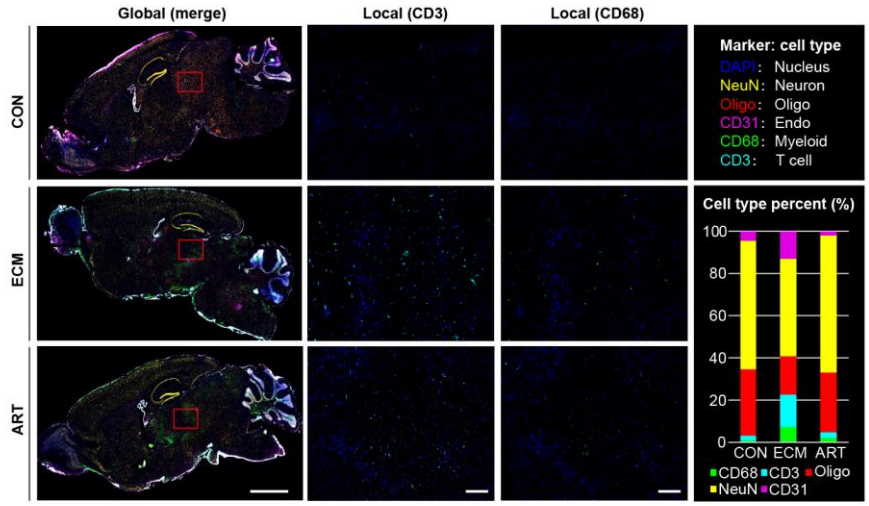


Fig. 6h

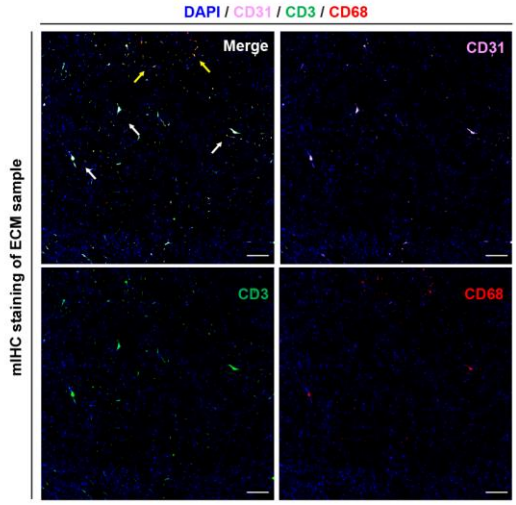


Fig. 6i

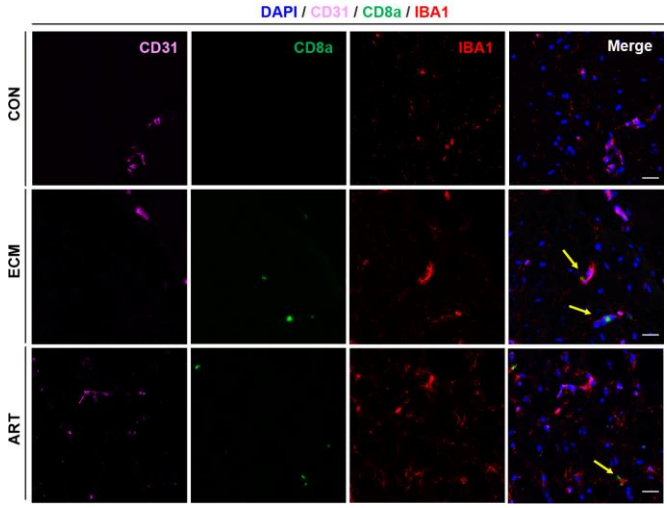


Fig. 2f Six-plex mIHC staining panels show the cellular components and spatial distribution of the sagittal planes (left panel, scale bar = 1mm), CD3 and CD68 markers (middle panel, scale bar = 100μm), and cellular proportions (right panel) among 3 groups, colored by cell types. **Fig. 6h** The representative mIHC staining of ECM sample (DAPI, blue; CD31, pink; CD3, green; CD68, red), the white arrows indicates the co-location of T cell and myeloid cell within the microvessels, and the yellow arrows indicates the co-location outside the microvessels, scale bar = 100 μm. **Fig. 6i** IF staining shows the co-location of CD8+ T cell, microglia, and vascular endothelial cells (DAPI, blue; CD31, pink; CD8a, green; IBA1, red) among three groups (n = 3 samples per group), the interactions were highlight in yellow arrow, scale bar = 20 μm.

R2_2. Whilst the authors do acknowledge some of the relevant literature, they don't take into account reports where microglia cells are depleted and where this does not significantly affect the development of ECM. As such, the relative importance of some of the inferred interactions between T cells and microglia in development of ECM is still unclear, and there is little experimental evidence that suggest that monocytes and microglia, at the time point of ECM development, contribute to disease pathogenesis as outlined in lines 530-552. Without mechanistic testing, this paragraph should be tempered.

✓ **Response:**

Thanks for your kind suggestions. In our findings, we observed that inflammatory microglia enhance antigen presentation pathway such as MHC-I to $CD8^+$ cytotoxic T cells. The latter underwent an inflammatory state transition with up-regulated cytokine expression and cytotoxic activity. To highlight the relative importance of some of the inferred interactions between T cells and microglia during ECM pathogenesis, we have examined several relevant literature and performed necessary experiments to support our assumption. However, whether and how the monocytes and microglia depletion's location and the time points affect the ECM development, are needed in-depth exploration and validation.

Taken your valuable advice, we acknowledged that this limitation in our current study, and tempered the paragraph about the role of microglia cells during ECM development (Line 576-580). In short, understanding the role of microglia in the pathogenesis of cerebral malaria could lead to the development of new therapeutic strategies, while mechanistic investigation is beyond the scope of the current study, but warrants future investigation.

R2_3. Line 7-10 within abstract could be rewritten for clarity.

✓ **Response:**

Thanks for your valuable suggestion. We have rewritten the Abstract part to improve the clarity of our revised manuscript, and the content are as follows: " Cerebral malaria (CM) is a severe encephalopathy caused by *Plasmodium* parasite infection, resulting in thousands of annual deaths and neuro-cognitive sequelae even after anti-malarial drugs treatment." (Line 5-7).

Reviewer #3

Overall, I believe that Chen, Bai, He, et al., have effectively addressed the majority of my concerns. I would like to emphasize the significant effort invested in reperforming the single-cell RNA sequencing (scRNA-seq) analysis on a new extracellular matrix (ECM) sample and thoroughly reanalyzing the resulting data. Additionally, I commend the authors for their responsiveness to the reviewer's feedback, as evidenced by their incorporation of most suggestions, including expanding certain analyses and condensing or removing others deemed less pertinent to the narrative. The manuscript has undergone notable enhancements since its initial submission. However, there remain some minor suggestions for improvement. I strongly urge the authors to rectify any inaccuracies in relation to the previous literature.

We appreciate your efforts in reviewing our manuscript and thank you for your positive and detailed feedback. We fully agree with your kind comments and understand your concerns about the probe selectivity and specificity in the current manuscript. Based on your suggestions, we have addressed your concerns as shown below.

R3_1. New paragraph in intro: Lines 44-51. The distinction between ECM and (human) CM is still not clear. I would suggest the following modification. "Furthermore, the host's innate and adaptive immune responses in experimental cerebral malaria (ECM) are activated under the induction of the parasite biomass, accompanied by the recruitment of various immune cells such as T cell and monocytes and the release of cytokines. Alternatively, remove the sentence that refers to ECM as until now, the intro only covers disease in humans.

✓ **Response:**

Thanks for your constructive suggestion on improving the accuracy of our manuscript. We have modified our description by remove the sentence and reference about ECM, to emphasize the distinction between ECM and HCM as you suggested.

The content are as follows: "Furthermore, innate and adaptive immune responses in patients with HCM to *Plasmodium* infection unfold at the same time as endothelial dysfunction and disruption of the BBB (**Hadjilaou A, et al.** ***Nat Rev Neurol***). The patients' immune responses are activated under the induction of the parasite biomass, accompanied by the recruitment of various immune cells and the release of cytokines (**Dorovini-Zis K, et al.** ***Am J Pathol.,*** **Hochman SE, et al.** ***mBio.***). "(Line 40-43).

Reference:

[1] Hadjilaou, Alexandros et al. Pathogenetic mechanisms and treatment targets in cerebral malaria. ***Nat Rev Neurol***. 2023 Nov;19(11):688-709.

[2] Dorovini-Zis K, et al. The neuropathology of fatal cerebral malaria in malawian children. ***Am J Pathol.*** 2011 May;178(5):2146-58.

[3] Hochman SE, et al. Fatal Pediatric Cerebral Malaria Is Associated with Intravascular Monocytes and Platelets That Are Increased with HIV Coinfection. ***mBio***. 2015 Sep 22;6(5):e01390-15.

R3_2. Line 66: remove iRBC sequestration. The sequestration levels in ECM are much lower than in humans.

✓ **Response:**

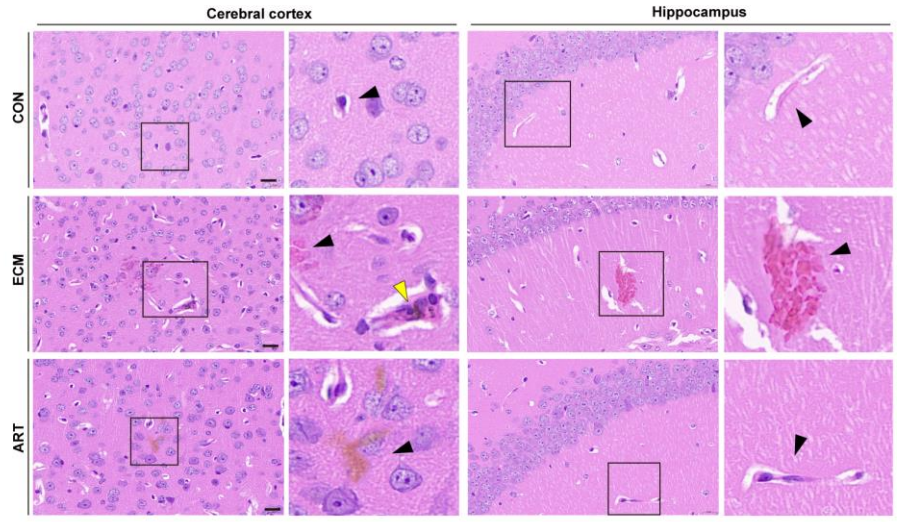
Thank you for pointing this out. We have removed remove iRBC sequestration in the revised manuscript.

R3_3. Fig S1: Describe what are the arrows pointing in panel G

✓ **Response:**

Thank you for your kind suggestion, we have supplemented the description about the arrows pointing in **Supplementary Fig. 1g** as follows: "Black arrows indicate hemorrhage or blood vessels and yellow arrow indicate iRBC".

Supplementary Fig. 1g



Supplementary Fig. 1g H&E staining results show the cerebral cortex (left panel) and hippocampus (right panel) region of brain tissues in CON (top panel), ECM (middle panel) and ART (bottom panel) groups. Black arrows indicate hemorrhage or blood vessels and yellow arrow indicate iRBC, scale bar

= 20 μm.

R3_4. Line 142: "These results suggest" instead of "indicate".

✓ **Response:**

Thanks for your valuable comments, we have replaced the "indicate" with "suggest" in Line 141.

R3_5. Line 180: The result of the endo and immune cell distribution in panel 2C is interesting and barely described in the text.

✓ **Response:**

Thanks for your nice suggestions. We used the annotated scRNA-seq dataset to deconvolute the ST-seq data via cell2location analysis, and identified the spatial localization pattern of main cell types. We have supplemented the description about the co-location of Endo and Immune cells as follows: "Some of Immune spots co-localized with the Endo spots, suggesting the recruitment and infiltration of Immune cells in the cerebral microvasculature, with potential interaction with Endo cells during ECM development, consistent with previous reported (**Shafi AM, et al.** ***Front Immunol***, **Howland SW, et al.** ***PLoS Pathog***)." We have added the detail description in Line 181-183.

References:

[1] Shafi AM, et al. Brain endothelial cells exposure to malaria parasites links type I interferon signaling to antigen presentation, immunoproteasome activation, endothelium disruption, and cellular metabolism. ***Front Immunol.*** 2023 Mar 13;14:1149107.

[2] Howland SW, et al. Activated Brain Endothelial Cells Cross-Present Malaria Antigen. ***PLoS Pathog.*** 2015 Jun 5;11(6):e1004963.

R3_6. Fig 2: The resolution of panel 2f is not good. I would suggest picking better images.

✓ **Response:**

Thanks for your valuable comments. We have replaced the former images with better images with good resolution in **Fig. 2f.**

Fig. 2f

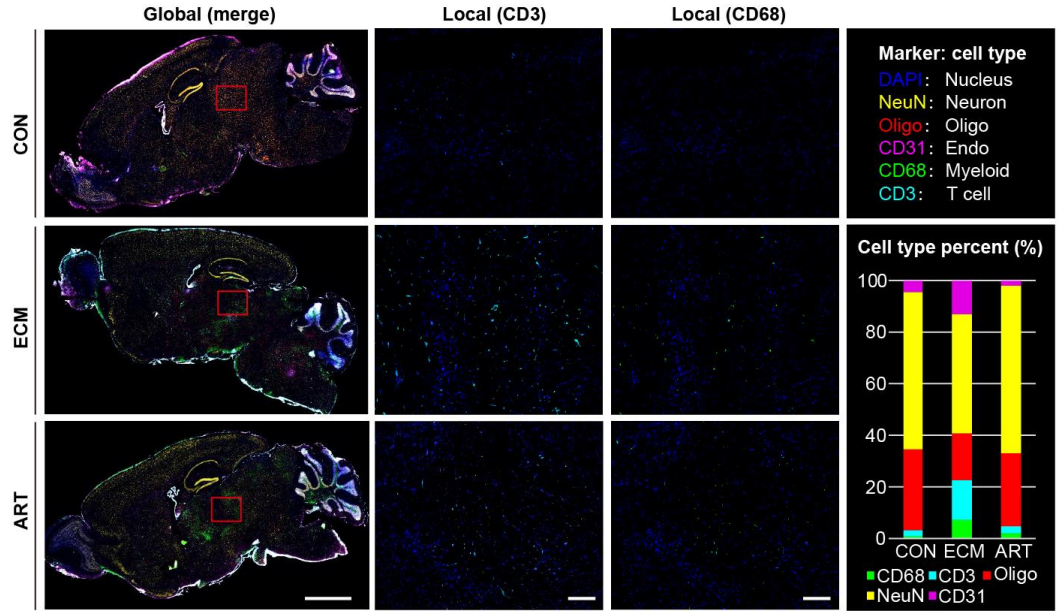


Fig. 2f Six-plex mIHC staining panels show the cellular components and spatial distribution of the sagittal planes (left panel, scale bar = 1mm), CD3 and CD68 markers (middle panel, scale bar = 100μm), and cellular proportions (right panel) among 3 groups, colored by cell types.

R3_7. Line 203: I would not qualify the percent of PbA in ethe ECM group is dramatically higher given that only 14 iRBC were identified.

✓ **Response:**

Thanks for your valuable comments. Considering that the low numbers of iRBC identified in the ECM group, we have deleted the description about the percent of PbA in the ECM group is dramatically higher than other two groups.

R3_8. Line 215-218: Rephrase for accuracy. PfEMP1 does not have orthologous in P. berghei. The transcripts identified are orthologous of transcripts/proteins that quite likely contribute to trafficking of proteins expressed at the red blood cell surface.

✓ **Response:**

Thanks for your valuable comments. After examination, we acknowledge that transcripts/proteins (such as *PBANKA_1145800*, and *PBANKA_1101300*) were not PfEMP-1 orthologues genes. Thus, we have modified our description about these genes as follows: "We also identified the expression of *PBANKA_1145800* (encodes membrane associated histidine-rich protein 1a, MAHRP1a) and *PBANKA_1101300* (encodes

skeleton-binding protein 1, SBP1) in iRBC with PbA parasites, which contributes to trafficking of proteins expressed at the red blood cell surface." (Line 216-219).

R3_9. Fig 4 and 6 and 7 and other supplementary. MVC and AVC are not clear as they do not correspond with the new term associated with the ECM condition, I would suggest to spell the comparisons or find a new acronym.

✓ **Response:**

Thanks for your suggestions. In the text, we defined comparisons of ECM versus CON group as MVC, and ART versus CON group as AVC. To make it clearer, we have changed the acronyms of MVC and AVC to ECM vs. CON and ART vs. CON, respectively, in the text and figures of revised manuscript.

R3_10. Line 235 and line 298: Spell Endo-inflam and micro_inflam on the title.

✓ **Response:**

Thanks for your nice suggestions. We have added up the full spelling of Endo-inflam and micro_inflam on the subtitle (Line 237 and Line 305, respectively).

R3_11. Line 334 to 343. I acknowledge the authors extending their analysis on neutrophils. I would suggest to move the description of the results at the end of the section, as now it is breaking the flow on microglia associated changes.

✓ **Response:**

Thanks for your kind advice. We have moved the description of the results on neutrophils at the end of the section, to improve the description flow on microglia associated alterations (Line 355-364).

R3_12. Line 382-388 and Fig5e and f. Would it be possible that the authors depict C1, C2 and C3 in the pseudotime in 5e? What are the groups being compared in 5E in the DEG analysis?

✓ **Response:**

Thanks for your kind question. Along with the pseudotime trajectory, we categorize genes into three clusters and depicted as follows: "Cluster 1 genes (n = 58, represented by *Klre1*, *Klrk1,* and *Xcl1*), highly expressed at beginning of the trajectory, participating in leukocyte mediated cytotoxicity and cell killing. Cluster 2 genes (n = 59) were enriched in the middle part, which mainly associated with leukocyte migration and T

cell activation. Previous studies have reported that granzyme B (*Gzmb*) from the infiltrating $CD8^+$ T cells plays a critical role in the effector phase during ECM infection (**Haque A, *et al.* *J Immunol***), and granzyme K (*Gzmk*) from NK cells was up-regulated in the murine liver by blood-stage malaria of *Plasmodium chabaudi* (**Araúzo-Bravo MJ, *et al.* *Vaccines (Basel).***).Cluster 3 genes ($n = 47$) such as *Icos*, *Cd28,* and signal transducer and activator of transcription 3 (*Stat3*), were highly expressed at the end of the trajectory, participating in antigen receptor-mediated signaling and lymphocyte differentiation.". We have modified the description of the part in the revised manuscript (Line 390-399).

According to the official tutorial of Monocle2 package, we selected the union significant DEGs detected from ECM vs. CON and ART vs. CON comparisons to sort cells for trajectory construction. For CD8_CTL among three groups, each cell was assigned a "pseudotime" value to represent their relative progress through the dynamic process. In the pseudotime-DEGs analysis, which is not directly performed by comparing groups, we identified the pseudotime-dependent genes via *differentialGeneTest* function. Monocle2 can test against changes as a function of this value via VGAM package, and genes that co-vary across trajectory were identified in pseudotime analysis. We have added up the description of the part in the revised manuscript (Line 928-930).

References:

[1] Haque A, *et al.* Granzyme B expression by CD8+ T cells is required for the development of experimental cerebral malaria. ***J Immunol.*** 2011 Jun 1;186(11):6148-56.

[2] Araúzo-Bravo MJ, Delic D, Gerovska D, Wunderlich F. Protective Vaccination Reshapes Hepatic Response to Blood-Stage Malaria of Genes Preferentially Expressed by NK Cells. ***Vaccines (Basel).*** 2020 Nov 13;8(4):677.

R3_13. Line 428: replace "indicating" by "suggesting".

✓ **Response:**

Thanks for your valuable comments, we have replaced the "indicating" with "suggesting" in Line 441.

R3_14. Line 433-435: some brain regions are not spelled (HC and MB).

✓ **Response:**

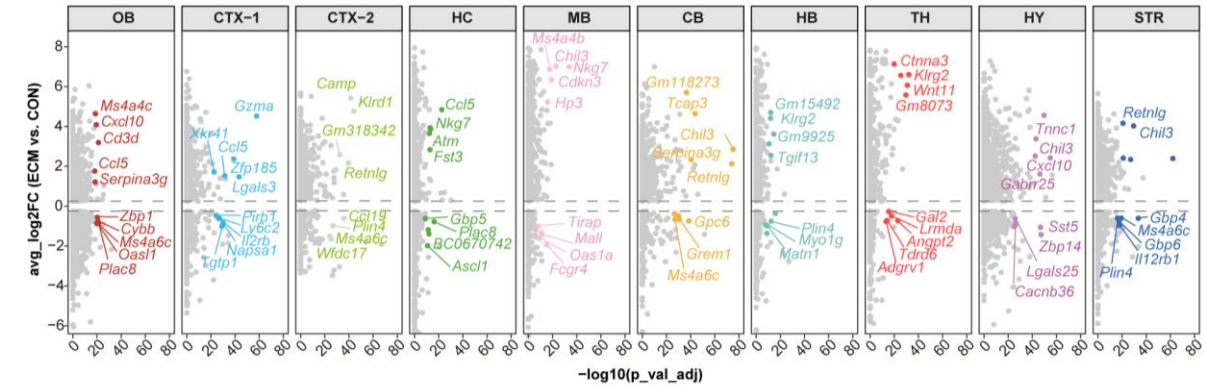
Thanks for your nice suggestions, the spell of hippocampus (HC) have been provided in the Line 104, we have added up the full spelling of midbrain (MB) in Line 447.

R3_15. Figure 7e and suppl 7b. Some font colors are difficult to read when printed (CTX-2, MB, HY).

✓ **Response:**

Thanks for your valuable comments. We have changed the font color (such as CTX-2, MB, HY) in **Fig. 7e** and **Supplementary Fig. 7b**.

Fig. 7e



Supplementary Fig. 7b

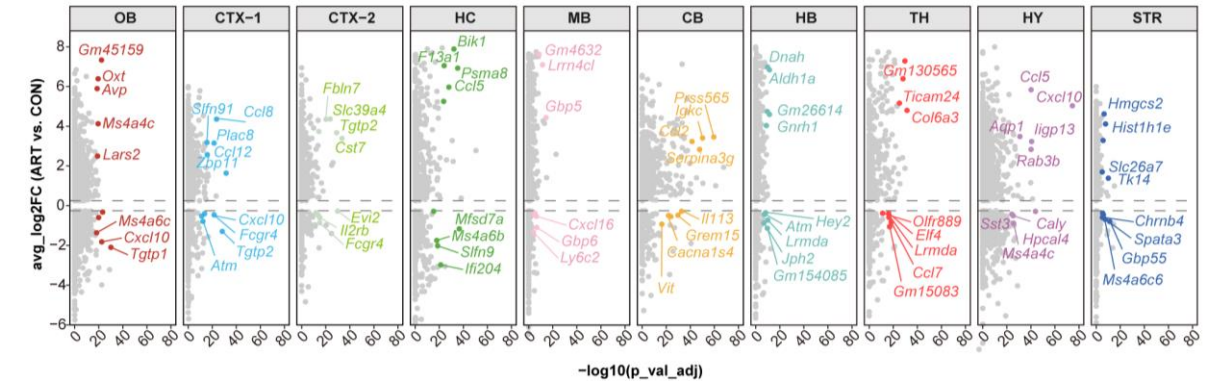


Fig. 2f Volcano plots show the DEGs of ECM vs. CON in each neuron region. The representative up-regulated and down-regulated genes were labeled. **Supplementary Fig. 7b** Volcano plots show the DEGs of ART vs. CON in each neuron region. The representative up-regulated and down-regulated genes were labeled.

R3_16. Lines 464-473. This is not necessary, but I would suggest to integrate this paragraph with the section below as they both focus on IFNg response in neurons.

✓ **Response:**

Thanks for your kind reminding. Although both two paragraphs have reported the activation of IFN-γ response in neurons, the scRNA-seq part additionally profiled the neuron cell subtypes, cellular composition, and their spatial distributed abundance in ECM and ART groups, which should be considered as an independent section. Thus, we prefer to separate these two paragraph rather than to integrate them into one

section.

R3_17. Line 490-492: This sentence is confusing "We also found the persisted activation of the IFN-γ response in the ART group even after the expression of IFN-γ decreased after ART treatment (Supplementary Fig. 8f)." I don't see persistence of the IFNg activation on the western blot.

✓ **Response:**

Thanks for your kind reminder. After consideration, we found that we might not able to determine the relationship of the activated IFN- γ response with the IFN-γexpression level during ECM and ART treatment using the scRNA-seq and ST-seq dataset. Thus, we remove the related results and description in the revised manuscript. Mechanistic investigation is beyond the scope of the current study, but warrants future investigation.

R3_18. Line 511 and 521: spell endothelial; spell inflammatory.

✓ **Response:**

Thanks for your suggestions. We have added up the spell of endothelial and inflammatory in Line 526, Line 544 and 550, respectively.

R3_19. Line 515-519. The reference cited in this paragraph is kind of outdated. Since 2013 there have been multiple evidences that binding to EPCR is quite likely very relevant for cerebral malaria pathogenesis. This interaction does not occur on humans. I would suggest the authors to frame their results in the relevant literature and to clearly state when they are referring to ECM or (human) CM.

✓ **Response:**

Thanks for your constructive suggestion on improving the quality of our manuscript. We have added up the description of the part as follows: "In HCM, iRBC sequestration is mediated by specific interactions between PfEMP1 family's members and receptors on the endothelial receptor such as ICAM-1, and endothelial protein C receptor (EPCR) (**Turner L, et al. *Nature*, Bengtsson A, et al. *J Immunol***), Several studies have emphasized the critical role of EPCR in the pathogenesis of severe malaria (**Kessler, A. et al. *Cell Host Microbe***), as the PfEMP-1 binding to EPCR blocks the binding of protein C (PC) and activated PC (APC) to the EPCR, thus reducing PC activation and dampening APC-induced anti-inflammatory and barrier-protective effects, which promotes proapoptotic, proinflammatory, and barrier-disruptive signaling pathways in endothelial cells (**Mosnier LO, et al. *Thromb Res***). However, the ECM model infected with *P.*

berghei ANKA does not express PfEMP-1 molecules and thus does not epitomize the sequestration of infected RBCs in the microvasculature comparable to human *P. falciparum* malaria. Therefore, the profiling of iRBC genes and pathways might shed light on the interaction and cytoadhesion of iRBC and endothelium during ECM development."

We acknowledge this limitation and supply this to the discussion part of our revised manuscript, we also frame our results in the relevant literature and clearly state when they are referring to ECM or HCM (Line 527-538).

References:

[1] Turner L, et al. Severe malaria is associated with parasite binding to endothelial protein C receptor. ***Nature***. 2013 Jun 27;498(7455):502-5.

[2] Bengtsson A, et al. A novel domain cassette identifies Plasmodium falciparum PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. ***J Immunol***. 2013 Jan 1;190(1):240-9.

[3] Kessler, A. et al. Linking EPCR-binding PfEMP1 to brain swelling in pediatric cerebral malaria. ***Cell Host Microbe***. 2017 Nov 8;22(5):601-614.e5.

[4] Mosnier LO, et al. The role of EPCR in the pathogenesis of severe malaria. ***Thromb Res***. 2016;141 Suppl 2(Suppl 2):S46-S49.

R3_20. 530: remove one of the two "depletion".

✓ **Response:**

Thanks for your nice suggestion. We have removed the one of the two "depletion" in the revised manuscript.

R3_21. Line 535 and 536: spell neutrophil.

✓ **Response:**

Thanks for your nice suggestion. We have added up the spelling of neutrophil in Line 560, Line 561 and Line 562.

R3_22. Line 569-584: Caution should be taken when drawing conclusions on pathogenic transcriptomic signatures in neurons. As the authors acknowledge in the point by point response to reviewers "Thanks for your valuable question. Indeed, brain tissue mainly consists of neuron and glial cells, while the

proportion of neuron cells in most of our scRNA-seq datasets is much fewer than expected. The main reasons were: (1) neuron cells can easily cause plugging, due to their special protruding structure resulting in a very large size (up to 100μm or more); (2) neuron cells are relatively fragile and easily damaged during the brain tissue dissociation (Tasic B, et al. Nature. 2018 Nov;563(7729):72-78.; Armand EJ, et al. Neuron. 2021 Jan 6;109(1):11-26. 3). Thus, we obtained lower numbers of neuron cells in the scRNA-seq dataset when compared with that in the ST-seq dataset (without tissue dissociation step). I would suggest to acknowledge the limitation on this paragraph or in the paragraph below (600-613).

✓ **Response:**

Thanks for your reminder. We have added the limitation of scRNA-seq in capturing neuron cells as follows: "Thirdly, the captured number of neuron cells in scRNA-seq dataset was much fewer than expected, which might be attributed to their vulnerability and fragility during tissue dissociation. Although scRNA-seq dataset have revealed the transcription reprograms of neuron cells, with high concordance with results on ST-seq dataset, other technologies such as single nucleus RNA sequencing that obtain more neuron nucleus, should be employed in the future for systematically uncover the pathogenic transcriptomic signatures in neurons." (Line 640-645).

R3_23. Line 569: "Previous research has suggested that brain swelling and cerebral herniation are the primary causes of neuron cell death in ECM, which is similar to observations in HCM patients (85)." I would suggest to rephrase this sentence, a reference is missing on the first part and the reference given for HCM is a seminal paper that described for the first time the association between brain swelling and cerebral hernation in fatal cerebral malaria, but the manuscript did not explore a relationship with neuron cell death.

✓ **Response:**

Thanks for your kind reminder. Thanks for your kind reminder. We have rephrased this sentence and quoted a reference in the first part, which reported a relationship between brain swelling and cerebral hernation with neuron cell death. And the content in our revised manuscript are as follows: "In ECM model, Swanson PA et al. has suggested that host's death from ECM is associated with vascular leakage and neuronal cell death in the brainstem (**Swanson PA et al, *PLoS Pathog.***), which is similar to observations of the brain swelling and cerebral herniation occurred in children with HCM (**Seydel, Karl B et al. *The New England journal of medicine.***)." (Line 598-600).

References:

[1] Swanson, Phillip A 2nd et al. CD8+ T Cells Induce Fatal Brainstem Pathology during Cerebral Malaria via Luminal Antigen-Specific Engagement of Brain Vasculature. ***PLoS pathogens***. 2016 Dec 1;12(12):e1006022.

[2] Seydel, Karl B et al. Brain swelling and death in children with cerebral malaria. ***The New England journal of medicine.*** 2015 Mar 19;372(12):1126-37.

R3_24. Line 575: Spell the neuron subtypes.

✓ **Response:**

Thanks for your kind suggestions. We have added up the full spelling of neuron subtypes (mature excitatory neurons and mature inhibitory neurons) in Line 605.

In conclusion, according to your comments, we have made a detailed revision of the original manuscript. At the same time, we have thoroughly revised the manuscript in order to express our ideas and describe our results more clearly. We believe the manuscript has been greatly improved. Once again, thank you for the kind advice.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

I thank the authors for responding to my comments. I have assessed the IF images and I still have reservations regarding the data shown in Figure 6H. Whilst the different cell types may be proximal to each other, the staining shows complete overlap on the cells. The results in Figure 2F are difficult to analyse, particularly when evaluating the alignment in CD68 staining between the large field of view and the focussed region of interest.

It is also debatable whether the additional text in the discussion sufficiently represents the overall understanding of the role of microglial cells in development of ECM, at the time point of development of the syndrome.

Reviewer #3 (Remarks to the Author):

I would like to acknowledge Chen an authors for their manuscript and addressing all reviewers comments and suggestions. However, the manuscript is very heavy on data, with analysis being done on many cell types. The use of cell abbreviations through the text make the reading difficult, even after multiple reads. I fully endorse the publication of the article but in my opinion, all cell names should be fully spelled for clarity.

Point-by-point response to the comments

We thank the reviewers for the insightful comments and constructive suggestions that have helped us with the preparation of a revised manuscript. We have performed additional experiments to address all the concerns and comments raised by our reviewers. Here we submitted a substantially improved manuscript along with our point-by-point response.

On the following pages, the comments of reviewers #2, and #3 are presented below in purple, italicized font text point-by-point. Our response is given in black, normal font text, with the figure numbers or revised positions highlighted in red. All the authors have read the revised manuscript and agreed with the contents.

Reviewer #2

I thank the authors for responding to my comments.

We greatly appreciate your professional review work on our article. The reviewer has given an accurate summary of our work and brought forward constructive questions. According to your excellent suggestions, we have made extensive corrections to our previous draft. The detailed corrections are listed below.

R2_1. I have assessed the IF images and I still have reservations regarding the data shown in Figure 6H. Whilst the different cell types may be proximal to each other, the staining shows complete overlap on the cells.

✓ **Response:**

Thanks for your valuable comments. The overlapped signals from different cellular markers might be attributed to the low imaging resolution of multiplex immunohistochemistry (mIHC) results. In addition, considering that the cellular markers in mIHC experiment represent the major cell types (i.e. CD31 for endothelial cell, CD3 for T cell, CD68 for myeloid cell) rather than subtypes (i.e. $CD8^+$ T lymphocyte and microglial cells that we want to emphasize and validate), it seems indirect and redundant to use the mIHC experiment to validate such cell-cell interaction.

To further elaborate the interaction between $CD8^+$ T lymphocyte and microglial cells within the brain parenchyma, we have performed and immunofluorescent staining (IF) experiments with higher resolution. Compared with the previous mIHC results, it's clear enough to distinguish $CD8^+$ T lymphocyte, microglial,

and endothelial cells. IF assay also directly validates the interactions among these cell types of cerebral samples in the ECM and ART groups, in agreement with our scRNA-seq and ST-seq analysis results. To further confirm these findings, we replicated the IF experiment in this revision with an additional murine group. Reassuringly, similar results were obtained, underscoring the reliability and robustness of our original IF data. Since IF experiments provide standalone conclusions and due to limitations in the low resolution of mIHC images, we excluded mIHC staining to enhance clarity and avoid potential confusion in the manuscript.

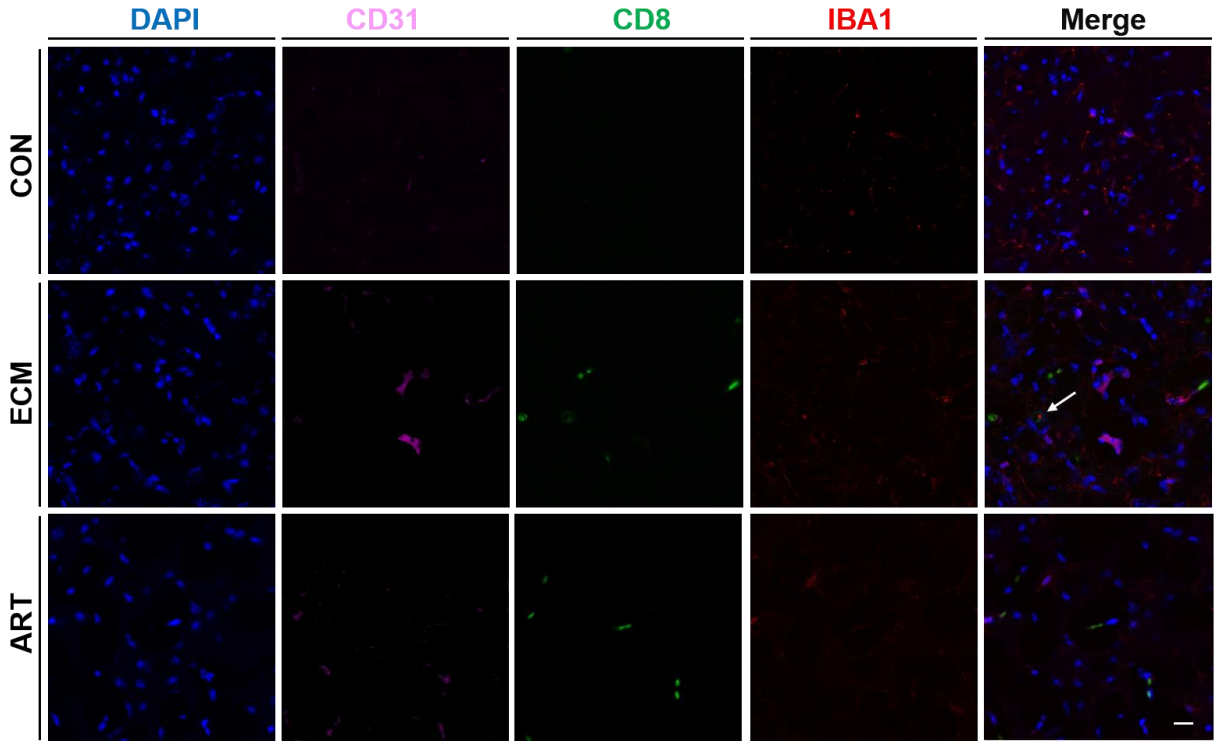


Figure. IF staining shows the co-location of $CD8^+$ T cell, microglia, and vascular endothelial cells (DAPI, blue; CD31, pink; CD8a, green; IBA1, red) among three groups (n = 3 samples per group), the interactions were highlight in white arrow, scale bar = 20 μm.

R2_2. The results in Figure 2F are difficult to analyze, particularly when evaluating the alignment in CD68 staining between the large field of view and the focused region of interest.

✓ **Response:**

Thanks for your valuable comment. For interpreting our mIHC result, we have evaluated the relative abundance of five main cell types (including neuron, oligodendrocyte, endothelial, myeloid, and T cell) among three groups. We observed the increased abundance of T cell and myeloid cell in both ECM and

ART samples compared with that in the CON group, which in agreement the proportional changes of immune cells in both of ST-seq and scRNA-seq datasets.

In particular, we apologized for the carelessness and misalignment of CD3/CD68 staining in the **Fig. 2f**. In the revised manuscript, we have carefully checked and ensured that the local region of interest (highlighted in red square, and marked by CD3/CD68 in the column 2/3) of view matches the large field of view (column 1) for each mIHC slide. Thanks again for you pointing this out to avoid mistakes in figures and improve the quality of our manuscript.

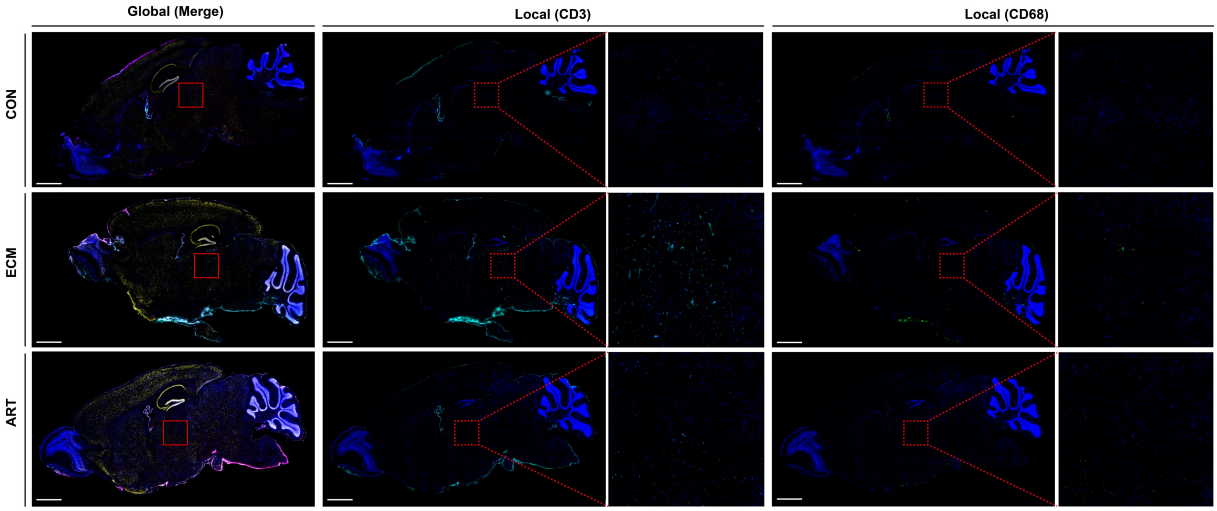


Figure. Six-plex mIHC staining panels show the spatial distribution of the sagittal planes (left panel, scale bar = 1mm), CD3 and CD68 markers (middle and right panels, scale bar = 1mm) among 3 groups, the regions of interest were highlighted in red square.

R2_3. It is also debatable whether the additional text in the discussion sufficiently represents the overall understanding of the role of microglial cells in development of ECM, at the time point of development of the syndrome.

✓ **Response:**

Thanks your valuable advice. In our study, we have employed scRNA-seq and ST-seq to dissect the heterogeneity of microglia subtypes, and identified that inflammatory microglia with increased antigen presentation activity during ECM development. However, the critical role of microglial cells as well as subtypes during ECM progression still needed mechanistic testing and in-depth validation. We have taken account that it's still debatable about the role of microglial cells in development of ECM, as you mentioned.

To address this issue, we have retrieved the relevant reports in recent years. Although several studies have emphasized the important role of microglia during the pathogenesis of both murine and human host with cerebral malaria [1-3], perturbation studies have reported its undetermined role at the time point of development of the syndrome. In particular, Shaw et al. have reported that the targeted depletion of the CX3CR1^{+} cells (including perivascular and meningeal macrophages and microglia) from day 5 p.i. failed to protect mice against ECM development, and specific depletion of CX3CR1 expressing cells from day 3 p.i. utilizing CX3CR1-iDTR mice was not to confer protection from ECM [4-5]. Alternatively, previous studies investigating the role of monocytes and macrophages in ECM pathogenesis, have employed systemic administration of clodronate liposomes, CCR2 and Gr1 depleting antibody to ECM mice [6-8]. Most of these interventions successfully prevented ECM development when given at the onset of disease symptoms rather than the late course of PbA infection.

Therefore, the depleted location and timepoint of microglia cells might be critical elements for the effect of ECM intervention, while mechanistic investigation is beyond the scope of the current study, but warrants future investigation. We acknowledged that this limitation in our current study and tempered the paragraph about the role of microglia cells during ECM development. We also added up additional discussion about this part in our revised manuscript (Line 565-578).

Reference:

[1] Capuccini B, Lin J, Talavera-López C, Khan SM, et al. Transcriptomic profiling of microglia reveals signatures of cell activation and immune response, during experimental cerebral malaria. ***Sci Rep***. 2016. doi: 10.1038/srep39258.

[2] Medana IM, Hunt NH, Chan-Ling T. Early activation of microglia in the pathogenesis of fatal murine cerebral malaria. ***Glia***. 1997. doi: 10.1002/(sici)1098-1136(199702)19:2<91::aid-glia1>3.0.co;2-c.

[3] Andoh NE, Gyan BA. The Potential Roles of Glial Cells in the Neuropathogenesis of Cerebral Malaria. ***Front Cell Infect Microbiol.*** 2021. doi: 10.3389/fcimb.2021.741370.

[4] Shaw TN, Stewart-Hutchinson PJ, Strangward P, et al. Perivascular Arrest of CD8+ T Cells Is a Signature of Experimental Cerebral Malaria. ***PLoS Pathog***. 2015. doi: 10.1371/journal.ppat.1005210.

[5] Parkhurst CN, Yang G, Ninan I, et al. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. ***Cell***. 2013. doi: 10.1016/j.cell.2013.11.030.

[6] Pai S, Qin J, Cavanagh L, et al. Real-time imaging reveals the dynamics of leukocyte behaviour during experimental cerebral malaria pathogenesis. ***PLoS Pathog***. 2014. doi: 10.1371/journal.ppat.1004236.

[7] Howland SW, Poh CM, Gun SY, et al. Brain microvessel cross-presentation is a hallmark of experimental cerebral malaria. ***EMBO Mol Med***. 2013. doi: 10.1002/emmm.201202273.

[8] Schumak B, Klocke K, Kuepper JM, et al. Specific depletion of Ly6C(hi) inflammatory monocytes prevents immunopathology in experimental cerebral malaria. ***PLoS One***. 2015. doi: 10.1371/journal.pone.0124080.

Reviewer #3

I would like to acknowledge Chen and authors for their manuscript and addressing all reviewers comments and suggestions. However, the manuscript is very heavy on data, with analysis being done on many cell types. The use of cell abbreviations through the text makes the reading difficult, even after multiple reads. I fully endorse the publication of the article but in my opinion, all cell names should be fully spelled for clarity.

✓ **Response:**

Thank you for your appreciation and suggestions on our manuscript. Totally, we identified over 50 cell types/subtypes of ECM brain in this study, we acknowledged that the use of cell abbreviations through the text makes the reading difficult in deed. As you suggested, we have removed the cellular abbreviations and updated the full spelling of cell types/subtypes throughout the manuscript, to make it more concise and clearer. But we still retain the use of the abbreviation of red blood cell (i.e. RBC) section, we think it might be redundant if we use the full spellings of this cell type.

In conclusion, according to your comments, we have made a detailed revision of the original manuscript. At the same time, we have thoroughly revised the manuscript in order to express our ideas and describe our results more clearly. We believe the manuscript has been greatly improved. Once again, thank you for the kind advice.