nature portfolio

Peer Review File

Spatial transcriptomics uncovers vasculature-centered cellular interactions driving Japanese encephalitis progression in mice

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Summary:
Ou et al. utilized spatial transcriptomics to examine gene expression dynamics in mouse brains infected with JEV at 3, 5, and 7 d post-infection. By employing both a polyT probe for host transcripts and a JEV-specific probe targeting the NS5 region, the authors reported temporal and spatial mapping of viral and host gene expression. Their bioinformatics analyses revealed widespread viral dissemination across multiple brain regions, including the thalamus and cortex. Monocytes were identified as the initial targets of infection, followed by involvement of various cell types, including endothelial cells. The study described dynamic, time-dependent transcriptional changes, with immune-related gene upregulation particularly enriched in vascular endothelial cells. The authors propose that Ackr1$^+$ endothelial cells facilitate immune cell recruitment and amplify inflammation, while Ccl12$^+$ microglia are activated and expand in response to infection. They also reported increased necroptosis and pyroptosis in the meninges and neurons, contributing to tissue damage. Additionally, host factors potentially associated with JEV-induced neurological dysfunction were suggested. Overall, the authors propose a model in which monocytes and endothelial cells synergistically drive disease progression.

Comments:
1) Descriptive nature of the study: While the study employs a comprehensive suite of bioinformatics tools, the conclusions are based entirely on transcriptomic data. The functional roles of key cell types (e.g., Ackr1$^+$ endothelial cells and Ccl12$^+$ microglia) are inferred rather than experimentally validated. Functional assays are essential to confirm the biological relevance of these findings, particularly for publication in a high-impact journal.

2) Novelty of the proposed model: The proposed model—emphasizing endothelial activation, blood-brain barrier disruption, monocyte infiltration, and immune-mediated cell death—aligns with existing paradigms in JEV pathogenesis. The manuscript would benefit from a more explicit discussion of how these findings refine or extend current models.

3) Route of virus inoculation: The use of intraperitoneal inoculation raises concerns, as it does not mimic the natural route of JEV transmission via mosquito bite. The authors should justify this choice and discuss its implications for disease modeling and relevance to natural infection.

4) Spatial resolution and validation: The spatial resolution of the transcriptomics platform may not be sufficient to resolve cell-type-specific expression in complex brain regions. Key spatial findings should be validated using other techniques such as in situ hybridization or immunohistochemistry.

5) Sample size and reproducibility: The study includes only three mice per time point, which limits statistical power and raises concerns about reproducibility. Have the authors validated their findings in independent cohorts?

6) Cell type annotation: Cell type identification based on transcriptomic signatures can be ambiguous, especially in infected tissues where gene expression is altered. Were annotations cross-validated using canonical markers?

7) JEV probe and viral RNA detection: Lines 111–115: This reviewer is somewhat confused. JEV expresses only its

genomic RNA as mRNA and does not produce multiple distinct mRNAs. Also, the limitations of spatial transcriptomics in quantifying viral load should be acknowledged.

8) Translational relevance: Findings from a mouse model may not fully translate to human JEV infection due to species-specific differences in immune responses and brain architecture. Are any human data or comparative analyses included to support translational relevance?

9) Taxonomic nomenclature: Line 90: The manuscript should reflect the updated taxonomy, noting that JEV is now classified under the Orthoflavivirus genus, in accordance with current nomenclature recommendations.

Reviewer #2

(Remarks to the Author)
In the paper," Spatial transcriptomics elucidates the central role of cerebral vasculatures in the progression of Japanese encephalitis" Ou et al investigate the temporal dynamics of the immune response in the brain following peripheral JEV infection in a mouse model using spatial transcriptomics. The basic experimental setup is sound, including appropriate controls, the different time points and three biological replicates per time point. They detect virus infection in monocytes the brain at 3 days post i.p. inoculation and the virus then spread to other cell types during day 5 and 7. The results show the dynamics and transient nature of interferon and immune responses in the brain, highlights the importance of the meninges and identifies several infiltrating/activated cell populations that likely play an important role in driving immunopathology during JEV infection. This study is well performed and adds significantly to the current knowledge about flavivirus infection in the CNS of mice. While the experimental setup and analysis is sound, the study is missing some complementary experiments to validate key observations to further strengthen the conclusions. For example, fluorescence imaging of brain slices or FACS analysis of infiltrating immune cells. These analyses are important to verify the transcriptional findings as RNA and protein levels are not always comparable. Additionally, if the study was better put into the context of similar studies for other neurotropic viruses this would add meaning to the results and facilitate for the reader to understand the importance of the results presented.

Major:
- Please add clinical characteristics (weight loss/symptoms) for the model used (mouse strain, inoculation route and infectious dose) since now it is difficult to correlate how the observations correlate with disease progression, especially the apparent reduction in inflammatory response seen between day 5 and day 7.
- Complementary experiments validating some of the key observations are necessary.
o Please validate the immune cell infiltration in the brain Fig 2e with FACS analysis.
o Please show the Necroptosis and Pyroptosis in brain slices with immunofluorescence imaging.
o Verify the high infection rate of the immune cells by FACS analysis.
o Quantify the IFN response with Q-PCR over time to see that the slices are representing the brain.
o Verify the localization of tight junction proteins, cell adhesion molecules and chemokine receptors over time using fluorescent imaging of brain slices.
- On line 227 authors write that disruption of the BBB is crucial for immune cell infiltration and virus invasion. However, there is no data supporting this claim although RNA data indicate it. Please investigate the BBB integrity with fluorescent dextran or Evans blue 3, 4, 5, and 6 days post infection in combination with FACS analysis of infiltrating immune cells and viral load with q-PCR.
- The study and results need to be better put into the larger context of neurotropic virus infection both in the introduction and the discussion. For example, the virus distribution and cellular tropism (Fig 1c-d), the role/importance of inflammatory response in the meninges and the specific cell populations highlighted to potentially be driving the inflammatory response. There are several reports that the choroid plexus is highly responsive to virus infection by upreglating type I IFNs.

Minor:
Row 66-67: "Without 67 timely treatment, the condition progresses to encephalitis…", makes it sound like treatment can prevent encephalitis, rephrase, or clarify which treatment with reference.
Row 92-97: Mention the model system used (mice).
Row 116-117: Add rout of infection in the main text line, for clarity.
Row 123-125: Mention if any cells were positive for JEV in the control as QC/validation of the method/threshold selected.
Row 191-194: Add statistical test and indicators of significance in the figure (Fig 2e) to support this statement.
Row 194-196: "We also observed increased signals of endothelial cells (Fig. 2e), 195 which was probably due to vascular dilation caused by immune infiltration or tissue 196 edema, instead of cell proliferation" – elaborate what this statement is based on.
Row 199-200: "JE progression" indicates worsened disease, requires clinical scoring or similar for the mice, else rephrase to immune response.
Row 210-213: Cell population mentioned as significant in the text differs from those labeled as significant in the figure (Fig 3c)
Row 220-222: Strengthen some of these observation with e.g. IF (Fig 3c).
Row 287-288: "Only necroptosis and pyroptosis were found to be associated with JEV infection (Fig. 5a-d, Extended Data Fig. 6a-d)", unclear which figure is showing the association only with necroptosis and pyroptosis. Method/statistical test to measure association?
Row 299-300: Mentions different regions to be highly infected than previously (row 131), hindbrain instead of cerebral cortex.

Row 369-383: Difficult section with no clear message/result.
Row 409- 434: Can this section be shortened and more result-focused, briefly describing how the results support the suggested model.
Fig 1a: Reduce repetition of methodology.
Fig 1b: consider including a sketch of a brain slice to highlight some of the main brain regions mentioned in the text to guide the reader. Can the slices be rotated/aligned in a way to make the data more accessible?
Fig 2a-b: Why is the pathway analysis for upregulated genes divided by timepoint (2a) but downregulated genes all time points are grouped together (2b)?
Fig 2c: Important figure that is very difficult to read/interpret. Consider making the scale the same across all conditions for one gene and have the legend only once. Make text/legends larger/readable. Highlight in the figure legend that the coloring represent the brain parts as described in Fig1b.
Fig2f: Avoid implying results in the figure legend. Why is only one sample chosen here, and additionally a sample with a much higher intensity flow for the CCL pathway than the other two samples from that time point (D7_1, Fig 2d). Would it not be better to pool all samples from d5 and/or d7 or make an average?
Fig 7c: Change the colouring, the yellow letters on white background is very hard to see.

Figures general: Ensure all text is readable and improve the style formatting of headings/legends, e.g. change "_" for spaces, capitalize the first letter etc.

Reviewer #3

(Remarks to the Author)
The manuscript presented by Zhihua Ou and colleagues presents for the first time a spatial atlas of the neuropathology caused by the Japanese Encephalitis Virus. Firstly, I would like to commend the authors for providing such as concise and well-presented manuscript, with some novel insights derived from their dataset. I would also like to apologise for the time that took me to review this manuscript (personal circumstances precluded me from doing this sooner).

This is an incredible resource for the scientific community, as it provides novel insights about the expression pattern of candidate genes of interest that could be the focus of much needed therapeutic applications. The comments below are geared towards increasing the impact of the findings presented here, and I hope the authors find it useful. My major recommendations are to include independent validation of potential key findings (e.g., immune infiltrates, changes in vascular permeability, gliosis, demyelination) to increase the impact of the manuscript. These are listed in my questions below:

Line 105: How was the 1:10 ratio chosen to create the virus-compatible Stereo-seq chip?

Line 130: Does the accumulation of viral transcripts in these brain regions correlate with what is known in the field? In other words, can these observations be used to benchmark the newly developed platform?

Figure 1d: Is it not surprising to find adipocyte signatures in the brain? Can these cells be mesenchymal progenitors wrongly annotated as such? What are the markers genes used to identify the cell types in this figure? As far as I know there are not adipocytes in the brain parenchyma.

Figure 2a: The gene signatures observed here are quite consistent with potential adaptive immune cell infiltrates into the brain parenchyma, which is not surprising due to the viral infection going on. Is this known in JEV infection? If not, I think a validation of these findings by flow cytometry and/or IFA would be advantageous and would certainly elevate the paper. In this regard, it would be interesting to determine whether: i) the dynamics of T and NK cell accumulation in the brain parenchyma (as shown in Figure 2e), and ii) which of these subsets produce IFNg?

Figure 2c: It is interesting to see that the genes associated with lymphocyte trafficking and recruitment (Cxcl10, Ccr2, Ccl5) seem to precede IFN-mediated signatures. Have the authors try to run a pseudotime analysis to better resolve the order of events leading to the robust IFN signature observed in the brain?

Line 199: The choroid plexus also seems to display inflammatory signatures, in particular at d7pi (Figure 2e). Given the possibility that infected cells might exploit both routes to colonise the brain parenchyma, I wonder if the authors have compared meninges vs choroid plexus to determine region-specific differences, or lack of?

Line 220: I find it intriguing to see so many chemokine interactions with Acrk1, which is thought to bind to CXCL12. Is this expected? Have the authors considered validating these findings using imaging techniques (e.g., IFA or RNAscope) to determine if Cxcl9/10+ cells cluster around Ackr1+ ECs in JEV-infected brain specimens?

Given that this manuscript is likely to set a standard for others in the field trying to replicate these findings, or use similar methods for their own studies, I'd encourage the authors to include a supplementary figure highlighting the optimisation workflow. For instance, if possible, I'd like to see the permeabilization time course and the bioanalyzer profile of the resulting RNA/libraries, etc. This could be really informative to the scientific community

Line 289: The choroid plexus also shows cell death signatures observed in the meninges, but overall, the signals shown in figure 5b and 5d are not the most convincing are certainly not limited to the regions described in the text. For instance, there

is a clear enrichment for pyroptosis-related genes in the brainstem area and the midbrain, whereas the necrosis seems more prominent in the external capsule. I believe this is further discussed in figure 5. I believe that validating these findings in independent experiments would be extremely informative to determine whether various types of cell death occur in a region-specific manner.

Line 405: The results presented here are indeed quite interesting and might suggest that the neuronal death results in a loss of MBP (and potential demyelination) and gliosis. Given that this is critical to understand the neuropathology of JEV infection, I think some validation using independent samples of i) demyelination using Luxol Fast blue (or related) and ii) gliosis would be critical.

Figure 7: Does the vascular changes observed at the transcriptional level correlate with changes in vascular permeability? I think experiments using labelled dextran with various molecular weights would help clarify whether the overall vascular permeability changes in response to infection.

Line 413: gene symbols in this line are not italicised. Please amend.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Summary
I would like to commend the authors for their thorough and thoughtful revision, which has substantially strengthened the manuscript. The additional experimental validation, expanded discussion, and clarifications have addressed nearly all of the initial concerns, resulting in a version that is considerably more rigorous and compelling. I offer below a set of points that the authors may consider in further revision.

Comments
1) The study used BALB/c mice inoculated with 10^6 PFU of the Nakayama strain via the intraperitoneal route. While many strains (e.g., BALB/c, C57BL/6, and C3H/He) are susceptible to JEV infection, the use of such a high dose in BALB/c mice warrants further discussion. It would be helpful if the authors could elaborate on the rationale for this choice and comment on how results might differ with other strains.

2) The data suggest that JEV RNA was not detected in granulocytes or B cells. It would be valuable to discuss whether this finding implies that these cell types are not susceptible to JEV infection.

3) Please clarify the distinction between Ly6c^{+} and Ly6c2^{+}, and consider using one consistent designation throughout the manuscript if appropriate.

4) Virus taxonomy terminology should be standardized. Please use Orthoflavivirus, orthoflaviviruses, and orthoflaviviral consistently throughout.

5) Lines 73–93: The paragraph describing the current working hypothesis of JEV pathogenesis could be revised for clarity. The findings of this study align well with existing concepts of viral amplification, brain invasion, and pathology, while specifying the involvement of endothelial cells and macrophages.

6) Line 130: The detection of JEV RNA in blood at 1 day post-infection may reflect residual input virus, given the high inoculum (10^6 PFU), which can persist for about 2–4 days without replication. Please clarify this point.

7) Lines 161–189: JEV expresses only one mRNA, the genomic RNA. Please revise the paragraph to reflect this fact accurately.

8) Lines 226–233: The statements referring to Supplementary Fig. 2b (L229, L233) do not appear to be supported by the data. Please review and correct.

9) Line 297: The emphasis on IFN-γ is not explained or justified. A brief rationale for focusing on this cytokine would be helpful.

10) Line 303: Please revise “NK cells (Fig. 2i) for 6.3–6.9%” to “NK cells for 6.3–6.9% (Fig. 2i).”

11) Line 319: The statement “the intense involvement of the meninges in JE progression (Fig. 2a–d)” would benefit from further elaboration.

12) Lines 443–464: Could the authors clarify whether they can differentiate direct virus-induced cell death from bystander cell death?

13) One of the most important findings appears to be the specific involvement of Ly6c$^+$ monocytes and Ackr1$^+$ endothelial cells in disease progression. It would be valuable to elaborate on whether this observation can be translated to human JE or other neuroinvasive orthoflaviviruses. Testing the functional importance of these cell types or their interactions would further strengthen the study.

14) Lines 465–490: This section is somewhat difficult to follow. Please revise for clarity.

15) Lines 491–570: The section describing virus–host interactions should explicitly state that these are “predicted” protein-protein interactions.

16) Lines 592–594: This reviewer feels that the use of terms such as “groundbreaking” and “unprecedented” is overstated. I recommend toning down this language here and throughout the manuscript to maintain a balanced and objective tone.

17) Line 595: The authors state, “Demonstrating JEV's dual neuroinvasion pathways while mapping region-specific viral tropism across distinct brain areas.” Could the authors clarify what is meant by “dual neuroinvasion pathways” and specify the nature of the “region-specific viral tropism”?
18) Figures (e.g., Fig. 1): Abbreviations for brain regions make interpretation difficult. Please spell out the names of brain regions in figures and throughout the manuscript.

19) Supplementary Fig. 2b: Please verify whether the y-axis labeling is correct.

20) The section “Establishment of a murine JE model” distracts from the main points of the paper. Consider moving this section to the Supporting Information.

Reviewer #2

(Remarks to the Author)
I am very satisfied with the revision, and all my comments have been addressed. Only two minor points that I would like the authors to change.

First, please add example FACS plots showing the gating strategy for the different cell populations mentioned in line 928-931, as supplemental figure.

Secondly, line 294 and line 668 both states that 60% of the living cells in the flow cytometry analysis is monocytes however, the panel Fig 2e shows 0.6% monocytes day 7.

Reviewer #3

(Remarks to the Author)
Many thanks to the authors for addressing my questions. The revised manuscript is certainly much improved and I am sure will be of interest to a broad readership.

Reviewer #1: Summary: Ou et al. utilized spatial transcriptomics to examine gene expression dynamics in mouse brains infected with JEV at 3, 5, and 7 d post-infection. By employing both a polyT probe for host transcripts and a JEV-specific probe targeting the NS5 region, the authors reported temporal and spatial mapping of viral and host gene expression. Their bioinformatics analyses revealed widespread viral dissemination across multiple brain regions, including the thalamus and cortex. Monocytes were identified as the initial targets of infection, followed by involvement of various cell types, including endothelial cells. The study described dynamic, time-dependent transcriptional changes, with immune-related gene upregulation particularly enriched in vascular endothelial cells. The authors propose that Ackr1⁺ endothelial cells facilitate immune cell recruitment and amplify inflammation, while Ccl12⁺ microglia are activated and expand in response to infection. They also reported increased necroptosis and pyroptosis in the meninges and neurons, contributing to tissue damage. Additionally, host factors potentially associated with JEV-induced neurological dysfunction were suggested. Overall, the authors propose a model in which monocytes and endothelial cells synergistically drive disease progression.

Comments:

1) Descriptive nature of the study: While the study employs a comprehensive suite of bioinformatics tools, the conclusions are based entirely on transcriptomic data. The functional roles of key cell types (e.g., Ackr1⁺ endothelial cells and Ccl12⁺ microglia) are inferred rather than experimentally validated. Functional assays are essential to confirm the biological relevance of these findings, particularly for publication in a high-impact journal.

Dear reviewer, thank you very much for this constructive feedback. In accordance with your and the other reviewers' recommendations, we have now performed a very comprehensive validation of the key sequencing findings using multiple experimental techniques, specifically including:

(1) validate the infiltration (Fig. 2e), JEV infection (Supplementary Fig. 2b-e), and IFN-γ secretion (Fig. 2f-k) of different types of immune cells in the mouse brain using flow cytometry;
(2) validate the IFN-γ secretion function of monocytes by conducting JEV infection experiment on cells isolated from human and mouse PBMCs (Fig. 2l-o);
(3) confirm meningeal inflammation using immunofluorescence assay (Fig. 2c, Supplementary Fig. 5a,b);
(4) confirm the activation of *Ackr1⁺* endothelial cells, the down-regulation of tight junction proteins, and the up-regulation of crucial immune cytokines using immunofluorescence assay (Fig. 3h,k,m, Supplementary Fig. 7b,c,e,f);
(5) confirm the dynamic alteration of blood-brain barrier permeability using Evans blue staining (Fig. 3l);
(6) confirm the activation of *Ccl12⁺* microglial cells using immunofluorescence assay (Fig. 4f);
(7) verify the necroptosis and pyroptosis of immune cells using immunofluorescent co-staining of specific biomarkers (Fig. 5c,f,i,l);
(8) confirm the pathological damage including neuron cell death, vasodilation with

inflammatory cells adhering to the vascular endothelial cells, myelin loss, and gliosis in brain using H&E staining (Supplementary Fig. 1f) and Luxol Fast Blue-Eosin staining (Supplementary Fig. 15e);

(9) validate the dynamic expression of viral genes, interferons and down-stream transduction genes, chemokine genes, cell death and other damage releated genes in JEV-infected mouse brains using RT-qPCR (Fig. 3n, Fig. 4d-e, Fig. 5d-j, Supplementary Fig. 4c, Supplementary Fig. 7g, Supplementary Fig. 15b-d).

The newly acquired data have been incorporated into the revised manuscript and are highlighted in red. For further details, please refer to the point-by-point responses addressing the comments from other reviewers. We sincerely thank you for your valuable feedback, which have significantly improved the reliability, coherence, and overall readability of our manuscript.

2) Novelty of the proposed model: The proposed model—emphasizing endothelial activation, blood-brain barrier disruption, monocyte infiltration, and immune-mediated cell death—aligns with existing paradigms in JEV pathogenesis. The manuscript would benefit from a more explicit discussion of how these findings refine or extend current models.

Dear reviewer, thank you very much for your constructive suggestions. While our findings align with existing knowledge at the macroscopic level, this study provides a spatially resolved qualitative and quantitative comparison of the cell populations involved in JE. We further identify central cellular players and their key interacting molecules, thereby offering a more precise and comprehensive dissection of the disease pathogenesis.

Regarding the pathogenesis of flavivirus encephalitis, the most critical questions include: 1. How does the virus enter the brain tissue? 2. Which brain regions and cell types are primarily infected by the virus in the brain tissue, and how do these infected cells die? 3. How do myeloid immune cells enter the brain tissue, and what role do they play in the pathogenesis of encephalitis? 4. After the massive death of neuronal cells, how does the brain tissue repair itself, and what is the mechanism leading to clinical manifestations when repair fails? Although research has been conducted on these key scientific questions, the current understanding remains limited due to differences in research methods and focuses. There is still a lack of a comprehensive analysis of the pathogenesis of flaviviral encephalitis.

In this study, we provide groundbreaking insights into JEV pathogenesis through integrated multi-omics analysis, revealing unprecedented spatial and cellular resolution of the brain microenvironment during infection. Our key findings include: 1) Demonstrating JEV's dual neuroinvasion pathways while mapping region-specific viral tropism across distinct brain areas; 2) Demonstrating that the monocytes are the major carrier of JEV and also serve as the primary source of IFN-γ within the brain parenchyma, challenging existing paradigms of neuroinflammatory responses. 3) Deciphering the complete gene regulatory networks underlying the activation and function of *Ackr1*$^+$ endothelial cell and microglia; 4) Identifying specific receptor-ligand pairs that mediate immune cell recruitment by *Ackr1*$^+$ endothelial cells, especially those between *Ackr1*$^+$ endothelial cells and *Ly6c*$^+$ monocyte; 5) Revealing pyroptosis and necroptosis as the dominant cell death modes, particularly concentrated in meningeal regions and potentially mediated by monocyte-derived signals;

6) Establishing the vasculature, particularly the meninges, as the organized inflammatory and cell death hubs within brain parenchyma; 7) Pinpointing a panel of injury-associated gene signatures as potential intervention targets for JE sequelae. These findings collectively provide a comprehensive spatial-cellular atlas of JEV pathogenesis, offering new paradigms for understanding and treating neurotropic viral infections. This content has been added to the first paragraph of Discussion (Lines 592-610).

Detailed discussion about these key findings were also presented in the remaining part of the Discussion (Lines 611-768).

3) Route of virus inoculation: The use of intraperitoneal inoculation raises concerns, as it does not mimic the natural route of JEV transmission via mosquito bite. The authors should justify this choice and discuss its implications for disease modeling and relevance to natural infection.

Dear reviewer, thank you for your insightful comment. We understand your concerns regarding the use of an intraperitoneal injection route in the murine model of JEV infection. Bharucha *et al*. have conducted a systematic review of currently available JEV mouse infection models reported in the literature. Their analysis identified eight different routes of viral administration, including intracerebral, subcutaneous, intranasal, conjunctival, intravenous, intramuscular, intraperitoneal, and intraperitoneal with sham intracerebral. Among these, the intraperitoneal route was employed in the overwhelming majority of studies. Notably, there is no significant difference in mortality among mice challenged via different routes of administration (Bharucha *et al*., PLoS Negl Trop Dis. 2022 PMID: 35143497).

The intraperitoneal route in fact presents several advantages: (1) It allows administration of a sufficient viral dose. Immunocompetent mice are generally not very susceptible to JEV, therefore a large inoculum is required to reliably establish infection and induce encephalitis; in practice, this is only feasible via the intraperitoneal route. (2) The peritoneal cavity contains abundant innate immune cells, such as peritoneal macrophages, monocytes, and dendritic cells, which are primary targets for JEV infection. The types of target cells are comparable to those at mosquito bite sites in skin in natural infection, thus enabling simulation of early viral infection, dissemination, and immune responses at the initial infection site. This feature cannot be replicated by intracerebral or intravenous inoculation. (3) The intraperitoneal route is minimally invasive for mice. (4) The procedure is technically convenient. However, a clear limitation of this route is that it differs from the natural transmission mode of JEV (i.e., skin inoculation by mosquito bite), which is an inherent drawback.

Intradermal inoculation is a route that more closely mimics natural JEV transmission. However, a major limitation of this method is that only a very small volume of virus inoculum can be introduced, leading to heterogeneous clinical presentations in mice and a low incidence of encephalitis, which is inadequate for the study of disease pathogenesis. In our preliminary experiments, we evaluated footpad intradermal injection as a route of JEV infection using the highest available dose (3×10^5 PFU per 60 μL per two hind footpads). However, this approach resulted in a very low level of viremia (Fig. R1A), a very small body weight loss (Fig. R1B), a low score of clinical severity (Fig. R1C), and a low incidence of

encephalitis (Fig. R1D) in mice, making it unsuitable for subsequent mechanistic studies of encephalitis.

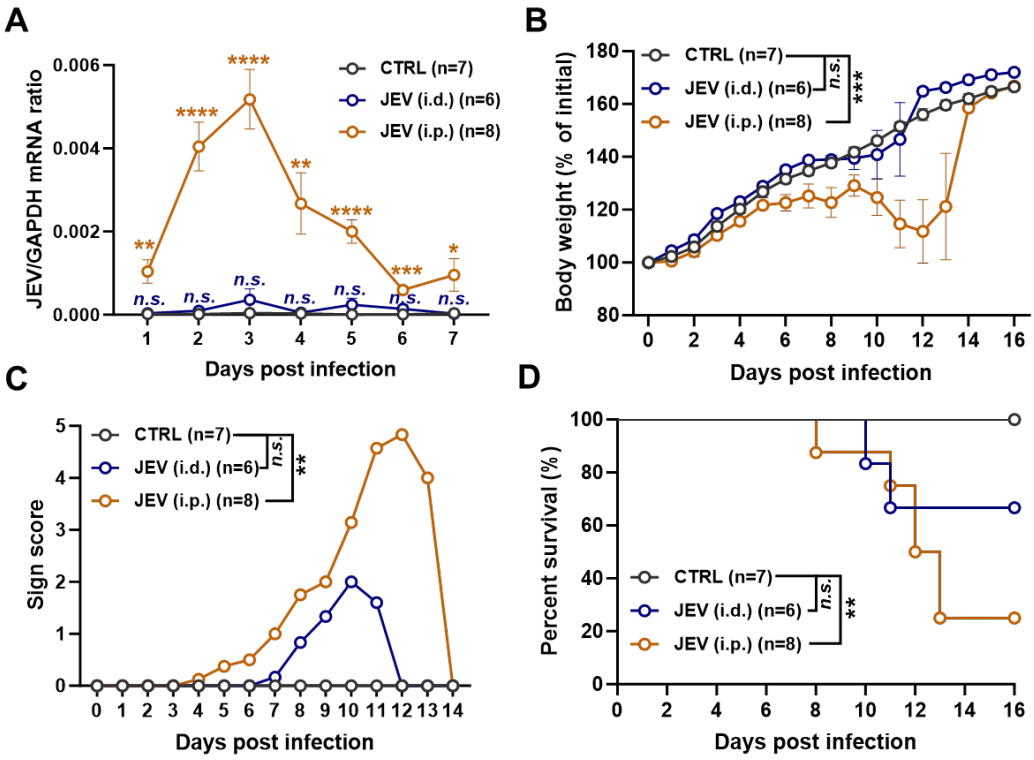

Figure R1. Viremia, body weight change, clinical sign score and survival of mice following JEV infection via different routes. Eighteen four-week-old female Balb/c mice were randomly assigned to three groups. The JEV (i.d.) group (n=6) received an intradermal injection of 3 × 10^5 PFU JEV per 60 μL per two hind footpads (the highest available dose via intradermal injection); the JEV (i.p.) group (n=8) received an intraperitoneal injection of 1 × 10^6 PFU JEV in 200 μL PBS; the control (CTRL) group (n=7) received an intraperitoneal injection of 200 μL PBS. (A) Blood viral loads of JEV-infected mice. (B) Body weight changes of JEV-infected mice. (C) Clinical sign scores of JEV-infected mice. (D) Survival curves of JEV-infected mice. *n.s.*, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

To better simulate the pathogenesis of JEV infection and encephalitis under natural conditions, we employed immunocompetent adult mice. In order to achieve consistent disease manifestations (viremia, weight loss, and encephalitis) following JEV challenge, it was necessary to administer a relatively high viral dose (1 × 10^6 PFU). The volume associated with this dose is substantial, limiting practical administration to the intraperitoneal route. Although i.p. injection has inherent limitations with regard to modeling the earliest events of peripheral tissue viral infection, it has minimal impact on studies of encephalitic pathogenesis. Our research specifically focuses on the cascade of infection, immunological, and cellular events within the brain following JEV infection, and in this context, the use of the intraperitoneal route represents an acceptable compromise. Mechanistic insights gained from this model may provide valuable references for understanding the pathogenesis of human JEV encephalitis in natural settings.

We have discussed the physiological relevance and limitations of our chosen JEV

infection model in the Discussion section of the manuscript (Lines 770–788).

4) Spatial resolution and validation: The spatial resolution of the transcriptomics platform may not be sufficient to resolve cell-type-specific expression in complex brain regions. Key spatial findings should be validated using other techniques such as in situ hybridization or immunohistochemistry.

Thank you for these valuable comments. We have validated the key findings from our spatial transcriptomic study using *in situ* immunofluorescence assay and other methods, and the results are consistent with our spatial transcriptomics analyses. These includes:

(1) validate the infiltration (Fig. 2e), JEV infection (Supplementary Fig. 2b-e), and IFN-γ secretion (Fig. 2f-k) of different types of immune cells in the mouse brain using flow cytometry;
(2) validate the IFN-γ secretion function of monocytes by conducting JEV infection experiment on cells isolated from human and mouse PBMCs (Fig. 2l-o);
(3) confirm the activation of *Ackr1*$^+$ endothelial cells, the down-regulation of tight junction proteins, and the up-regulation of crucial immune cytokines using immunofluorescence assay (Fig. 3h,k,m, Supplementary Fig. 7b,c,e,f);
(4) confirm the activation of *Ccl12*$^+$ microglial cells using immunofluorescence assay (Fig. 4f).

The detailed validation data have been incorporated into the Results section and are highlighted in red.

5) Sample size and reproducibility: The study includes only three mice per time point, which limits statistical power and raises concerns about reproducibility. Have the authors validated their findings in independent cohorts?

Dear reviewer, thank you for your insightful comment.

(1) In addition to the spatial transcriptomic data, we have performed an independent mouse experiment that includes immunostaining and flow cytometry validation and the results fully recapitulate the transcriptomic findings.
(2) Furthermore, the single-cell RNA-seq data analyzed in our study were obtained from a published dataset generated by an external group using a different JEV strain (GSE237915, PMID: 38532383); which confirm the presence of *Ccl12*$^+$ microglia (Lines 403-406, Supplementary Fig. 9c-e) identified by our spatial transcriptomics data.

Collectively, this evidence underscores the robustness and reproducibility of our conclusions.

6) Cell type annotation: Cell type identification based on transcriptomic signatures can be ambiguous, especially in infected tissues where gene expression is altered. Were annotations cross-validated using canonical markers?

Dear reviewer, we appreciate your attention to the potential ambiguity in transcriptomic signature-based identification. Following your suggestion, we have supplemented the analysis by examining the expression of canonical cell type marker genes, and these results are now included in Supplementary Fig. 2a. To further validate the cell annotation

by transcriptomic signatures, we have employed flow cytometry to detect the infiltrated immune cell types in brain at 3, 5, and 7 dpi, and the results are well consistent with the analyses of transcriptomic annotation, these results have been incorporated in Fig 2b-e and described in Lines 226-235 in the Result section. These supplementary analyses help validate the reliability of our cell type annotations. Thank you again for your insightful feedback, which has prompted us to strengthen the rigor of our results.

7) JEV probe and viral RNA detection: Lines 111–115: This reviewer is somewhat confused. JEV expresses only its genomic RNA as mRNA and does not produce multiple distinct mRNAs. Also, the limitations of spatial transcriptomics in quantifying viral load should be acknowledged.

Dear reviewer, thank you for raising this important point. While it is true that JEV expresses only a single genomic RNA that serves as mRNA and does not generate multiple distinct transcripts, we nevertheless examined whether the probe design exhibits any capture bias across different regions of the viral genome. When quantifying JEV expression, we assumed that each detected viral gene fragment corresponds to a single mRNA molecule. Although virus-specific probes were included in the chips, the total number of probes that can be anchored on each spot is limited and that the probe density in certain capture spots may be lower than the actual number of viral mRNAs present in the tissue. In addition, steric hindrance may prevent a virus-targeted probe from hybridizing to its cognate mRNA. Consequently, our spatial transcriptomic data provide a qualitative map of JEV distribution rather than an absolute quantification of viral load within each region. We apologize that our original description caused confusion. We have now revised the relevant text for clarity. (Lines 174-179, 183-189).

8) Translational relevance: Findings from a mouse model may not fully translate to human JEV infection due to species-specific differences in immune responses and brain architecture. Are any human data or comparative analyses included to support translational relevance?

Dear reviewer, thank you for your insightful comment. We fully acknowledge the limitations of translating findings from mouse models to human JEV infection due to species-specific differences in immune responses and brain architecture. While we were unable to access clinical data from the limited human JEV cases in China for direct validation, we conducted experiments using human peripheral blood mononuclear cells (PBMCs) to validate the IFN-γ secretion function of monocytes following JEV infection. This approach provides a bridge between our mouse model and human immune responses, partially supporting the translational relevance of our findings.

Actually, mice are a well-characterized and widely accepted animal model for JEV research, studying viral infection and disease pathogenesis, as well as evaluating the safety and efficacy of potential medical countermeasures at a preclinical stage of development. The mouse model has many advantages over other animal models (PMID: 37242385). By integrating our findings with relevant literature, we compared the clinical manifestations and neuropathological changes observed in mice infected with JEV to those seen in humans, in order to better elucidate the translational relevance of our study.

Clinical manifestations: Both mice and humans infected with JEV exhibit symptoms such as fever, reduced appetite, body weight loss, and decreased activity. When encephalitis occurs, both may present with altered consciousness, coma, limb paralysis, and seizures, hallmarks of central nervous system infection, that can ultimately lead to death in severe cases (PMID: 29697099; PMID: 37242385). Regarding viremia, immunocompetent mice and humans infected with regular doses of JEV both develop low levels of viremia (PMID: 37242385). In terms of disease outcome, some human JE patients recover with intensive treatment, but approximately half are left with severe sequelae, including permanent paralysis, cognitive impairment, and neuropsychiatric disorders (PMID: 37242385). In mouse models, however, the natural course of JE is typically observed without intervention, and mice that develop encephalitis almost invariably die, with little chance of spontaneous recovery.

Neuropathological changes: The clinical manifestations of encephalitis are directly related to the pathological changes in brain tissue. In mouse models of JE, the affected cell types, regional distribution of infection, neuronal death, glial proliferation, blood-brain barrier disruption, and inflammatory infiltration closely resemble those observed in human JE pathology (PMID: 37242385; PMID: 29697099; PMID: 32611752; PMID: 33534086; PMID: 33724154). Therefore, the mouse model serves as a robust platform for studying the pathogenesis of JE as well as for evaluating vaccines and antiviral therapies.

In summary, the mouse model of JEV infection effectively recapitulates key aspects of human disease, including neuropathology, viremia, and clinical symptoms. Consequently, the pathogenic mechanisms identified in our mouse model have translational potential, though extrapolation to humans should be approached with caution and supported by additional evidence-based studies. We have included this discussion in the revised manuscript (Lines 770-788).

9) Taxonomic nomenclature: Line 90: The manuscript should reflect the updated taxonomy, noting that JEV is now classified under the Orthoflavivirus genus, in accordance with current nomenclature recommendations.

Dear reviewer, thank you for pointing our mistake. We have corrected the term (Line 112).

Reviewer #2 (Remarks to the Author): In the paper," Spatial transcriptomics elucidates the central role of cerebral vasculatures in the progression of Japanese encephalitis" Ou et al investigate the temporal dynamics of the immune response in the brain following peripheral JEV infection in a mouse model using spatial transcriptomics. The basic experimental setup is sound, including appropriate controls, the different time points and three biological replicates per time point. They detect virus infection in monocytes the brain at 3 days post i.p. inoculation and the virus then spread to other cell types during day 5 and 7. The results show the dynamics and transient nature of interferon and immune responses in the brain, highlights the importance of the meninges and identifies several infiltrating/activated cell populations that likely play an important role in driving immunopathology during JEV infection. This study is well performed and adds significantly to the current knowledge about flavivirus infection in the CNS of mice. While the experimental setup and analysis is

sound, the study is missing some complementary experiments to validate key observations to further strengthen the conclusions. For example, fluorescence imaging of brain slices or FACS analysis of infiltrating immune cells. These analyses are important to verify the transcriptional findings as RNA and protein levels are not always comparable. Additionally, if the study was better put into the context of similar studies for other neurotropic viruses this would add meaning to the results and facilitate for the reader to understand the importance of the results presented.

Major: - Please add clinical characteristics (weight loss/symptoms) for the model used (mouse strain, inoculation route and infectious dose) since now it is difficult to correlate how the observations correlate with disease progression, especially the apparent reduction in inflammatory response seen between day 5 and day 7.

Dear reviewer, thank you for this valuable suggestion. We have added clinical data for the mouse experiment. (Lines 120-160, Supplementary Fig. 1a-f)

- Complementary experiments validating some of the key observations are necessary.
o Please validate the immune cell infiltration in the brain Fig 2e with FACS analysis.

Dear reviewer, thank you for this valuable suggestion. We have validated immune cell infiltration in the brain at 3, 5, and 7 dpi. Consistent with the results of the spatial transcriptomic analysis, we observed a marked increase in the proportions of monocytes, NK cells, T cells, and macrophages in the brains of JEV-infected mice compared to controls. The proportions of these immune cell populations increased since 3 dpi and peaked at 7 dpi or 5 dpi. The flow cytometry data robustly corroborate the findings of the spatiotemporal transcriptomics. The results of these validation experiments have been incorporated into Fig. 2e-k and Supplementary Fig. 2b-e and are described in Lines 226-235 and Lines 292-306 of the revised manuscript.

o Please show the Necroptosis and Pyroptosis in brain slices with immunofluorescence imaging.

Dear reviewer, thank you for this helpful suggestion. Mlkl (mixed lineage kinase domain-like) is a key protein involved in the execution of necroptosis. During necroptosis, Mlkl is phosphorylated by activated Ripk3, which induces conformational changes in Mlkl. The phosphorylated Mlkl (p-Mlkl) then translocates to the plasma membrane, where it forms pores, leading to the leakage of cellular contents and ultimately triggering necroptotic cell death. Therefore, p-MLKL is considered a reliable marker for the detection of necroptosis. Gsdmd (Gasdermin D) is an essential effector protein in the execution of pyroptosis, and consists of two major functional domains: an N-terminal domain responsible for pore formation and a C-terminal domain that acts as an auto-inhibitory region under physiological conditions. Upon cleavage by specific proteases, such as caspase-3, the N-terminal domain of Gsdmd is released and translocates to the cell membrane, where it forms pores, resulting in cell membrane rupture and the release of cellular contents, thereby mediating pyroptotic cell death. Thus, the N-terminal fragment of Gsdmd serves as an ideal marker for detecting pyroptosis using immunofluorescence staining.

Accordingly, we employed immunofluorescence staining to assess the expression

levels of p-MLKL and Gsdmd-N in brain tissue sections from both control and JEV-infected mice, in order to evaluate necroptosis and pyroptosis, respectively. Elevated expression of p-MLKL was observed primarily in the meninges of JEV-infected mice compared to controls. While Gsdmd-N showed stronger signals than p-MLKL in multiple brain regions. At 3 dpi, pyroptotic signals first appeared in the meningeal tissues and some areas of the cerebral cortex. By 5 and 7 dpi, pronounced pyroptotic signals were observed in the meninges, cerebral cortex, olfactory bulb, hippocampus, cerebral nuclei, midbrain, and hindbrain, with the strongest signals detected in the meninges, cerebral cortex, and olfactory bulb. These findings further demonstrate that JEV infection can induce necroptosis and pyroptosis in brain cells, particularly those located in the meninges. These results have been incorporated in Fig. 5c,f,i,l and descried in Lines 430-440.

o Verify the high infection rate of the immune cells by FACS analysis.

Dear reviewer, thank you for this important suggestion. We have verified the high infection rates of immune cells in the brain by flow cytometry. The results showed that, at different time points following JEV infection, a proportion of NK cells, T cells, monocytes, and macrophages in brain tissue were infected with JEV. The median infection rates of these immune cell types detected by flow cytometry were consistent with those obtained from spatiotemporal transcriptomic analysis. When considering the percentage of infected cells within each immune cell type, NK cells and macrophages exhibited the highest proportions of JEV infection among infiltrating immune cells in the brain. In contrast, when assessing the proportion of infected immune cells relative to the total brain cell population, which better reflected the overall contribution of each immune cell type, monocytes were the predominant JEV-infected immune cells in the brain at both days 3 and 5 post-infection, which is consistent with transcriptomic findings. In summary, our flow cytometric validation confirms that monocytes are the key immune cell type with JEV infection in the brain. These results have been incorporated in Supplementary Fig. 2b-e and descried in Lines 226-235.

o Quantify the IFN response with Q-PCR over time to see that the slices are representing the brain.

Dear reviewer, thank you for this valuable suggestion. We have assessed the mRNA levels of Toll-like receptor (TLR) genes including *Tlr2, Tlr3, Tlr7*, and *Tlr8*, interferon genes including *Ifn-α*, *Ifn-β*, and *Ifn-γ*, as well as downstream targets of interferons such as *Ifitim3*, *Isg15*, *Irf7*, and chemokines including *Cxcl10*, *Ccl2,* and *Ccl5* using RT-qPCR. Consistent with the results of the spatial transcriptomic analysis, the expression of TLRs, interferons, their downstream target genes (*Ifitim3*, *Isg15*, *Irf7*), and chemokines (*Cxcl10*, *Ccl2*, *Ccl5*) was markedly upregulated in the brains of JEV-infected mice at day 3 post-infection, peaking at day 5 and/or day 7. These qPCR validation results further demonstrate that JEV infection in the brain robustly stimulates immunoinflammatory responses. The relevant results have been incorporated into Supplementary Fig. 4c, with the corresponding description provided in Lines 261-262.

o Verify the localization of tight junction proteins, cell adhesion molecules and chemokine receptors over time using fluorescent imaging of brain slices.

Dear reviewer, we have examined the spatial and temporal expression patterns of two key tight junction proteins (Claudin-5 and Zo-1, also known as Tjp-1), two essential cell adhesion molecules (Icam1 and Vcam1), and three important chemokines (Cxcl9, Cxcl10, and Ccl2) in the brain using *in situ* immunofluorescent staining and qRT-PCR. Consistent with the results obtained from spatiotemporal transcriptomics analysis, Claudin-5 and Tjp-1 were robustly expressed in vascular endothelial cells in the brain of control (CTRL) mice. In contrast, their expression was markedly reduced in the brain of JEV-infected mice at days 5 and 7 post-infection, suggesting disruption of tight junction integrity and, consequently, impairment of the blood–brain barrier during JEV infection.

Similarly, the expression of the adhesion molecules Icam-1 and Vcam-1 was primarily localized to Ackr1$^+$ endothelial cells in the meninges (as indicated by Ackr1 staining). In JEV-infected mice, their expression was significantly upregulated at day 3, peaked at day 5, and remained elevated but slightly decreased by day 7 compared to controls. The mRNA levels of Icam-1 and Vcam-1 measured by RT-qPCR were well consistent with the immunofluorescent staining results. These findings mirror those from the spatiotemporal transcriptomic analysis and indicate a pivotal role for brain Ackr1$^+$ endothelial cells in orchestrating immune cell recruitment during JEV infection.

Regarding chemokines, the expression of Cxcl9, Cxcl10, and Ccl2 was predominantly observed in meninges of JEV-infected mice. All these chemokines were markedly induced beginning at day 3, with peak expression at day 5 and a slight reduction by day 7, yet levels remained elevated compared to controls. The mRNA levels of Cxcl9, Cxcl10, and Ccl2 measured by RT-qPCR were well consistent with the immunofluorescent staining results. These results closely match the transcriptomics data and demonstrate that JEV infection elicits pronounced chemokine expression in the brain.

Collectively, these immunofluorescent and RT-qPCR validation experiments corroborate the spatiotemporal transcriptomics findings and provide further evidence for dynamic alterations in tight junction integrity, adhesion molecule expression, and chemokine-mediated chemotaxis in the brain during JEV infection. The relevant results have been incorporated into Fig. 3h,k,m,n and Supplementary Fig. 7b,c,f,g with the corresponding description provided in Lines 343-346, 355-361.

- On line 227 authors write that disruption of the BBB is crucial for immune cell infiltration and virus invasion. However, there is no data supporting this claim although RNA data indicate it. Please investigate the BBB integrity with fluorescent dextran or Evans blue 3, 4, 5, and 6 days post infection in combination with FACS analysis of infiltrating immune cells and viral load with q-PCR.

Dear reviewer, thank you for this valuable suggestion. We have now assessed blood–brain barrier (BBB) permeability in control mice and JEV-infected mice at days 1, 3, 5, and 7 post infection by using a widely used Evans blue assay. We observed a significant increase in BBB permeability beginning at 5 dpi, which was further elevated at 7 dpi in JEV-infected mice compared to CTRL mice (Fig. 3i, LInes 356-359). These findings are in strong concordance with the results obtained from our spatial transcriptomics analysis.

Additionally, we have validated the infiltration of immune cells into the brain following JEV infection at 3, 5, and 7 dpi by flow cytometry. In agreement with our transcriptomics

data, there was a significant increase in the proportions of monocytes, NK cells, T cells, and macrophages in the brains of JEV-infected mice compared to controls (Fig. 2e, Lines 292-296). Notably, these immune cell populations increased from 3 dpi and peaked at 5 dpi or 7 dpi, paralleling the observed trend in BBB permeability. The percentage of JEV infection rate for different immune cell types were also determined by flow cytometry (Lines 226-235, Supplementary Fig. 2b-e)

We also quantified JEV viral load in the brains of infected mice using RT-qPCR at 3, 5, and 7 dpi (Supplementary Fig. 1b, Lines 133-134). The results demonstrated that JEV was detectable in the brain of JEV-infected animals since 3 dpi, with viral loads further increasing at 5 dpi and 7 dpi in a manner that correlated closely with the observed changes in BBB permeability.

Collectively, these experimental validations further substantiate the findings from our spatiotemporal transcriptomics analysis, highlighting JEV-induced BBB disruption, subsequent infiltration of inflammatory cells, and progressive viral invasion of the brain.

- The study and results need to be better put into the larger context of neurotropic virus infection both in the introduction and the discussion. For example, the virus distribution and cellular tropism (Fig 1c-d), the role/importance of inflammatory response in the meninges and the specific cell populations highlighted to potentially be driving the inflammatory response. There are several reports that the choroid plexus is highly responsive to virus infection by upregulating type I IFNs.

Dear Reviewer, thank you very much for your constructive suggestions. We have thoroughly revised both the Introduction and Discussion to sharpen the rationale, clarify the knowledge gap, and better integrate our findings with the broader context of neurotropic virus infection. The specific changes are highlighted in red in the revised manuscript.

In the introduction, we introduced more details about the infection and immune reponse process of JEV (Lines 73-88), quoted as follows:

> "JEV initiates infection following a skin bite, where it infects susceptible cells including dendritic cells, macrophages and monocytes in the bloodstream and establishes a viremia[9–12]. This enables the virus to disseminate to the brain via circulation, ultimately causing encephalitis. JEV can infect many brain parenchymal regions such as thalamus, brainstem, and hippocampus[13,14]. The replication of JEV in neurons can lead to direct neuronal death, while activation of microglia/glial cells and infiltrating inflammatory monocytes lead to indirect killing of neurons via pro-inflammatory cytokines such as IL-6 and TNF-α[15,16]. Studies have identified monocytes as an essential cell type contributing to JE neuropathology[17–19]. Inflammatory Ly6c^{hi} monocytes have paradoxical roles in viral encephalitis. On one hand, monocytes could differentiate into effector cells and produce reactive oxygen species (ROS) which repress and clear the virus. On the other hand, unbalanced and uncontrolled migration and infiltration of monocytes will lead to tissue damage and destruction20. In this process, the meninges and choroid plexus play critical roles in the central nervous system immune response, including the secretion of cytokines such as Cxcl10 and Tnf-α[21–24]. "

In Lines 94-106, We also put in the context of neutropic viruses to state the novelty of our technology platform:

"Recent investigations into the pathogenesis of neurotropic viruses has been advanced by single-cell RNA sequencing (scRNA-seq), which elucidates the immune functions of microglia, monocytes, and other immune cells in the cerebrospinal fluid and brain tissues[16,25–27]. However, scRNA-seq requires tissue dissociation, a process that can lead to the loss of low-viability cells and poses significant challenges for the effective isolation of neurons. Consequently, this approach may fail to provide a complete picture of brain pathology. In contrast, spatial transcriptomics can capture transcripts in situ within intact tissue architectures. This technology can therefore elucidate the regional tropism of viral infection, the spatial infiltration patterns of immune cells, the extent of infiltration by various immune subsets, the degree of tissue damage and the neighborhood interactions between different cell types, providing high-resolution molecular evidence for deciphering the pathogenic mechanisms of neuroinvasive viruses[28]. "

In Discussion (Lines 790-807), we further emphasize the transferable potential of our technical framework:

"By leveraging high-resolution spatial transcriptomics complemented by confirmatory assays, we have delineated the alterations in the abundance and physiological states of diverse cell populations within distinct brain regions following JEV infection. This approach has allowed us to unravel, at an unprecedented resolution, the intricate interactions between endothelial cells and immune cells, particularly monocytes, which are central to the ensuing neuroinflammatory cascades. The pathology induced by JEV is shared by other neurotropic viruses. A key gap in the field lies in quantifying the specific contributions of various brain-infiltrating or resident cells and defining the principal cellular communication pathways that orchestrate neuropathology in situ. For example, it has long been known that microglia and monocytes are essential in the neuropathology of multiple neurotropic viruses such as JEV, West Nile virus (WNV), tick-borne encephalitis virus (TBEV), and herpes simplex virus (HSV)[25,59,93,94]. However, with the high detection throughput and spatial resolution power of spatial technologies, the interactive network of monocytes can be more thoroughly dissected, as demonstrated for JEV. The spatial transcriptomics workflow established herein is directly applicable to closing the knowledge gap for a spectrum of neurotropic viral infections, offering a new paradigm for elucidating their pathogenesis."

Minor:

Row 66-67: "Without 67 timely treatment, the condition progresses to encephalitis…", makes it sound like treatment can prevent encephalitis, rephrase, or clarify which treatment with reference.

Dear Reviewer, thank you very much for your comment. We have rephrased the sentence as "After infection, the condition can progress to encephalitis, characterized by severe fever, neck stiffness, and seizures." (Lines 65-66)

Row 92-97: Mention the model system used (mice).

Dear Reviewer, thank you very much for your comment. We have now added a description of the animal model in this paragraph: "In this study, we construct a BALB/c mouse infection model and design Stereo-seq chips with JEV probes that ..."(Lines 107). We've also added detailed description of the clinical symptoms observed in the infected mice (Lines 120-160).

Row 116-117: Add rout of infection in the main text line, for clarity.

Dear Reviewer, thank you for your comment. We have now added a description for the infection route: "Mice in the JEV infection group were intraperitoneally injected with 10^6 PFU of the JEV Beijing-1 strain per animal, while mice in the control group received an equal volume of PBS." (Line 125)

Row 123-125: Mention if any cells were positive for JEV in the control as QC/validation of the method/threshold selected.

Dear Reviewer, thank you very much for your comment. We have now added a description for the control group: "The spatial transcriptomics data showed that the JEV genes were expressed in approximately 0.04%, 3.49%, and 4.25% area of the brain tissues on 3, 5, and 7 dpi respectively, reaching a peak on 7 dpi; while no virus positive bins were identified in the control samples." (Lines 195-196)

Row 191-194: Add statistical test and indicators of significance in the figure (Fig 2e) to support this statement.

Thank you for your valuable suggestion. We have performed statistical tests for the data in Fig 2e (now Extended Data Fig. 5c). However, as the differences did not reach statistical significance, we did not mark significance indicators in the figure. Following your comment, we have supplemented the corresponding figure legend with a description of this information to ensure transparency.

Row 194-196: "We also observed increased signals of endothelial cells (Fig. 2e), 195 which was probably due to vascular dilation caused by immune infiltration or tissue 196 edema, instead of cell proliferation" – elaborate what this statement is based on.

Dear Reviewer, we observed an expansion of bins containing endothelial-cell-specific signals, indicating a larger spatial distribution of the corresponding RNAs. Coupled with the subsequent loss of tight junction and increase in endothelial permeability (Fig. 3k,l, Supplementary Fig. 7e), we interpret this change because of vasodilation rather than an increase in endothelial cell number.

Row 199-200: "JE progression" indicates worsened disease, requires clinical scoring or similar for the mice, else rephrase to immune response.

Dear Reviewer, based on our daily health monitoring (Supplementary Fig. 1c-e), the majority of infected mice succumbed by day 10 post-infection; we therefore believe that the term "JE progression" accurately reflects the course of disease in our model.

Row 210-213: Cell population mentioned as significant in the text differs from those labeled as significant in the figure (Fig 3c)

Dear Reviewer, thank you for pointing out this mistake. We have carefully revised the relevant text (Lines 331-333) to ensure full consistency between the figure and the description.

Row 220-222: Strengthen some of these observation with e.g. IF (Fig 3c).
【Spatially, we observed strong co-localization signals for ligand-receptor pairs including Ccl7-Ccr2, Ccl2-Ccr2, Ccl5-Ccr1, Ccl2-Ccr5 and Cxcl9/Cxcl10/Ccl7/Ccl5/Ccl2-Ackr1 in the meningeal region on 5 and 7 dpi】
Dear Reviewer, thank you for this valuable suggestion. We have now performed the requested immunofluorescence validation; the data are presented in Fig. 3h and Supplementary Fig. 7b,c, described in Lines 343-347.

Row 287-288: "Only necroptosis and pyroptosis were found to be associated with JEV infection (Fig. 5a-d, Extended Data Fig. 6a-d)", unclear which figure is showing the association only with necroptosis and pyroptosis. Method/statistical test to measure association?
Dear Reviewer, thank you for this insightful comment. We applied GSVA to score death-pathway gene sets in every brain region of each individual sample. Because the comparison is across regions/cell types within single specimens, which entails thousands of bins, resulting in ubiquitously small p-values in standard statistical tests that we considered unreliable; consequently, no p-values are displayed. Across repeated analyses, only two cell-death pathways produced robust RNA signatures whose marker genes were also visibly expressed. These signatures were subsequently validated by immunofluorescence and qPCR. We apologize for the redundancy in our original presentation. We have now removed the original Extended Data Fig. 6 and modified the text to avoid confusion (Lines 421-422).

Row 299-300: Mentions different regions to be highly infected than previously (row 131), hindbrain instead of cerebral cortex.
Dear Reviewer, thank you very much for your meticulous comparison and valuable feedback. We did analyze the cortex in this section; the description was just omitted in the original text. We have now revised the sentence to "We further focused on the brain regions with a high viral load including the thalamus, cerebral cortex, cerebral nuclei, and hindbrain, to investigate the impact of viral infection on the death of neurons in these regions (Stereo-seq bin35 data)." (Line 444-445)

Row 369-383: Difficult section with no clear message/result.
Dear Reviewer, thank you for your comment. Because the Protein-Protein Interaction (PPI) network was constructed from genomic data, we wished to test–at the transcriptome level–whether the predicted interactions are accompanied by measurable changes in gene expression. We therefore tested two potential strategies:

1) For genes directly implicated in virus-host interactions, we asked whether their transcripts are significantly up- or down-regulated. Despite integrating single-cell and spatial transcriptomes with the PPI data, no statistically significant shifts were detected;

this negative result is stated in Lines 520-534.

2) For genes that act indirectly in virus-host interactions, we examined the expression of their downstream targets. In the following paragraph we show that these PPI-associated regulons are indeed altered, and that the affected genes are enriched for immune-cell chemotaxis and neurological-damage pathways. We have now added explanatory sentences to this paragraph to clarify the rationale and outcome of each analysis, ensuring that readers can readily follow our logic (Lines 515-520).

Row 409- 434: Can this section be shortened and more result-focused, briefly describing how the results support the suggested model.

Dear Reviewer, thank you very much for your comment. We have revised and reorganized the content according to your suggestions (Lines 570-587) and quoated as follows:

"*Ly6c*$^+$ monocytes and *Ackr1*$^+$ endothelial cells synergize to fuel JE. Our data suggest a central role of JEV-infected Ly6c+ monocytes in spreading the virus to the CNS and initiating encephalitis (Fig. 7e). In addition to being the primary viral carriers into the CNS, these cells represent the predominant immune population in the infected brain, where they secrete abundant inflammatory mediators like IFN-γ, orchestrating neuroinflammation and modulating cell deaths, particularly pyroptosis. The vascular network, including the meninges and choroid plexus, is distributed throughout the brain, where it normally shields neural tissue and delivers nutrients. Upon infection, this same network is converted into a sensor and amplifier of immune signals: *Ackr1*$^+$ endothelial cells become inflamed, BBB permeability increases, and both virus and immune mediators disseminate more freely. Intense immune cell infiltration and cell damage follows; once vessels are injured, nutrient transport is impaired and post-acute repair is dampened, culminating in severe neurological deficits and death. During JE development, the interplay between *Ly6c*$^+$ monocytes and *Ackr1*$^+$ endothelial cells plays a dual role, mediating host defense against viral infection while simultaneously driving tissue destruction. The resolution of inflammation targeting these two cell types within the vascular system may be essential in mitigating JE progression."

Fig 1a: Reduce repetition of methodology.

Dear Reviewer, thank you very much for your constructive feedback. We have revised the descriptions of the figure legend.

Fig 1b: consider including a sketch of a brain slice to highlight some of the main brain regions mentioned in the text to guide the reader. Can the slices be rotated/aligned in a way to make the data more accessible?

Dear Reviewer, thank you for your valuable suggestion. We have revised the figure as recommended:

- A schematic sketch of the brain slice has been added to highlight the key brain regions mentioned in the text (Fig. 1a), which we hope will help guide readers in contextualizing the data.
- The brain slices have been rotated and aligned uniformly to ensure consistency

across samples (Fig. 1b), making the spatial distribution of the data more intuitive and accessible.

Fig 2a-b: Why is the pathway analysis for upregulated genes divided by timepoint (2a) but downregulated genes all time points are grouped together (2b)?

Dear Reviewer, thank you very much for your insightful comment. We appreciate your careful attention to the details of our pathway analysis. Regarding your question about the different grouping strategies for upregulated and downregulated genes: the reason for this approach is that the number of downregulated genes in the D3 and D5 groups compared to the control group is relatively small (only 2 and 6 genes, respectively). Given the limited number of downregulated genes at these early time points, we combined the downregulated differential genes from D3, D5, and D7 (relative to the control group) into a union set for pathway enrichment analysis. This approach helps to ensure the robustness and reliability of the enrichment results by aggregating sufficient gene numbers, which would be challenging with the small individual datasets at D3 and D5. In contrast, the upregulated genes at each time point (D3, D5, D7) were sufficiently abundant to allow meaningful timepoint-specific pathway analysis, so we presented them separately.

Fig 2c: Important figure that is very difficult to read/interpret. Consider making the scale the same across all conditions for one gene and have the legend only once. Make text/legends larger/readable. Highlight in the figure legend that the coloring represent the brain parts as described in Fig1b.

Dear Reviewer, thank you for your constructive feedback on Fig 2c (now Fig 2a). We have implemented the following revisions: For each gene, the scale has been standardized across all conditions to facilitate direct comparison. The scale bar, text, and legend fonts have been enlarged to ensure better readability. For the brain region annotation, we have explicitly made sure that the coloring corresponds to the brain regions as described in Fig 1b, providing clearer contextual guidance.

Fig3f: Avoid implying results in the figure legend. Why is only one sample chosen here, and additionally a sample with a much higher intensity flow for the CCL pathway than the other two samples from that time point (D7_1, Fig 2d). Would it not be better to pool all samples from d5 and/or d7 or make an average?

Dear Reviewer, thank you for your insightful comments. Regarding the selection of a single D7 sample, this was due to space constraints, and the sample was chosen randomly. We have supplemented the figure with the average cell communication patterns of the CCL pathway across all samples in the D5 to provide a more comprehensive view of the pathway activity at this time point (Fig. 3f). We hope these revisions address your concerns and enhance the robustness of the presentation.

Fig 7c: Change the colouring, the yellow letters on white background is very hard to see.

Dear Reviewer, thank you for your feedback. As suggested, we have modified the coloring scheme to improve visibility—specifically, we have adjusted the text color into black to ensure better contrast with the background for Fig. 7c.

Figures general: Ensure all text is readable and improve the style formatting of headings/legends, e.g. change "_" for spaces, capitalize the first letter etc.

Dear Reviewer, thank you for your suggestion regarding the general formatting of figures. We have carefully revised all figures to ensure readability and consistency in style: All text within figures (including labels, headings, and legends) has been adjusted to ensure clarity and legibility. Formatting of headings and legends has been standardized: underscores ("_") have been replaced with spaces, and the first letter of each heading/legend entry is now capitalized.

Reviewer #3 (Remarks to the Author): The manuscript presented by Zhihua Ou and colleagues presents for the first time a spatial atlas of the neuropathology caused by the Japanese Encephalitis Virus. Firstly, I would like to commend the authors for providing such as concise and well-presented manuscript, with some novel insights derived from their dataset. I would also like to apologise for the time that took me to review this manuscript (personal circumstances precluded me from doing this sooner). This is an incredible resource for the scientific community, as it provides novel insights about the expression pattern of candidate genes of interest that could be the focus of much needed therapeutic applications. The comments below are geared towards increasing the impact of the findings presented here, and I hope the authors find it useful. My major recommendations are to include independent validation of potential key findings (e.g., immune infiltrates, changes in vascular permeability, gliosis, demyelination) to increase the impact of the manuscript. These are listed in my questions below:

Line 105: How was the 1:10 ratio chosen to create the virus-compatible Stereo-seq chip?

Dear Reviewer, given the high cost of the chip-based assay, we have evaluated the sensitivity of virus-specific primers (VSP), oligo-dT, and random hexamers for JEV detection under solution-phase conditions. When VSP and oligo-dT were mixed at a ratio of 1:10, the sensitivity for viral RNA exceeded that achieved with random hexamers or oligo-dT alone. When diluted to a 1:100 ratio, viral detection was poorer than with random hexamers. At both ratios, the threshold cycle for the housekeeping gene *Gapdh* remained unchanged. We therefore adopted the 1:10 VSP-to-PolyT ratio to maximize viral detection sensitivity. We have now incorporated the explanation into the revised text (Lines 168-170).

Line 130: Does the accumulation of viral transcripts in these brain regions correlate with what is known in the field? In other words, can these observations be used to benchmark the newly developed platform?

Dear Reviewer, thank you very much for this valuable comment. The temporal kinetics of JEV infection observed in the mouse brain were validated by qPCR (Supplementary Fig. 1b) and the spatial distribution patterns were consistent with previous reports. We have now added the corresponding references to substantiate the reliability of our findings (Line 205).

Figure 1d: Is it not surprising to find adipocyte signatures in the brain? Can these cells be

mesenchymal progenitors wrongly annotated as such? What are the markers genes used to identify the cell types in this figure? As far as I know there are not adipocytes in the brain parenchyma.

Dear Reviewer, thank you very much for your insightful questions. After carefully checking and verifying the expression of cell marker genes in the SingleR reference set, the expression of adipocyte marker genes in the Stereo-seq data, and the annotated quantity of adipocytes in each sample, we found that the Stereo-seq data barely express adipocyte marker genes (*Adipoq*, *Lep*, *Plin1*). Instead, there is a certain overlap in fibroblast marker genes between the SingleR reference set and the Stereo-seq data. Therefore, we believe the reason may be that due to signal contamination in the Stereo-seq data, coupled with the presence of adipocyte signals in the SingleR reference set itself, some spatial bins were annotated as adipocytes. We found that the number of adipocytes in 10 out of 12 samples is less than 1000 bins, with the overall number of adipocytes ranging from 53 to 2060. Given the low proportion of adipocytes and that they are not associated with our study focus here, we have reclassified adipocytes as "Other cells" to avoid causing confusion (Supplementary Fig. 2a). We sincerely appreciate your rigorous feedback, which has significantly improved the reliability of our results.

Figure 2a: The gene signatures observed here are quite consistent with potential adaptive immune cell infiltrates into the brain parenchyma, which is not surprising due to the viral infection going on. Is this known in JEV infection? If not, I think a validation of these findings by flow cytometry and/or IFA would be advantageous and would certainly elevate the paper. In this regard, it would be interesting to determine whether: i) the dynamics of T and NK cell accumulation in the brain parenchyma (as shown in Figure 2e), and ii) which of these subsets produce IFNg?

Dear Reviewer, thank you for this constructive suggestion. The traditional view holds that the brain is an immune-privileged site, mainly because it possesses highly sophisticated barrier systems, the most important of which is the blood-brain barrier (BBB). The BBB restricts certain factors in the blood from entering the brain parenchyma, thereby maintaining brain cells within a relatively stable environment. Evidently, this perspective considers the brain tissue as not being an immunologically active site (PMID: 28092374; PMID: 30310148). However, an increasing number of studies have demonstrated that the brain is not as immune-privileged as traditionally believed. On the contrary, during the course of encephalitis, numerous immune cells infiltrate the brain tissue to carry out cytotoxic and clearance functions, indicating that under pathological conditions, the brain is indeed an immunologically active site. In fact, our previous studies have shown that, during JEV infection, the integrity of the blood-brain barrier is compromised, facilitating the infiltration of various immune cells, such as $CD4^+$ T cells and $CD8^+$ T cells, into the brain (PMID: 32611752; PMID: 33534086). While these immune cells play a role in eliminating the virus and virus-infected cells, they may also exacerbate inflammation in the brain, representing a double-edged sword.

Our spatiotemporal transcriptomic studies have shown that, upon JEV infection, brain microvascular endothelial cells upregulate the expression of various adhesion molecules to promote immune cell adhesion, while simultaneously downregulating tight junction

proteins between endothelial cells, thereby increasing BBB permeability and further promoting the entry of immune cells from the blood into the brain parenchyma. Many of the immune cells that infiltrate the brain parenchyma can themselves become infected by the virus, bringing the virus into the brain in a "Trojan horse" manner. These immune cells, in the process of eliminating the virus and virus-infected neural cells, further amplify the inflammatory damage in the brain and worsen the progression of encephalitis. These findings further support our previous observations (PMID: 32611752; PMID: 33534086).

To corroborate our spatial transcriptomic findings, we validated immune cell infiltration into the brain following JEV infection at 3, 5, and 7 dpi. Consistent with the transcriptomic data, there was a significant increase in the proportions of monocytes, natural killer (NK) cells, T cells, and macrophages in the brains of JEV-infected mice compared to controls. Notably, these immune cell populations increased from 3 dpi and peaked at 5 or 7 dpi. These results are presented in Fig. 2e, Lines 292-296. The percentage of JEV infection rate for different immune cell types were also determined by flow cytometry (Lines 226-235, Supplementary Fig. 2b-e).

We also examined the types of cells capable of secreting IFN-γ in brain tissue and observed an intriguing phenomenon. On days 5 and 7 post-JEV infection, when the viral load in the brain tissue is high, immune cells accounted for up to 50% of all IFN-γ-secreting cells in the brain. Given that immune cells comprise only about 1% of total brain cells, this suggests that immune cells have a much stronger capacity for IFN-γ secretion compared to non-immune cells in the brain. Among IFN-γ-secreting immune cells (Ifn-$\gamma^{+}$$CD45^{+}$), T cells made up 3.6%–5.0%, while NK cells represented a slightly higher proportion at 6.3%–6.9%. Macrophages accounted for a similar proportion as T cells, at 2.5%–5.5%. Strikingly, monocytes comprised a very large proportion, 25.6%–28.3% of these IFN-γ-secreting immune cells. This finding suggests that during JEV infection, monocytes are the main source of IFN-γ in the brain, probably due to their massive infiltration into the brain during the encephalitis.

It is generally accepted that activated T cells and NK cells are the principal cell types responsible for IFN-γ secretion, while monocytes typically do not produce IFN-γ (PMID: 38635718). However, some studies have demonstrated that under certain stimulatory conditions, monocytes can also secrete IFN-γ and may even become the main source of IFN-γ. For instance, stimulation of peripheral blood mononuclear cells (PBMCs) with LPS has been shown to enhance IFN-γ production, and flow cytometric analysis reveals that the cells secreting IFN-γ are predominantly monocytes (including both classical and non-classical subsets), rather than lymphocytes or granulocytes (PMID: 31013450). In addition, in another study, Kraaij et al. isolated monocytes from the peripheral blood of healthy individuals and renal transplant recipients, and found that in vitro LPS stimulation was capable of inducing IFN-γ release from monocytes (PMID: 24680476). During the pathogenesis of Japanese encephalitis, monocytes in brain tissue are derived from circulating blood and are thus probably to secrete IFN-γ following JEV infection. To further validate the sequencing data and the results from flow cytometry, we isolated monocytes from both murine and human peripheral blood and infected them in vitro with JEV at 1 MOI. Supernatants and cell lysates were collected at 48 hours post-infection. IFN-γ content in supernatants was measured by ELISA, while IFN-γ RNA transcription level in cell lysates

was quantified by RT-qPCR. The results showed that, in both murine and human monocytes, the IFN-γ levels were significantly increased at both RNA and protein levels in JEV-infected groups versus control group. These findings further confirm that monocytes infected by JEV are capable of secreting IFN-γ, and also substantiate the flow cytometry results indicating that, during encephalitis, infiltrating monocytes in the brain are a major source of IFN-γ. These results have been incorporated into Fig. 2f-o, and the corresponding descriptions have been included in the Results (Lines 292-318) and Discussion section (Lines 677-693).

Figure 2c: It is interesting to see that the genes associated with lymphocyte trafficking and recruitment (Cxcl10, Ccr2, Ccl5) seem to precede IFN-mediated signatures. Have the authors try to run a pseudotime analysis to better resolve the order of events leading to the robust IFN signature observed in the brain?

Dear Reviewer, thank you very much for your comment. As shown in Extended Data Fig. 2e-2f, both IFN-related genes and lymphocyte trafficking/recruitment genes (*Cxcl10*, *Ccl5*) are already detectable at D3. For instance, *Ifna2* (an IFN gene) is expressed starting from D3, and classic ISGs such as *Cxcl10* and *Ccl5* are also expressed at this time point—with heatmap intensities indicating their expression levels are lower than those at D5 but higher than in the control group. Therefore, we cannot conclude that "lymphocyte trafficking and recruitment genes (*Cxcl10*, *Ccr2*, *Ccl5*) precede IFN-mediated signatures" due to the large sampling interval.

We have additionally performed pseudobulk analyses for each cell type to examine the expression patterns of gene modules, including PRRs, IFNs, IRFs, and ISGs. Their expression shows a consistent upward trend across control, D3, and D5 groups. Notably, the majority of cells at D5 simultaneously exhibit highest expression of PRRs (*Ddh58*, *Ifih1*), IFNs (*Ifna2*, *Ifnb*, *Ifng*), and ISGs (e.g., *Ifi27l2a*, *Ifitm3*, *Isg15*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Cxcl10*). It is difficult to infer which type of cell initiates the expression of IFNs/ISGs from this. Collecting samples earlier than D3 would help clarify whether ISG induction occurs prior to viral infection, potentially stimulated by blood-derived IFNs or infiltrating immune cells. However, due to the relatively large intervals between our sampling time points, data of this study fail to answer this question. Maybe we can address this question in our future work. Thank you again for your thoughtful input, which helps us better contextualize and interpret our findings.

Line 199: The choroid plexus also seems to display inflammatory signatures, in particular at d7pi (Figure 2e). Given the possibility that infected cells might exploit both routes to colonise the brain parenchyma, I wonder if the authors have compared meninges vs choroid plexus to determine region-specific differences, or lack of?

Dear Reviewer, thank you very much for your insightful comment. First, we would like to clarify a key annotation detail: in some samples, the CHP region may include both ependymal cells and the choroid plexus, while the meninges (MEN) region may contain ependymal cells and the pia mater. Therefore, these two regions, at least in our data, due to technical deficit, shared some overlapped RNA signals.

Functional enrichment analysis revealed that CHP-specific upregulated genes are

primarily enriched in innate immunity and response to Ifnb pathways, which are largely consistent with the pathways enriched in the meninges. Thus, the overall inflammatory pathways in these two regions show minimal differences.

Since the choroid plexus is an integral component of the vasculature system, with endothelial cells constituting a key cellular element. Therefore, we didn't exclude it from our conclusions. The choroid plexus may also contribute to the reception and amplification of inflammatory signals. However, due to its inconsistent detection across the collected brain sections and the limited number of specimens available for analysis, we did not perform a targeted, in-depth investigation of choroid plexus. We are sorry that we can't provide more detailed data for this tissue.

Line 220: I find it intriguing to see so many chemokine interactions with Acrk1, which is thought to bind to CXCL12. Is this expected? Have the authors considered validating these findings using imaging techniques (e.g., IFA or RNAscope) to determine if Cxcl9/10+ cells cluster around Ackr1+ ECs in JEV-infected brain specimens?

Dear Reviewer, we greatly appreciate your insightful perspective on this issue. Ackr1, also known as Darc, is an atypical chemokine receptor that regulates chemokine levels and localization by binding chemokines with high affinity without activating downstream signaling pathways. Instead, Ackr1 mediates chemokine sequestration, degradation, or transcytosis, and thus acts as a chemokine-scavenging or decoy receptor. Ackr1 displays broad ligand specificity, binding multiple inflammatory chemokines including Ccl2, Ccl5, Ccl7, Ccl11, Ccl13, Ccl14, Ccl17, Cxcl5, Cxcl6, Il8/Cxcl8, and Cxcl11. Ackr1 regulates chemokine bioavailability, and thereby leukocyte recruitment, through two distinct mechanisms: when expressed on endothelial cells, it mediates abluminal-to-luminal transcytosis of tissue-derived chemokines and presents them to circulating leukocytes; when expressed on erythrocytes, it acts as a circulating reservoir for its cognate chemokines and buffers potential surges in plasma chemokine levels (PMID: 24625696).

Currently, there are few studies investigating the role of ACKR1 in the pathogenesis of Japanese encephalitis (JE). However, during the pathogenesis of experimental autoimmune encephalomyelitis (EAE), ACKR1 expressed on brain vascular endothelial cells mediates the basolateral-to-luminal transport of inflammatory chemokines across the BBB, thereby enhancing transcellular T-cell diapedesis across the BBB and promoting EAE pathogenesis (PMID: 24625696; PMID: 34524684).

To validate our spatial transcriptomic results, we examined the protein expression and distribution of ACKR1, CXCL9, CXCL10, and CCL2 in brain tissues from JEV-infected and control mice by immunofluorescence staining. Compared to controls, the proportion of *Ackr1*$^{+}$ cells was markedly increased in the brains of JEV-infected mice at days 5 and 7 post-infection. Furthermore, co-localization of *Ackr1*$^{+}$ cells with *Cxcl9*$^{+}$, *Cxcl10*$^{+}$ and *Ccl2*$^{+}$ cells was readily observed. Relevant experimental results have been integrated into the Results section of the revised manuscript (Lines 343-337, Fig. 3h, Supplementary Fig. 7b,c).

Given that this manuscript is likely to set a standard for others in the field trying to replicate these findings, or use similar methods for their own studies, I'd encourage the authors to

include a supplementary figure highlighting the optimisation workflow. For instance, if possible, I'd like to see the permeabilization time course and the bioanalyzer profile of the resulting RNA/libraries, etc. This could be really informative to the scientific community.

Dear Reviewer, thank you very much for this constructive suggestion. In the present study we used fresh-frozen OCT-embedded brain tissue for spatial transcriptomic profiling; the RNA integrity number (RIN) values were consistently $\geqslant$7, confirming high RNA quality. Because the protocol for brain tissue is well established, we followed the manufacturer's standard workflow with only one optimization step, fixing the permeabilization time at 12 min (without too much effort), which is now explicitly stated in the Methods (Line 863). Given the tight word limit and the already extensive figures, we decided not to add additional methodological panels.

Line 289: The choroid plexus also shows cell death signatures observed in the meninges, but overall, the signals shown in figure 5b and 5d are not the most convincing are certainly not limited to the regions described in the text. For instance, there is a clear enrichment for pyroptosis-related genes in the brainstem area and the midbrain, whereas the necrosis seems more prominent in the external capsule. I believe this is further discussed in figure 5. I believe that validating these findings in independent experiments would be extremely informative to determine whether various types of cell death occur in a region-specific manner.

Dear Reviewer, thank you very much for your comment. We have investigated pyroptosis (marked by Gsdmd-N) and necroptosis (marked by p-Mlkl) in various brain regions at different time points following JEV infection using in situ immunofluorescence staining. Elevated expression of p-MLKL was observed primarily in the meninges of JEV-infected mice compared to controls. While Gsdmd-N showed stronger signals than p-MLKL in multiple brain regions. At 3 dpi, pyroptotic signals first appeared in the meningeal tissues and some areas of the cerebral cortex. By 5 and 7 dpi, pronounced pyroptotic signals were observed in the meninges, cerebral cortex, olfactory bulb, hippocampus, cerebral nuclei, midbrain, and hindbrain, with the strongest signals detected in the meninges, cerebral cortex, and olfactory bulb.

In summary, JEV infection induces early pyroptosis and necroptosis in the meningeal tissues. As the infection progresses, JEV triggers both pyroptosis and necroptosis across multiple brain regions, with cell death being most prominent in the meninges, cerebral cortex, and olfactory bulb, suggesting a degree of region-specificity in JEV-induced cell death. These results have been incorporated in Fig. 5c,f,i,l and descried in Lines 430-440.

Line 405: The results presented here are indeed quite interesting and might suggest that the neuronal death results in a loss of MBP (and potential demyelination) and gliosis. Given that this is critical to understand the neuropathology of JEV infection, I think some validation using independent samples of i) demyelination using Luxol Fast blue (or related) and ii) gliosis would be critical.

Dear Reviewer, thank you very much for your valuable suggestions. First, we assessed the mRNA expression levels of *Gfap*, *Tspo*, and *Mbp* by qPCR. The expression of *Gfap* and *Tspo* began to increase at 3 dpi, reached a peak at 5 dpi, and then slightly decreased at 7

dpi, although levels remained higher than those observed at 3 dpi. In contrast, the expression of *Mbp* exhibited a continuous decline following JEV infection (Supplementary Fig. 15b-d, Lines 558-563). These results strongly suggest that the neuronal death might result in a loss of myelin basic protein (MBP) and cause potential demyelination and gliosis. Thus, we performed Luxol Fast Blue (LFB)-Eosin staining to specifically label myelin sheaths in brain sections from both JEV-infected and control mice. The results showed that the myelin in the brain tissue of control mice was well stained blue, whereas in JEV-infected mice at days 5 and 7 post-infection, the blue staining of myelin was noticeably reduced, indicating significant myelin loss (Supplementary Fig. 15e, Lines 565-567).

In addition, we investigated gliosis in brain tissues using hematoxylin and eosin (HE) staining. In the brains of control mice, there was no neuronal death, inflammatory cell infiltration, or gliosis observed. However, at days 5 and 7 following JEV infection, there was obvious neuronal death, substantial infiltration of inflammatory cells, and evident gliosis in the brains of JEV-infected mice (Supplementary Fig. 1f, Lines 141-147, 568).

Figure 7: Does the vascular changes observed at the transcriptional level correlate with changes in vascular permeability? I think experiments using labelled dextran with various molecular weights would help clarify whether the overall vascular permeability changes in response to infection.

Dear Reviewer, thank you very much for your suggestion. Currently, the most commonly used method for assessing blood-brain barrier (BBB) permeability is the Evans Blue assay (PMID: 31483106; PMID: 37776457). Therefore, we evaluated BBB permeability in both JEV-infected mice (D1, D3, D5, and D7) and control mice using Evans Blue. The results showed a significant increase in BBB permeability on days 5 and 7 post-JEV infection, which corresponded precisely with the time points (D5 and D7) at which transcriptomic analysis revealed marked downregulation of tight junction proteins. In addition, both JEV viral loads and infiltrating immune cells in brain tissue were significantly increased at days 5 and 7 post-infection, which is closely associated with enhanced BBB permeability. The relevant results have been incorporated into Fig. 3l and the Results section of the manuscript (Lines 356-359).

Line 413: gene symbols in this line are not italicised. Please amend.

Corrected. Thank you for pointing out.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
Summary
I would like to commend the authors for their thorough and thoughtful revision, which has substantially strengthened the manuscript. The additional experimental validation, expanded discussion, and clarifications have addressed nearly all of the initial concerns, resulting in a version that is considerably more rigorous and compelling. I offer below a set of points that the authors may consider in further revision.
Thank you very much for your recognition of our revised manuscript and for your valuable suggestions for further improvement. We have addressed each of your comments point by point, and we kindly ask you to review our revisions.

Comments
Q1: The study used BALB/c mice inoculated with 10^6 PFU of the Nakayama strain via the intraperitoneal route. While many strains (e.g., BALB/c, C57BL/6, and C3H/He) are susceptible to JEV infection, the use of such a high dose in BALB/c mice warrants further discussion. It would be helpful if the authors could elaborate on the rationale for this choice and comment on how results might differ with other strains.
Thank you for the insightful comments. As you pointed out, BALB/c, C57BL/6, and C3H/He mice are all susceptible to JEV infection, with C3H/He mice displaying the highest susceptibility. This is likely due to their myeloid cells being more readily infected by JEV and their limited immune response capability (PMID: 21949747; PMID: 2165769). BALB/c and C57BL/6 are immunocompetent strains with good immune response to JEV infection (PMID: 12771426), and BALB/c mice in particular are known for their docile temperament, making them one of the most commonly used models for JEV infection. In the literature, the typical intraperitoneal challenge dose ranges from 10^5 to 10^8 PFU (PMID: 21949747; PMID: 18691668; PMID: 37071015; PMID: 41192153). At doses below 10^6 PFU, mice show very heterogeneous clinical symptoms, and the incidence of encephalitis drops significantly, often below 30% (PMID: 37071015). Since our study aimed to characterize the spatiotemporal features of JE, we selected a higher challenge dose within the commonly used range to increase the consistency and incidence of encephalitis, thereby ensuring that the results of our spatiotemporal transcriptomic analysis would be more representative.

Under the same inoculation route and dose, different mouse strains may exhibit significant differences in viremia, encephalitis incidence, and mortality. For example, C3H/He mice show higher rates of JEV-induced encephalitis and mortality compared to BALB/c, C57BL/6, and DBA/2 strains (PMID: 2853677; PMID: 21949747), likely due to their increased susceptibility of myeloid cells to

JEV and limited immune response to infection (PMID: 21949747). However, once encephalitis develops, the pathological changes in the brain are remarkably similar across mouse strains, effectively modeling the clinical features of Japanese encephalitis in humans (PMID: 37242385).

To explain the choice of BALB/c mice and use of 10^6 PFU dose to establish the JE model, we have included the relevant statements “BALB/c mice are a widely used immunocompetent inbred strain. Upon JEV infection, they display clinical symptoms and brain neuropathology similar to those seen in human JE. Their docile nature and affordability also make them a popular choice for establishing JEV infection models (PMID: 35143497; PMID: 37242385). Typical intraperitoneal challenge doses range from 10^5 to 10^8 PFU (PMID: 21949747; PMID: 18691668; PMID: 37071015; PMID: 41192153). To better characterize the spatiotemporal features of JE, we chose a higher dose within this range to improve consistency and incidence of JE in our study.” in the Supplementary Information (Lines 80-86). As requested in Comment 20, we have moved the section "Establishment of a murine JE model" to Supplementary Information.

2) The data suggest that JEV RNA was not detected in granulocytes or B cells. It would be valuable to discuss whether this finding implies that these cell types are not susceptible to JEV infection.

Thank you for your valuable comments. Currently, there are very few reports regarding the susceptibility of granulocytes or B cells to JEV infection. Research on the role of B cells has mainly focused on how B cell-mediated humoral immune responses or B cell-T cell interactions contribute to the pathogenesis of JE (PMID: 6300303; PMID: 21450826; PMID: 40473021). In our study, we found that JEV RNAs were not detected in B cells, suggesting B cells are not susceptible to JEV infection. To further investigate the susceptibility of B cells to JEV infection, splenic B cells were isolated from 4-week-old female BALB/c mice using a mouse pan B cell isolation kit (Biolegend, Cat# 480051) and seeded into 96-well plates at a density of 2×10^4 cells per well. The cells were infected with JEV at multiplicities of infection (MOI) of 0.1, 1, and 10 for 1.5 hours. Following infection, the inoculum was removed, cells were washed three times with PBS, and subsequently cultured in RPMI 1640 medium supplemented with 2% FBS for 72 hours. Supernatants and cells were collected at 72 hours post infection, and cells were washed three times with PBS. The viral load in both the culture supernatants and within the cells was quantified by qPCR. No JEV was detected in either the supernatant (Fig. R1A) or the cells (Fig. R1B) at the examined time point, indicating that B cells are not susceptible to JEV infection.

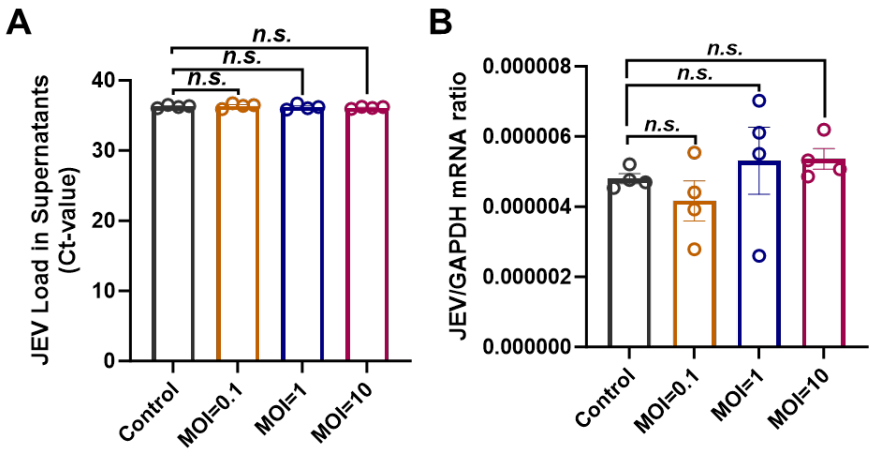

Fig. R1. In vitro infection of primary mouse B cells with JEV. (A) Viral load of JEV in B cell culture supernatants. (B) Intracellular viral load of JEV in B cells. *n.s.*, not significant ($p > 0.05$).

Regarding granulocytes, our study found that the primary infiltrating granulocytes in the brain tissues during JE were neutrophils and JEV RNA was not detected in neutrophils at both 3 dpi and 5 dpi. It is currently believed that JEV does not infect neutrophils; on the contrary, neutrophils can eliminate JEV through the production of reactive oxygen species (PMID: 10365083). We have included this discussion in the revised manuscript (Lines 616–630). and quoted as below:

> " In this study, JEV RNAs were not detected in B cells or granulocytes at both 3 dpi and 5 dpi (Fig. 1d), suggesting these cells may not be susceptible to JEV infection. Currently, there are very few reports regarding the susceptibility of B cells or granulocytes to JEV infection. During JEV infection, research on the role of B cells has mainly focused on how B cells mediate humoral immune responses[64–66]. Inoculation of JEV with splenic B cells also failed to yield reproductive progenies (data not shown), indicating that the B cells were not permissive to JEV replication. Regarding granulocytes, the primary infiltrating granulocytes in the brain tissues during JE may be neutrophils, as they highly expressed marker genes including S100a8 and S100a9 (Supplementary Fig. 2a). It is currently believed that JEV does not infect neutrophils; on the contrary, neutrophils can eliminate JEV through the production of reactive oxygen species[67]. Notably, because viral RNA engulfed by the immune cells may also be captured by Stereo-seq technology, the detection of viral RNA in the granulocytes at 7dpi does not guarantee that these cells can support JEV replication."

3) Please clarify the distinction between Ly6c⁺ and Ly6c2⁺, and consider using one consistent designation throughout the manuscript if appropriate.

Thank you for this important reminder. In our text, Ly6c is the same as Ly6c2. We apologize for the typo and have unified the designation as Ly6c2 throughout

the revised manuscript.

4) Virus taxonomy terminology should be standardized. Please use Orthoflavivirus, orthoflaviviruses, and orthoflaviviral consistently throughout.
Thank you for this kind reminder. We have unified the designation as orthoflavivirus throughout the revised manuscript.

5) Lines 73–93: The paragraph describing the current working hypothesis of JEV pathogenesis could be revised for clarity. The findings of this study align well with existing concepts of viral amplification, brain invasion, and pathology, while specifying the involvement of endothelial cells and macrophages.
Thank you very much for your insightful comments. We have revised this section to more clearly delineate the disruption of the blood-brain barrier (BBB), viral invasion and infection of brain tissue, immune cell infiltration, the critical role of monocytes, and the involvement of the meninges during the pathogenesis of Japanese encephalitis (JE). The revised content highlights the roles of meningeal tissues and monocytes in mediating BBB disruption, viral entry, and regulation of neuroinflammation, as well as key scientific questions that warrant further investigation. We believe these revisions enhance the logical flow and readability of the manuscript. Please refer to Lines 71–110 in the revised manuscript.

6) Line 130: The detection of JEV RNA in blood at 1 day post-infection may reflect residual input virus, given the high inoculum (10^6 PFU), which can persist for about 2–4 days without replication. Please clarify this point.
Thank you for this comment. We agree with your point of view. Therefore, in the revised version, we have added the following statement: “While the detection of JEV RNA in blood at 1 dpi may reflect residual input virus, the peak of viremia marked a period when the virus could more readily invade the brain, establish infection, and induce encephalitis.” (Supplementary Information Line 94-96).

7) Lines 161–189: JEV expresses only one mRNA, the genomic RNA. Please revise the paragraph to reflect this fact accurately.
Dear reviewer, based on your comments and feedback from our preprint, we realize that the description of the original Supplementary Fig. 1h, which we used to show the binding preference of the viral probe, has caused a lot of confusion. Therefore, we have removed this figure and the associated description in this paragraph. We also clearly state that "During viral replication, JEV expresses only a single genomic RNA that serves as mRNA and does not generate multiple distinct transcripts. When quantifying JEV expression, we assumed that each detected viral RNA fragment corresponds to an intact JEV RNA." (Lines 163-166)

8) Lines 226–233: The statements referring to Supplementary Fig. 2b (L229,

L233) do not appear to be supported by the data. Please review and correct.
Thank you very much for your helpful reminder. We have carefully reviewed all the images and corresponding raw data for Supplementary Fig. 2b–2e, and we confirm that both the figures and the data are correct (Lines 211-219). We sincerely apologize for the confusion caused by our unclear explanation.

Specifically, when considering the percentage of JEV-infected cells within each immune cell type, the highest infection rates observed for monocytes, T cells, NK cells, and macrophages were 5.7% at 5 dpi, 4.4% at 5 dpi, 15.4% at 7 dpi, and 16.8% at 5 dpi, respectively. In the previous version of the manuscript, we stated: “When considering the percentage of JEV-infected cells within each immune cell type, NK cells and macrophages exhibited the highest proportions of JEV infection (>10%) among infiltrating immune cells in the brain (Supplementary Fig. 2b).” To improve accuracy, we have revised this statement to: “When considering the percentage of JEV-infected cells within each immune cell type (Supplementary Fig. 2b-e, upper panel), macrophages (16.8% infection rate at 5 dpi) and NK cells (15.4% infection rate at 7 dpi) exhibited the highest JEV infection rates among all infiltrating immune cells in the brain.” (Lines 211-214)

Furthermore, when assessing the proportion of infected cells relative to the total brain cell population, which better reflects the overall contribution of each immune cell type to brain infection, the highest percentages of JEV-infected monocytes, T cells, NK cells, and macrophages were 0.08% at 5 dpi, 0.003% at 5 dpi, 0.001% at 5 dpi, and 0.0005% at 5 dpi, respectively. Taken together, these data indicate that monocytes represent the predominant JEV-infected immune cell population in the brain (Supplementary Fig. 2b, lower panel). We originally stated: “when assessing the proportion of infected cells relative to the total brain cell population, which better reflected the overall contribution of each immune cell type, monocytes were the predominant JEV-infected immune cells in the brain at 3 and 5 dpi (Supplementary Fig. 2b)” For clarity, we have now revised this to: “when assessing the proportion of JEV-infected immune cells relative to the total brain cells (Supplementary Fig. 2b-e, lower panel), which better reflected the overall contribution of specific immune cell types to the brain infection, JEV^+ monocytes were the predominant infected immune cells, accounting for around 0.08% of the total living cells in the brain at 5 dpi.” (Lines 215-219)

Thank you again for your insightful comments and suggestions.

9) Line 297: The emphasis on IFN-γ is not explained or justified. A brief rationale for focusing on this cytokine would be helpful.
Thank you very much for your suggestion. IFN-γ is a critical immunomodulatory cytokine that coordinates both innate and adaptive immune responses and also

exerts powerful antiviral function (PMID: 29856726). Studies have also shown that IFN-γ is a key cytokine mediating blood-brain barrier disruption and immune cell infiltration in the pathogenesis of JE (PMID: 34379515; PMID: 25762733). Therefore, identifying the specific cell types in the brain that secrete IFN-γ would help reveal the drivers of JE immunopathology. We have added above rationale to the revised manuscript (Lines 283-287).

10) Line 303: Please revise "NK cells (Fig. 2i) for 6.3–6.9%" to "NK cells for 6.3–6.9% (Fig. 2i)."
Thank you! We have revised this issue (Line 293).

11) Line 319: The statement "the intense involvement of the meninges in JE progression (Fig. 2a–d)" would benefit from further elaboration.
Thank you for your suggestion. We have revised this sentence to "Our results showed that the meninges were the hotspot of immune response, and the excessive immune infiltration also caused tissue damage (Fig. 2). To further understand the transcriptional changes associated with this pathological process, we extracted the spatial transcriptomics data of meninges from all samples for in-depth investigation." (Line 309-312).

12) Lines 443–464: Could the authors clarify whether they can differentiate direct virus-induced cell death from bystander cell death?
Thank you for raising this point. We have clarified this in Lines 445-450 and quoted below:

> "Although the JEV-negative neurons had lower death signals, the uninfected neurons may also be killed by the bystander effect. Unfortunately, because our virus probes may be insufficient to capture all JEV RNA in each cell, a JEV-negative cell may be JEV-infected, making it hard to define a true bystander cell. Therefore, our detection sensitivity can't support the discrimination between direct virus-induced and bystander cell death."

13) One of the most important findings appears to be the specific involvement of Ly6c⁺ monocytes and Ackr1⁺ endothelial cells in disease progression. It would be valuable to elaborate on whether this observation can be translated to human JE or other neuroinvasive orthoflaviviruses. Testing the functional importance of these cell types or their interactions would further strengthen the study.
Thank you very much for your insightful and crucial comment. We have incorporated more discussion on *Ly6c⁺ monocytes and Ackr1⁺ endothelial cells* into the revised manuscript (Lines 645-713 & 832-836), also quoted below:

> "Our findings show that *Ly6c2*⁺ monocytes can activate *Ackr1*⁺ ECs through *Cxcl10*/*Cxcl9*/*Ccl2*/*Ccl5*/*Ccl7-Ackr1* interactions. Ackr1, also known as Darc, is an atypical chemokine receptor that regulates chemokine levels

and localization by binding chemokines with high affinity without activating downstream signaling pathways[68]. Instead, Ackr1 mediates chemokine sequestration, degradation, or transcytosis, and thus acts as a chemokine-scavenging or decoy receptor. Ackr1 displays broad ligand specificity, binding multiple inflammatory chemokines including Ccl2, Ccl5, Ccl7, Ccl11, Ccl13, Ccl14, Ccl17, Cxcl5, Cxcl6, Il8/Cxcl8, and Cxcl11. Ackr1 regulates chemokine bioavailability, and thereby leukocyte recruitment, through two distinct mechanisms: when expressed on endothelial cells, it mediates abluminal-to-luminal transcytosis of tissue-derived chemokines and presents them to circulating leukocytes to enhance leukocyte chemotaxis; when expressed on erythrocytes, it acts as a circulating reservoir for its cognate chemokines and buffers potential surges in plasma chemokine levels[69]. Currently, there is no study pertaining to the role of *Ackr*$^{+}$ meningeal endothelial cells in the pathogenesis of JE. However, during the pathogenesis of experimental autoimmune encephalomyelitis (EAE), ACKR1 expressed on brain vascular endothelial cells mediates the basolateral-to-luminal transport of inflammatory chemokines across the BBB, thereby enhancing transcellular T-cell diapedesis across the BBB and promoting EAE pathogenesis[69,70]. Accordingly, we speculate that ACKR1^{+} meningeal endothelial cells may play the same or similar roles in the pathogenesis of human encephalitis induced by JEV or other orthoflaviviruses. Intriguingly, in COVID-19 survivors, elevated anti-ACKR1 autoantibodies were linked to endothelial dysfunction and vascular complications[71], indicating the involvement of ACKR1 in a broader range of infectious diseases. Currently, few drugs targeting ACKR1 are available. Potential interventions include administrating antibodies targeting the extracellular domain of ACKR1, inhibiting the ACKR1 interaction axes, or disrupting ACKR1's chemokine-shuttling function at the BBB to mitigate excessive inflammatory response.

Monocytes play a controversial role in the pathogenesis of orthoflavivirus infection. Firstly, they are target cells of orthoflaviviruses, facilitating virus dissemination to various organs[72–74]. Secondly, they can exert pro-inflammatory effects, clearing viruses and dead cells[75,76]. However, the associated excessive infiltration and pyroptosis of immune cells can disrupt normal organ function. Our flow cytometry data showed that the monocytes accounted for over 0.6% of the living cells in JEV-infected mouse brain on 7 dpi (Fig. 3b), underscoring their dominant role in immune cell infiltration within the brain. The monocytes may accumulate around the blood vessels through multiple chemokine-ACKR1 axes, including CCL2, CCL5, CCL7, CXCL9 and CXCL10 (Supplementary Fig. 6a). The monocytes can also be recruited by endothelial cells through the VLA-4-VCAM-1 and LFA-1-ICAM-1 axes to exert transendothelial effects[77]. Kim et al. found that Ly6c^{hi} monocytes can be recruited into CNS by the CCR2-CCL2 axis and that the

ablation of CCR2 could restrict CCR2$^+$ monocytes entry and enhance the resistance to JE in a mouse model[25]. Therefore, dampening the excessive recruitment of monocytes can ameliorate the hyperinflammation triggered by orthoflavivirus infection. For instance, bindarit[78–80] has been identified as an inhibitor for CCL2, CCL7, and CCL8. It's also reported that the Janus kinase inhibitors, tofacitinib and upadacitinib, can help reduce the expression of CXCL10 in patients with drug-induced hypersensitivity syndromes[81]. These may potentially serve as candidate drugs reducing monocyte infiltration into the CNS during JE."

"Our data revealed that, in both murine and human monocytes, the IFN-γ levels were significantly increased at both RNA and protein levels after JEV infection. What's more, the IFN-γ-secreting monocytes accounted for around 25% of the IFN-γ-secreting immune cells in JEV-infected mouse brain on 5 and 7 dpi (Fig. 3h), demonstrating monocytes as a major source of IFN-γ in the brain. It has been shown that IFN-γ is a critical cytokine mediating BBB disruption and immune cell infiltration into the brain tissue[48,49], indicating that monocytes may be the key immune cells causing BBB breakdown and JE immunopathology. As monocytes contribute to the pathogenesis of multiple orthoflaviviruses, it would be valuable to explore whether their IFN-γ secretion capacity during infection by the other neuroinvasive viruses align with the findings of this study."

"The prominent roles of *Ly6c2*$^+$ monocytes and activated *Ackr1*$^+$ endothelial cells revealed by our analysis present promising therapeutic targets. Future studies should prioritize targeted intervention experiments, such as employing neutralizing antibodies against Ackr1 or depleting *Ly6c2*$^+$ monocytes, to directly assess the therapeutic potential of disrupting this pathogenic crosstalk."

14) Lines 465–490: This section is somewhat difficult to follow. Please revise for clarity.

Thank you for your suggestion. We have added a brief explanation (Lines 456-460): "While DEG analysis can identify lists of individually altered genes, we would like to find out coordinately expressed gene modules associated with the neurological disorders caused by JEV infection. To achieve this, we used hdWGCNA (high-dimensional Weighted Gene Co-expression Network Analysis) to detect the corresponding functional programs in specific cell types[60]") to help the readers to follow and revised lines 468-472 to guide better understanding of this section, highlighted in red.

15) Lines 491–570: The section describing virus–host interactions should explicitly state that these are "predicted" protein-protein interactions.

Thank you for pointing out. We have revised the title of this section into

"Predicted Interactions between JEV and host factors" (Line 483) and also stated this fact clearly in the paragraph, with the revised words highlighted in red (Lines 490-507).

16) Lines 592–594: This reviewer feels that the use of terms such as "groundbreaking" and "unprecedented" is overstated. I recommend toning down this language here and throughout the manuscript to maintain a balanced and objective tone.

Dear reviewer, thank you for instructive comment. We have removed these words in this paragraph and also revised similar phrases throughout the manuscript to maintain an objective tone (Lines 584-586).

17) Line 595: The authors state, "Demonstrating JEV's dual neuroinvasion pathways while mapping region-specific viral tropism across distinct brain areas." Could the authors clarify what is meant by "dual neuroinvasion pathways" and specify the nature of the "region-specific viral tropism"?

Thank you for your instructive comment. Our initial statement lacked clarity and contained some inaccuracies, particularly regarding the description of viral tropism. We have changed this sentence to "Demonstrating JEV may enter the CNS by directing infecting BMECs and through the Trojan horse form" (Line 586-587).

18) Figures (e.g., Fig. 1): Abbreviations for brain regions make interpretation difficult. Please spell out the names of brain regions in figures and throughout the manuscript.

Thank you for your helpful suggestion. We have now replaced all brain-region abbreviations in all the figures with their full names.

19) Supplementary Fig. 2b: Please verify whether the y-axis labeling is correct.

Thank you very much for your helpful suggestion. We have thoroughly examined all the images and corresponding raw data in Supplementary Fig. 2b–2e, and we can confirm that both the figures and the data are accurate. As this issue is similar to Comment 8, we kindly refer you to our response to Comment 8 for additional details.

20) The section "Establishment of a murine JE model" distracts from the main points of the paper. Consider moving this section to the Supporting Information.

Dear reviewer, thank you for your suggestion. We have moved this section to Supporting Information.

Reviewer #2 (Remarks to the Author):

I am very satisfied with the revision, and all my comments have been addressed. Only two minor points that I would like the authors to change.

Thank you very much for your recognition of our revised manuscript and for your valuable comments for further refinement. We have further revised the manuscript according to your suggestions. Please find our point-by-point responses below for your reference.

First, please add example FACS plots showing the gating strategy for the different cell populations mentioned in line 928-931, as supplemental figure.
Thank you for this important suggestion. We have now added a Supplementary Fig. 15 showing example FACS plots for the gating strategy for the different cell populations.

Secondly, line 294 and line 668 both states that 60% of the living cells in the flow cytometry analysis is monocytes however, the panel Fig 2e shows 0.6% monocytes day 7.
Thank you very much for your careful review of our data. We apologized for misinterpreting the y axis when writing the previous version. We have now corrected the description to “0.6%” (Line 280 & 678) to accurately reflect the data presented in Fig 3b (originally Fig. 2e). Furthermore, we have carefully reviewed the descriptions of the percentages for all other immune cell populations and verified that they are accurate. We thank you again for drawing our attention to this issue.

Reviewer #3 (Remarks to the Author):
Many thanks to the authors for addressing my questions. The revised manuscript is certainly much improved and I am sure will be of interest to a broad readership.
Thank you very much for your valuable suggestions and for recognizing our revised manuscript. We sincerely hope that this work will be read and appreciated by a broad audience.