nature portfolio

Peer Review File

Microglia are prominent producers of inflammatory cytokines during the hyperacute phase of ischemic stroke

Corresponding Author: Professor Connie Wong

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #2

(Remarks to the Author)
Review of Microglia are prominent producers of inflammatory cytokines during the hyperacute phase of ischemic stroke

This study aimed to highlight resident neuroinflammatory mechanisms at both the hyper acute (3 hour) and acute (24 hour) time points under the suggestion that potential treatments beyond this critical period are less effective. Using a mouse model of stroke, they interrogate the microglial involvement at these time points. They also block the infiltration of neutrophils and monocytes to demonstrate no alteration in the inflammatory response. They measured global cytokine levels and used multiphoton imaging of microglia to describe how the morphology of these labeled cells change over time. They also suggest that microglia and neurons are the primary producers of cytokines 3 hours post-stroke. This study is well-done and very interesting, particularly with adding to the body of literature about the hyper acute neuroinflammatory response to stroke.

Major Comments
1. 3 hours post-stroke is referred to in the abstract and the paper as a clinically relevant time point. But there is a significant amount of data suggesting that patients are not arriving at the hospital and being assessed for treatment within this time frame. Authors should please describe or reference more detail about why they think this is a clinically relevant time point.
2. Please describe the stereological approach to counting and analyzing cells via 2-photon microscope. When imaging ex vivo tissue, one is restricted by the thickness of the tissue (z plane) and structure of interest (x and y planes). Was there a well-defined region of interest when imaging through a cranial window? How did you control for the volume of tissue measured across each animal?
3. What brain structure were the microglia imaged in with 2-photon microscopy? The methods say image location was determined by the presences of vessel-occlusive thrombus in post-stroke mice. But it does not describe how this was used as a reference to take the image. Were the images taken only in cortical tissue? There is conclusive data to suggest heterogeneity of glial cells in different brain structures. What was the distance from the stroke border? There is a significant amount of published data about how microglia morphology change, even a short distance from the border. Was this controlled for? Please provide regional clarity for imaging and tissue collection. The anatomical locations where images were taken and tissue was collected (contralateral, peri-infarct, infarct core) aren't clearly defined in any figure or legend. A schematic or more precise description would help, especially for distinguishing ipsilateral vs peri-infarct tissue. Was a laser dissection microscope used to separate these two areas?
4. There are a number of significant problems with using evans blue (EB) as a marker for blood brain barrier integrity (see Saunders et al. 2015, Frontiers Neuroscience). Briefly summarized, it does not specifically bind to albumin, EB-blood level variability after injection, structure of the dye affected by vehicle solution, and potentially alternate binding. There are additional ways to measure the integrity of the blood brain barrier that are more appropriate.
5. The data in Figure 1c suggests that the vascular integrity in the stroke core and peri-infarct area is compromised as early as 30 minutes post-stroke. This directly counters the data that they collected about the timeliness of peripheral cell

infiltration. Additionally, the quantification of this method is relatively difficult to interpret, because even the representative sections provided are significantly manipulated in a way that utilizing a volumetric measurement would not be reliable. This is made further clear with the low n used (1 or 3) and the unbalanced groups. If you are not running analysis on these data, it may be best to just utilize the representative samples.
6. Based on the mechanistic data provided by the Barres laboratory suggesting that microglia release specific cytokines Il-1a, TNF, and C1q in abundance, I would have expected that these cytokine levels would have been measured or at least addressed (Fig 2b).
7. The interpretation that only microglia and neurons are responsible for cytokine release (section title) is not directly supported. Rather, it was demonstrated that there are four specific cytokines that are altered in microglia in neurons, but not other cell types. Additionally, the culturing of isolated cells from flow cytometry to measure cytokine production has inherent issues. For example, chemical and mechanical disruption of the cells is required with this technique. This interpretation would be circumvented if the methodology used to measure these cytokines was to sort cells or immunoprecipitate cell-specific RNA and then run sequencing to measure all cytokine production, instead of being selective. The authors need to update their analysis or word their interpretation much differently.
8. The interpretation that astrocytes are not early responders in direct contradiction to a host of other research suggesting that astrocytes respond within minutes and upregulate cytokines like IL-6, IL-1beta, TNF, IL-17, CXCL1, CXCL9, and NfkBeta. While this study only measured IL-6 (of this list), they interpreted that astrocytes are not a source of pro-inflammatory cytokines at 3 hours post-stroke. The authors should at the very least address this confliction between their data and the literature.
9. There are significantly more monocytes present at 3 h post stroke Fig 2L. While there are much fewer than later and much fewer than microglia at this point, it is still possible they are the primary producers of cytokines at this time point and are driving the pro-inflammatory escalation. If there is data about the ratio of cytokine release between activated microglia vs. monocytes, this should be cited and there should be room left in the interpretation for this possibility. On a related note, since you are eliminating all but about 20% of monocytes from the periphery (Fig 3a-c), it would be nice to have a representative picture (or quantification) of these cells in the infarct core and peri infarct area. If these small number of remaining cells are still penetrating the BBB, they may still be the primary producers of these cytokines.

Minor Comments
1. In the introduction, (line 87-88) it is suggested that the authors are taking a holistic approach to characterizing the entire immune composition in the brain, but this is not accurate. The authors are characterizing microglia and cytokines. While extremely important, the majority of their analyses do not include a number of other cells (ex. astrocytes, oligodendrocytes, endothelial cells, etc.) and molecules (ex. lipids, vesicles, reactive oxygen species) that are crucial for the neuroinflammatory response. Please rephrase the description of the approach.
2. While the introduction discusses clinical trial interventions regarding infiltrating immune cell interventions, they do not spend time setting up the body of literature about the timing when these cells infiltrate (either in mouse models or humans), which seems strange since the main goal of this paper is to examine the hyperacute and acute stages, a time when they claim these infiltrating cells are not present. At the very least, I would expect some description of this body of literature related to timeliness of cell infiltration.
3. Please provide justification for the use of young mice (7-12 weeks of age) for a mouse model of a human condition that primarily occurs in the later stages of life. There is data to suggest that there may be significant neuroinflammatory and pathological differences between younger mice and older mice. Therefore, 5 weeks is a wide range of mice to use to measure post-stroke pathology and assume that the response would be the same in 7-week-old mice and 12-week-old mice.
4. I understand why the markers for platelets and vasculature (and Nk cells) were used, but since there is no data or even qualitative mention of the relationship between them and microglia, why are they being displayed in Figure 5 and Supp Fig 8?
5. To interpret the microglia morphology data, it needs to be clear that the distribution of this fractalkine receptor is distributed across the membrane. If there have been other data suggesting Cx3cr1 distribution is consistent and spread equally, please cite it. Otherwise, dual labeling some microglia with Cx3cr1-GFP and another microglia marker like Iba1 (commonly used) might be helpful to demonstrate that the morphological assessment is similar.
6. Cx3cr1 expression occurs in resident microglia and peripheral monocytes. If the authors are using some type of inducible system (like cre/tamoxifen) and waiting for peripheral cells to overturn, this should be described in the methodology in detail. If they are not, they should describe how the GFP imaging done could be either infiltrating monocytes or resident microglia.
7. Sample size reporting is missing for Figure 5. The caption reports significance (e.g., $p < 0.001$), but we don't specify how many animals or cells were analyzed per group. Including n values (both animals and cells per region) would strengthen the statistical interpretation.
8. Image sampling for microglia morphology assessment. It's unclear how many images were collected per brain section and how many sections were analyzed per animal. This detail is important for assessing representativeness and should be added either in the figure legend or methods.
9. Was there an XY plane exclusion criterion used for analysis? In the methods, it says "cells were manually removed if they extended significantly beyond the XY plane." Was this determined manually or with a software-based cutoff? If manual, a brief explanation of the criteria used would improve transparency.
10. Thresholding and normalization: There's currently no detail on how thresholding was performed during 3D reconstruction (e.g., global vs region-specific), or whether any normalization was applied across samples. This is especially relevant since intensity and tissue integrity can vary quite a bit post-stroke, and these differences could bias dendrite complexity or volume measurements.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have carefully reviewed the revised manuscript by Bourne et al. and their detailed point-by-point rebuttal in response to the initial review. The study aims to dissect the temporal and cellular drivers of neuroinflammation in the hyperacute phase of ischemic stroke, a critical but under-investigated period with clinical relevance. Overall, the authors have provided substantial clarifications and experimental additions in response to the reviewers' comments.

Strengths and Improvements:

- Enhanced Justification of Timepoints: The authors provide a more solid rationale for selecting 3 and 24 hours post-stroke, emphasizing the relevance to the therapeutic window defined by the NINDS trial and subsequent clinical observations.
- Clarified Imaging Methodology: Concerns about regional specificity and stereological consistency have been addressed through additional details and supplementary figures.
- Additional Experimental Data: The inclusion of new data using CatchupIVM-red mice at 3h, as well as supplemental figures showing BBB leakage with dextran and immunohistochemical controls in sham animals, strengthens the technical rigor.
- Discussion of C1q and Astrocytic Responses: The authors provide a nuanced interpretation of C1q expression and acknowledge the complexities around astrocytic cytokine production, although certain gaps remain (see comments to authors).

Remaining Concerns:

- Interpretational Overreach: Despite rewording, the assertion that microglia are the primary cytokine producers still risks overgeneralization. While the methodology supports a dominant role, the limitations of ex vivo culture and intracellular staining merit a more cautious tone.
- Astrocytic Contribution: The field recognizes astrocytes as early responders. The data shown here are limited in scope (GLAST+ astrocytes, few cytokines), and alternative mechanisms (e.g., gliotransmission) remain unexplored.
- Microglial Heterogeneity: While the authors cite recent scRNA-seq studies (e.g., Patir et al. 2024, Paolicelli et al. 2022), they do not fully contextualize how hyperacute phenotypes may relate to known DAM, IFN-responsive, or PLX-resistant clusters.

Reviewer #2

(Remarks to the Author)
The authors have satisfied both my major and my minor comments. Thank you for detailed responses and collection of additional data to demonstrate and interpret your findings.

Point-by-point response

REVIEWER 1

In the manuscript entitled "Microglia are prominent producers of inflammatory cytokines during the hyperacute phase of ischemic stroke", Bourne et al. provide the molecular mechanisms underlying ischemic stroke-induced neuroinflammation, clarifying that microglia, rather than the infiltration of peripheral immune cells, are the primary producers of inflammatory cytokines during the hyperacute phase of ischemic stroke. The comments are given as follows.

(1) The study utilizes 3 hours post-stroke (hyperacute phase) and 24 hours post-stroke (acute phase) as experimental time points. However, they did not provide a clear rationale for the selection of these specific time points. It remains unclear whether these time points were chosen based on clinical considerations or derived from prior experimental findings. The authors should clarify the reasoning behind the selection of these time points.

RESPONSE: Three hours post-stroke is a clinically relevant timepoint because of its classical tie to the "therapeutic window" for effective treatment. Therapy by tPA was originally approved based on clinical trials (NINDS trial, 1995), which showed most efficacy if administered within 3 hours of stroke onset, albeit more recently this window has been shown to extend to 4.5 hours when aided with contrast imaging. In this study, we demonstrate that irrespective of therapeutic window expansion, neuroinflammation is already evident as early as 3 hours post-stroke, and is detrimental. This has now been clarified by inclusion of additional passage in the Introduction (P3 L46):

> "This is a clinically relevant timepoint because it aligns with the accepted "therapeutic window" for effective treatment via tissue plasmin activator (tPA). Intervention was originally approved based on the NINDS trial, which showed most efficacy if administered <3 hours of stroke onset."

Additionally, the 24 h timepoint was chosen as the beginning of the acute phase following stroke (Bernhardt, 2017 Neurorehab and Neural Repair). From our experience with other inflammatory diseases, such as infection, this is a timepoint we would expect to see peripheral immune infiltration into tissue.

(2) A significant portion of the article addresses the role of peripheral immune cell-derived cytokines in neuroinflammation during the hyperacute phase, arguing that these cytokines are not the primary drivers of neuroinflammation at this stage. From Figure 5 onwards, the study illustrates morphological changes in microglia, and in Figure 7, the upregulation of cytokine secretion by microglia is demonstrated. In the discussion, the authors mention the potential for targeting microglia removal to highlight their functional role. However, while the authors identify microglia as the source of neuroinflammation during the hyperacute phase of ischemic stroke, they do not propose any viable solutions or interventions to address this issue.

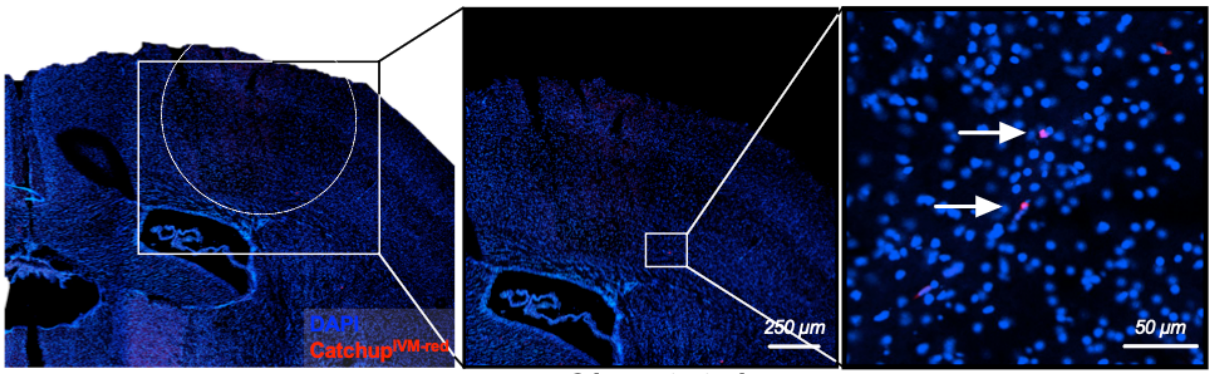
RESPONSE: We appreciate this comment and agree that a discussion of possible interventions targeting microglial-driven neuroinflammation in the hyperacute phase is important. In response, we have expanded the discussion (P16 L12):

> "Experimentally targeting microglia to limit neuroinflammation, such as blocking purinergic signaling or inflammasome activation has had preclinical success. However, therapeutically modulating microglia inflammatory activity, without impacting immune cells globally, remains a key translational challenge. Emerging techniques such as targeted nanoparticles (69), coupled with receptor-targeted peptides may allow for more precise and successful intervention in the future."

(3) In Figure 2I, the authors use CatchupIVM-red mice to observe neutrophil extravasation, but this experiment was conducted at 24 hours post-stroke, without a corresponding control experiment during the hyperacute phase. This creates some inconsistency with the claim in the manuscript that "peripheral immune cell infiltration occurs after 3 hours post-stroke, with inflammatory signals detected thereafter." Indeed, "the number of neutrophils in the ischemic tissue at 3 hours post-stroke was slightly increased compared to the contralateral or sham surgery control groups." Therefore, the authors should include complementary experiments conducted during the hyperacute phase to ensure the consistency and accuracy of the conclusion.

RESPONSE: We thank the reviewer for this suggestion. We have now included images of brain slices taken from CatchupIVM-red mice (neutrophil reporter) 3h post-stroke; images representative of 4 mice. As expected, neutrophils are scarce in brain tissue at this timepoint, which echoes our observation by flow cytometry. The very few neutrophils observed in the brain at this time have been highlighted by arrows. This data is now shown as Supplemental Figure 3a:

NEW Supp Figure 3a



(4) In Figure 2, the authors report that there is no significant change in the number of microglia in the stroke-affected hemisphere during the hyperacute phase. However, in Figure S7, the authors note that there is little to no change in the expression of classical pro-inflammatory (CD80, CD86, MHC-II, iNOS) or anti-inflammatory (CD206, Arg1, EGR2) markers in microglia on the ischemic side (IL) compared to the contralateral (CL) side or sham controls. Subsequently, the study investigates the morphological changes and cytokine secretion by microglia. Although these morphological and cytokine changes may reflect early

responses to microglial activation, they may not immediately correspond to the upregulation of classical markers such as CD80 or MHC-II. The authors should refine the discussion and ensure clearer connections between these observations.

RESPONSE: We agree that we have overlooked this in the Discussion. To address this, we have now added the following into the Discussion (P14 L27):

> "Our data indicate that microglia undergo early activation in the hyperacute phase of ischemic stroke, as evidenced by significant morphological remodeling and increased cytokine secretion. Interestingly, these functional and structural changes occur in the absence of significant alterations in microglial number (Figure 2) or in the expression of classical activation markers, such as CD80, MHC-II, or CD206 (Figure S7). This apparent discrepancy underscores the limitations of binary M1/M2 classification schemes, particularly at very early timepoints. Previous studies have demonstrated that morphological and secretory changes can precede transcriptional shifts or surface marker upregulation (Ransohoff, 2016; Masuda et al., 2020). Our findings suggest that during the hyperacute window, microglia may adopt a transitional activation state that is not well captured by canonical marker panels, highlighting the need for more temporally resolved and functionally nuanced definitions of microglial activation."

REVIEWER 2

Major Comments

1. 3 hours post-stroke is referred to in the abstract and the paper as a clinically relevant time point. But there is a significant amount of data suggesting that patients are not arriving at the hospital and being assessed for treatment within this time frame. Authors should please describe or reference more detail about why they think this is a clinically relevant time point.

RESPONSE: Three hours post-stroke is a clinically relevant timepoint because of its classical tie to the "therapeutic window" for effective treatment. Therapy by tPA was originally approved based on clinical trials (NINDS trial, 1995), which showed most efficacy if administered within 3 hours of stroke onset, albeit more recently this window has been shown to extend to 4.5 hours when aided with contrast imaging. In this study, we demonstrate that irrespective of therapeutic window expansion, neuroinflammation is already evident as early as 3 hours post-stroke, and is detrimental. This has now been clarified by inclusion of additional passage in the Introduction (P3 L46):

> "This is a clinically relevant timepoint because it aligns with the accepted "therapeutic window" for effective treatment via tissue plasmin activator (tPA). Intervention was originally approved based on the NINDS trial, which showed most efficacy if administered <3 hours of stroke onset."

2. Please describe the stereological approach to counting and analyzing cells via 2-photon microscope. When imaging ex vivo tissue, one is restricted by the thickness of the tissue (z

plane) and structure of interest (x and y planes). Was there a well-defined region of interest when imaging through a cranial window? How did you control for the volume of tissue measured across each animal?

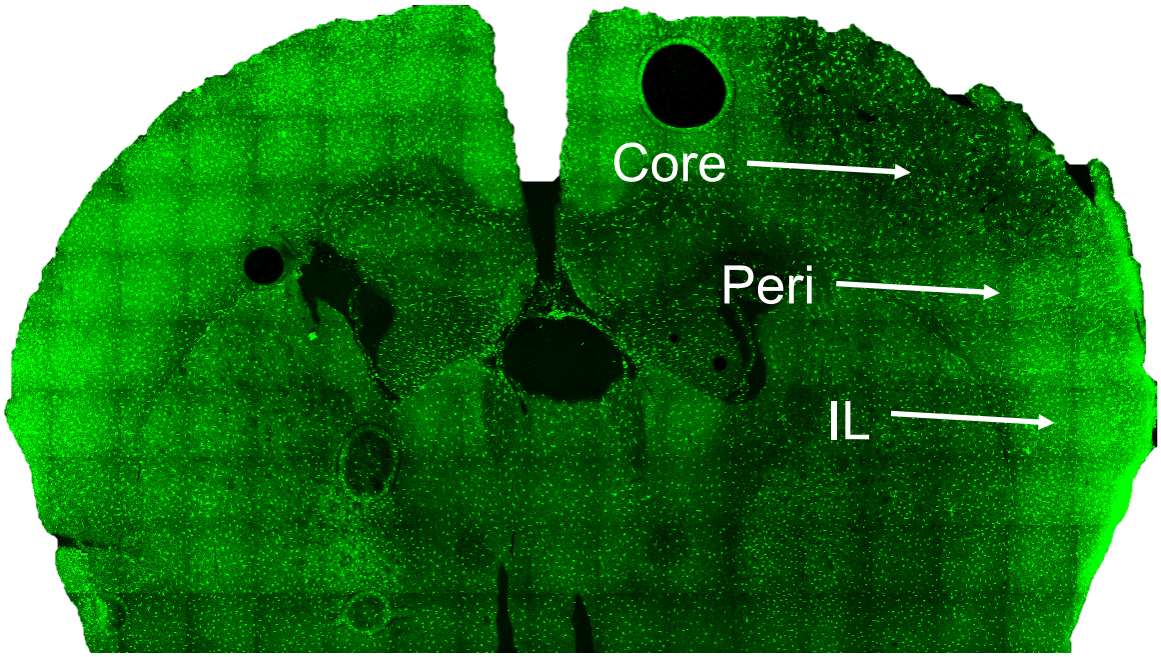
RESPONSE: For intravital 2-photon microscopy, the skull was thinned over the identical region in each animal, being the same area which has been illuminated previously to excite Rose Bengal (2 mm right and 1 mm posterior of bregma).

Intravital images were acquired in the center of this 3 mm x 3 mm thinned region. Following stroke induction, this region displayed abundant thrombi, consistent with the vascular response induced by Rose Bengal excitation. Z stacks were acquired at 0.5 µm intervals to a z-depth of 100 µm. For each image, acquisition commenced immediately below the dura layer, a structure which is easily distinguishable from brain tissue due to its thin, complex vasculature. This strategy aided consistent image acquisition across animals.

Further clarification has been added to the Methods section 'intravital two photon microscopy', beginning (page 8, line 37).

3. What brain structure were the microglia imaged in with 2-photon microscopy? The methods say image location was determined by the presences of vessel-occlusive thrombus in post-stroke mice. But it does not describe how this was used as a reference to take the image. Were the images taken only in cortical tissue? There is conclusive data to suggest heterogeneity of glial cells in different brain structures. What was the distance from the stroke border? There is a significant amount of published data about how microglia morphology change, even a short distance from the border. Was this controlled for? Please provide regional clarity for imaging and tissue collection. The anatomical locations where images were taken and tissue was collected (contralateral, peri-infarct, infarct core) aren't clearly defined in any figure or legend. A schematic or more precise description would help, especially for distinguishing ipsilateral vs peri-infarct tissue. Was a laser dissection microscope used to separate these two areas?

RESPONSE: For *ex vivo* histological cell analysis, we utilized frozen 30 µm coronal sections taken from the brains of *Cx3cr1*$^{gfp/+}$ mice, which were imaged by a 2-photon microscope. All images were acquired from either the primary motor cortex (core), primary somatosensory (peri-infarct) or supplementary somatosensory (ipsilateral) regions in the cortex. The stroke-induced infarct is identifiable by microscopy due to a change in tissue density, which is shown in Supp Fig 9, and reiterated in the figure below:

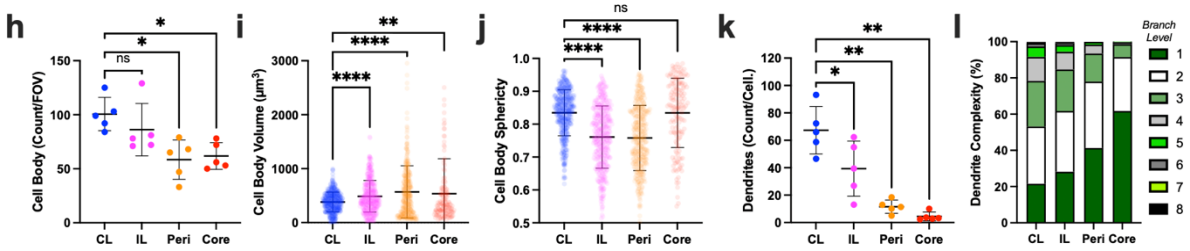


The images were acquired as per the following criteria:

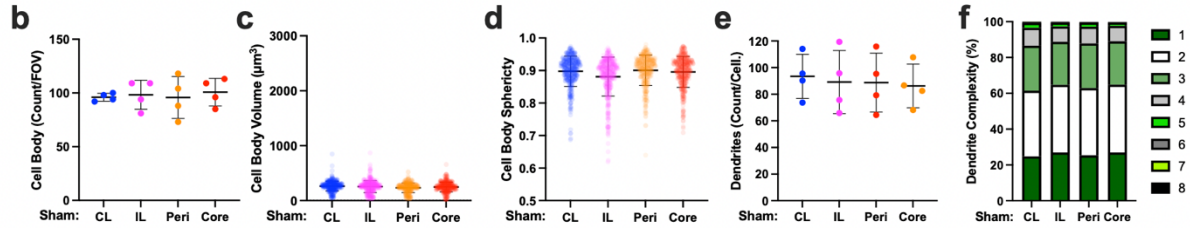
Infarct core	- a field of view in the center of the infarct.
Peri-infarct	- a field of view where the infarct edge intercepts the image.
Ipsilateral peri-infarct.	- this region was chosen as one full (20x) field of view away from the
Contralateral infarct core.	- a field of view in contralateral tissue mirroring the location of the

Whilst we acknowledge that there is regional disparity of microglia across different brain regions (i.e. between motor cortex and the thalamus), this is not expected within the motor/sensorimotor cortex. Nevertheless, as a way of controlling for potential regional differences in microglial morphology, we also analyzed microglia in the same regions of sham mice (NEW Supplemental Figure 10). With this approach, we demonstrate that the morphology of microglia does not differ across the regions of interest in sham control mice, unlike the observations from post-stroke mice (Figure 5).

FIGURE 5 – 24h post-stroke



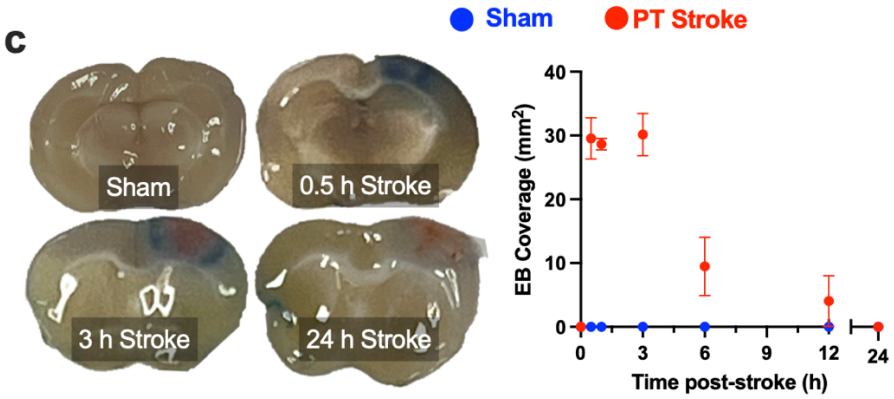
NEW SUPPLEMENTAL FIGURE 10 - SHAM



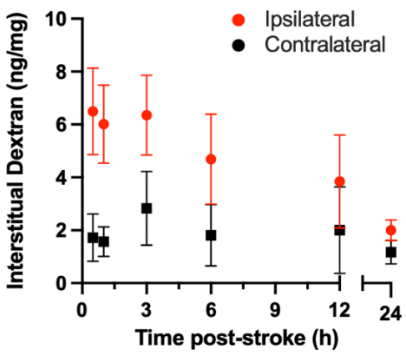
4. There are a number of significant problems with using evans blue (EB) as a marker for blood brain barrier integrity (see Saunders et al. 2015, Frontiers Neuroscience). Briefly summarized, it does not specifically bind to albumin, EB-blood level variability after injection, structure of the dye affected by vehicle solution, and potentially alternate binding. There are additional ways to measure the integrity of the blood brain barrier that are more appropriate.

RESPONSE: In the study, we have used Evans blue to visually demonstrate BBB leakage. We agree with the reviewers that Evans blue has limitations in quantifying the degree of vascular integrity breakdown, specifically the leakage of vascular proteins, such as albumin (67 kDa). To this end, we measured rhodamine B-70 kDa dextran accumulation in the tissue fluometrically in regions of brain affected by stroke (NEW SUP FIGURE 1). These data show that there is a loss of BBB integrity within 30 minutes of stroke induction, supporting the original observations generated using the Evans Blue technique (FIGURE 1).

FIGURE 1 – EVANS BLUE



NEW SUPPLEMENTAL FIGURE 1 – DEXTRAN 70 kDa



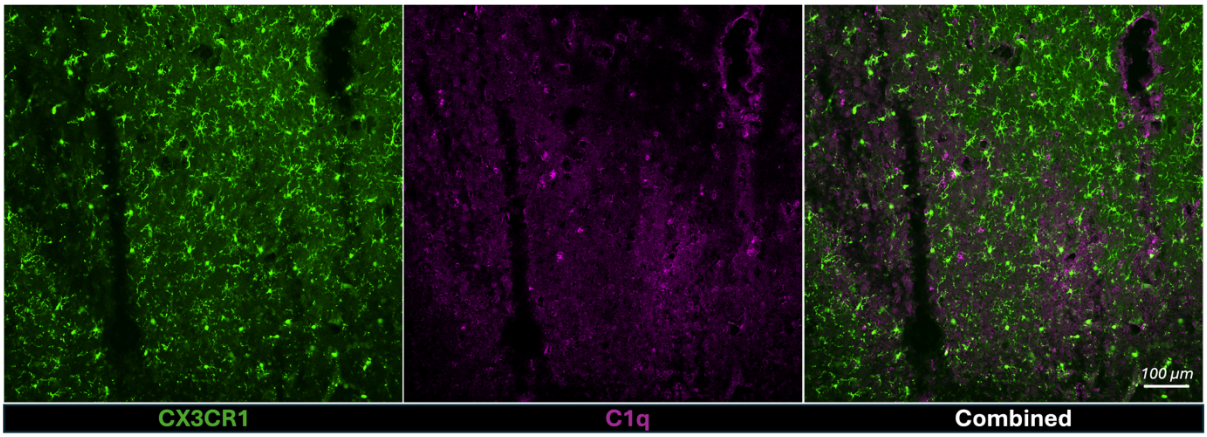
5. The data in Figure 1c suggests that the vascular integrity in the stroke core and peri-infarct area is compromised as early as 30 minutes post-stroke. This directly counters the data that they collected about the timeliness of peripheral cell infiltration. Additionally, the quantification of this method is relatively difficult to interpret, because even the representative sections provided are significantly manipulated in a way that utilizing a volumetric measurement would not be reliable. This is made further clear with the low n used (1 or 3) and the unbalanced groups. If you are not running analysis on these data, it may be best to just utilize the representative samples.

RESPONSE: It is critical to note here that the processes of increased vascular permeability and leukocyte infiltration are mechanistically distinct and do not automatically parallel each other. Increased vascular permeability is a passive process in which kDa-sized proteins normally retained within the bloodstream move out of the vasculature and into the brain tissue due to alterations in endothelial barrier function. In contrast, immune cell infiltration into the brain induced by inflammation or injury is an active process, whereby leukocytes moving rapidly in the bloodstream must first attach to the surface of the activated endothelium and then leave the vasculature via the dynamic process of diapedesis to infiltrate brain tissue. While it is not unusual for these processes to occur in tandem during an inflammatory response, a growing body of evidence indicates that they are mechanistically-distinct (Petri, Blood 2011; Wessel, Nat Immuno 2014). Given the independence of these processes, our evidence of BBB breakdown occurring earlier than the timing of leukocyte infiltration into the brain is not in conflict and is entirely plausible.

6. Based on the mechanistic data provided by the Barres laboratory suggesting that microglia release specific cytokines Il-1a, TNF, and C1q in abundance, I would have expected that these cytokine levels would have been measured or at least addressed (Fig 2b).

RESPONSE: IL-1α and TNF-α were both included in the panel shown in Figure 2b, 2c and 2e, and further investigated to a cellular level in Figure 7. We thank the reviewer for the suggestion of investigating complement protein C1q. This was not initially included as to our knowledge, C1q has not been reported to be expressed in microglia in the acute phase of neuroinflammatory disease. Reports of its expression in microglia were found in aging or associated neurodegenerative diseases (Liddelow, Nature 2017; Dejanovic, Nature Aging 2022; Scott-Hewitt, Cell 2024).

C1q is particularly difficult to quantify, and ELISA kits are only available commercially to quantify C1q in serum, not tissue homogenate. To this end, we stained and co-localized C1q (7H8, Invitrogen) to microglia in the brains of *Cx3cr1*$^{gfp/+}$ mice.



These images are representative of 3 mice, and were taken in the infarct core 3h post-stroke. It is of note that C1q was not detected in the contralateral tissue, nor in sham controls. Based on the fluorescence of C1q (magenta), it does appear that this complement factor is upregulated in response to stroke. However, the C1q detected does not co-localize with microglia (CX3CR1, green). As our study was focused on cytokine generation, and in the absence of a known cellular source, this data was not added to the main manuscript.

7. The interpretation that only microglia and neurons are responsible for cytokine release (section title) is not directly supported. Rather, it was demonstrated that there are four specific cytokines that are altered in microglia in neurons, but not other cell types. Additionally, the culturing of isolated cells from flow cytometry to measure cytokine production has inherent issues. For example, chemical and mechanical disruption of the cells is required with this technique. This interpretation would be circumvented if the methodology used to measure these cytokines was to sort cells or immunoprecipitate cell-specific RNA and then run sequencing to measure all cytokine production, instead of being selective. The authors need to update their analysis or word their interpretation much differently.

RESPONSE: We agree with reviewers that our study has limitations. We cultured cells to allow detection of cytokine protein production, rather than RNA levels, which is more biologically relevant. In order to quantify intracellular cytokines, cells need to be cultured in the presence of Brefeldin, to prevent cytokines release into their environment, allowing analysis of cytokine production to a cellular level. The aim of this study was to determine the source of the 4 cytokines which are most significantly upregulated during the hyperacute phase of stroke, which was achieved using this methodology.

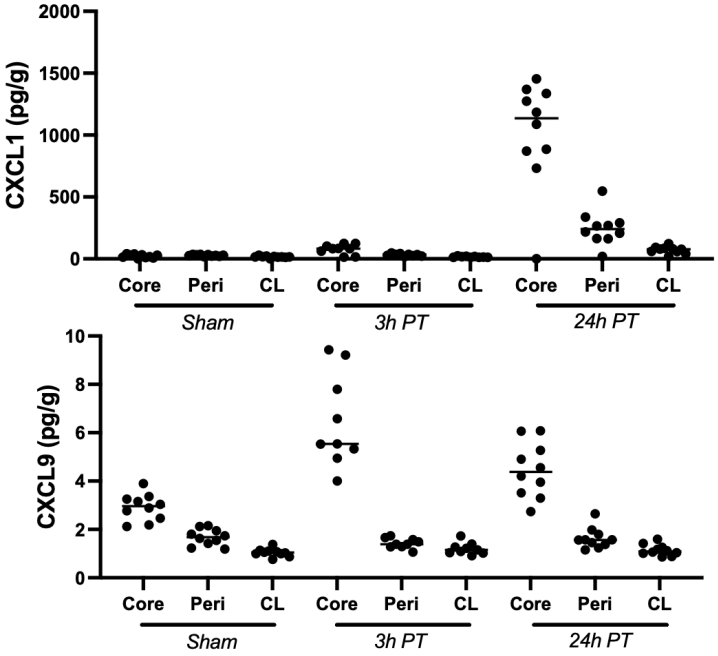
Single cell RNA sequencing of microglia is currently being planned in our lab for a future study, although similar to the cytokine studies described above, this technique also requires disruption of the tissues for cell isolation. For the purpose of this study, we have reworded our interpretation as advised by the reviewer in the introduction (p4 line 6):

> "Our study provides evidence that microglia are the key producers of the major inflammatory cytokines (TNF-α, IL-6, and IL-1β) detected within the hyperacute phase of ischemic stroke…"

8. The interpretation that astrocytes are not early responders in direct contradiction to a host of other research suggesting that astrocytes respond within minutes and upregulate cytokines like IL-6, IL-1beta, TNF, IL-17, CXCL1, CXCL9, and NfkBeta. While this study only measured IL-6 (of this list), they interpreted that astrocytes are not a source of pro-inflammatory cytokines at 3 hours post-stroke. The authors should at the very least address this confliction between their data and the literature.

RESPONSE: In contrast to the statement from the reviewer, we did measure IL-6, TNF-α and IL-1β specifically in astrocytes (GLAST-1+) in Supplemental Figure 11. IL-17A was included in the initial screen of cytokines in Figure 2, and was not detected in brain homogenate in the hyperacute, nor acute phases following stroke, hence was not further investigated.

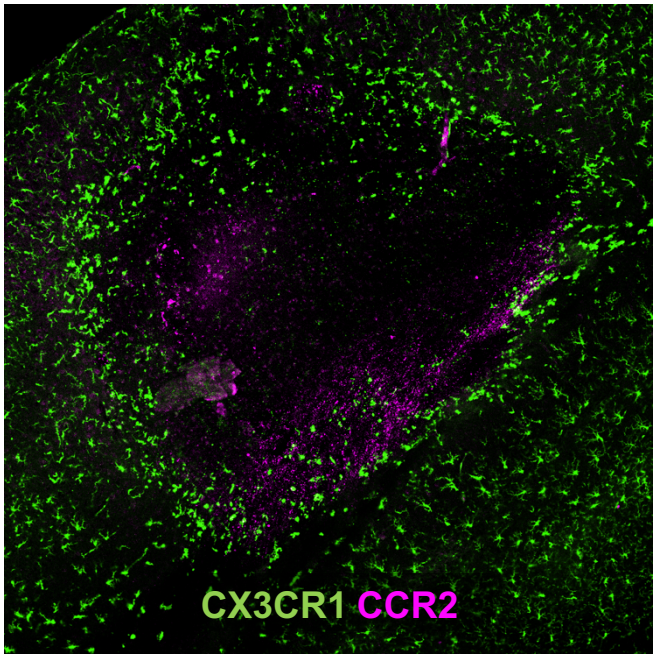
We have analyzed CXCL1 and CXCL9 in brain homogenate 3h and 24h post-stroke. CXCL1 in the infarct core was negligible 3h post-stroke, but notably increased in the acute phase (24h) post-stroke, drawing parallels with peripheral immune infiltration. CXCL9 was negligible in the brain following stroke onset up to 24h. This data is the basis of a future study, and was not included in the main text.

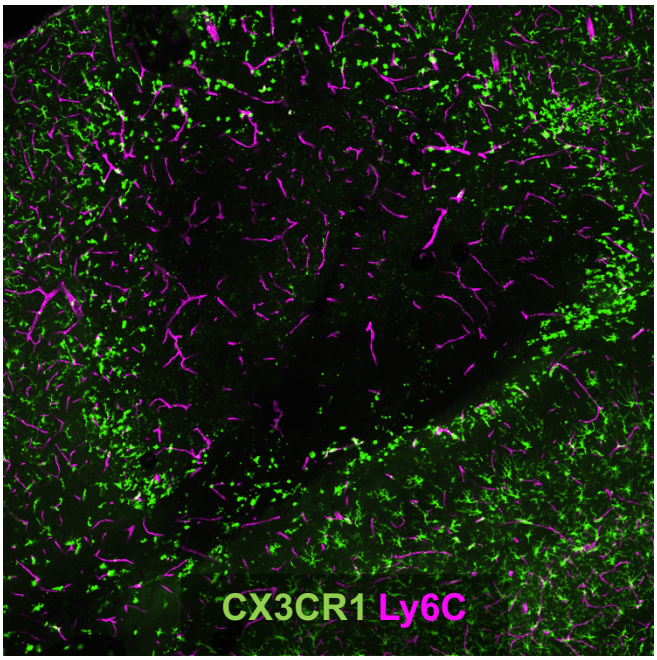


9. There are significantly more monocytes present at 3 h post stroke Fig 2L. While there are much fewer than later and much fewer than microglia at this point, it is still possible they are the primary producers of cytokines at this time point and are driving the pro-inflammatory escalation. If there is data about the ratio of cytokine release between activated microglia vs. monocytes, this should be cited and there should be room left in the interpretation for this possibility. On a related note, since you are eliminating all but about 20% of monocytes from the periphery (Fig 3a-c), it would be nice to have a representative picture (or quantification) of these cells in the infarct core and peri infarct area. If these small number of remaining cells are still penetrating the BBB, they may still be the primary producers of

these cytokines.

RESPONSE: We detected an average of 77 monocytes in the stroke-impacted brain hemisphere at 3h post-stroke. This is a 3-fold increase when compared to sham control at this timepoint (26/hemisphere), which is statistically significant. However, this number is overshadowed by the 55,000 microglia present in this region, bringing into question the relative biological contribution of monocytes at this time point, or indeed at the later time point of 24h, when monocyte infiltration was >1,100. With this in mind, it is unlikely that monocytes contribute to the inflammatory storm in the hyperacute phase following stroke. This is supported by the data in Figure 5, which demonstrates that the stroke-induced inflammatory storm persists in the absence of infiltrating monocytes (CCR2-KO mouse). Staining for monocytes is challenging, as the antigens utilized for their detection by flow cytometry are either shared by other cell types (Ly6C – endothelium) or short chemokine-receptor binding sites are masked by fixative (CCR2). Attempts are shown below:





For this reason, monocyte immunofluorescent staining was omitted from the manuscript, and quantification of their brain-infiltration was performed using flow cytometry alone.

Minor Comments

1. In the introduction, (line 87-88) it is suggested that the authors are taking a holistic approach to characterizing the entire immune composition in the brain, but this is not accurate. The authors are characterizing microglia and cytokines. While extremely important, the majority of their analyses do not include a number of other cells (ex. astrocytes, oligodendrocytes, endothelial cells, etc.) and molecules (ex. lipids, vesicles, reactive oxygen species) that are crucial for the neuroinflammatory response. Please rephrase the description of the approach.

RESPONSE: Thank you for this suggestion. We agree that we do not critically analyze all cell types in the brain. For this reason, in the revised Introduction, we have specified that we analyze the immune composition of the brain, and their inflammatory cytokine production. Updated (P3 L11):

> "…we propose that a characterization of the entire immune composition and cytokine levels in the brain during the hyperacute stage of stroke onset would provide insights into initiation the inflammatory response."

2. While the introduction discusses clinical trial interventions regarding infiltrating immune cell interventions, they do not spend time setting up the body of literature about the timing when these cells infiltrate (either in mouse models or humans), which seems strange since the main goal of this paper is to examine the hyperacute and acute stages, a time when they claim these infiltrating cells are not present. At the very least, I would expect some description of this body of literature related to timeliness of cell infiltration.

RESPONSE: Thank you for this suggestion. We agree that a clearer discussion of the timing of immune cell infiltration in the brain after stroke would be beneficial, especially given the focus of our study on the hyperacute and acute phases. In the revised manuscript, we have expanded the Introduction to include a brief overview of the temporal dynamics of immune cell infiltration observed in both preclinical models and clinical studies. This has been updated (P3 L21):

> "In animal models, neutrophils have been shown to accumulate in the perivascular space and adjacent parenchyma within 12–24 hours post-stroke, with more robust parenchymal infiltration typically evident at 1–3 days (10,14). Monocyte-derived macrophages tend to infiltrate more slowly, generally becoming prominent after 48 hours (8,15). In humans, while imaging and CSF data suggest a similar delayed infiltration, definitive timing remains difficult to resolve due to variability in sampling (16)"

3. Please provide justification for the use of young mice (7-12 weeks of age) for a mouse model of a human condition that primarily occurs in the later stages of life. There is data to suggest that there may be significant neuroinflammatory and pathological differences between younger mice and older mice. Therefore, 5 weeks is a wide range of mice to use to measure post-stroke pathology and assume that the response would be the same in 7-week-old mice and 12-week-old mice.

RESPONSE: We thank reviewers for noticing this. Mice were not used beyond 10 weeks old, and this has been updated in the Methods.

We acknowledge that stroke is classically an aged disease, and our group has previously demonstrated that aged mice (>12 months) have significantly increased neurological deficit compared to young mice post-stroke (Wen et al, Aging Cell 2019). That being said, in an effort to characterize the initiation of neuroinflammation post stroke, we elected to minimize the potential contribution from confounding inflammatory disorders associated with aging. Mice aged 1-4 months are classified as 'young adults' (Radulescu, Cell Calcium

2021), a stage of development which Jax Labs declare that mice are not damaged by inflammatory implications of aging. Therefore, we believe that using young mice is necessary to understand the impact of stroke, and not aging, on post-stroke neuroinflammation.

4. I understand why the markers for platelets and vasculature (and Nk cells) were used, but since there is no data or even qualitative mention of the relationship between them and microglia, why are they being displayed in Figure 5 and Supp Fig 8?

RESPONSE: Platelets and vasculature has now been mentioned in the text (P12 L37):

> "Interestingly, microglia in the infarct core and peri-infarct regions appear to be co-localized with thrombi-rich (GPIBβ, cyan) vasculature (Laminin, red) at hyperacute and acute timepoints."

5. To interpret the microglia morphology data, it needs to be clear that the distribution of this fractalkine receptor is distributed across the membrane. If there have been other data suggesting Cx3cr1 distribution is consistent and spread equally, please cite it. Otherwise, dual labeling some microglia with Cx3cr1-GFP and another microglia marker like Iba1 (commonly used) might be helpful to demonstrate that the morphological assessment is similar.

RESPONSE: Using the *Cx3cr1*$^{gfp/+}$ mice as a reporter for microglial cells has been done in many studies and is well accepted in the microglia community (Paolicelli, Neuron 2022).

Whilst the Iba1 is commonly used, detection of its expression by staining or genetic modification (reporter mice) is less optimal due to it being notably dimmer compared to the *Cx3cr1*$^{gfp/+}$ mice. For this reason, Iba1-staining or reporter mice is generally used for identification of microglia, and less so for microglial morphological analysis.

6. Cx3cr1 expression occurs in resident microglia and peripheral monocytes. If the authors are using some type of inducible system (like cre/tamoxifen) and waiting for peripheral cells to overturn, this should be described in the methodology in detail. If they are not, they should describe how the GFP imaging done could be either infiltrating monocytes or resident microglia.

RESPONSE: We agree with reviews on this point, and this has been added to limitations (P15 L 47):

> "Furthermore, we accept that expression of CX3CR1 is not limited to microglia, and is known to be presented on a sub-population of non-classical monocytes and monocyte-derived macrophages. However, these cell types are not common in the brain at the acute timepoints here chosen following stroke onset, and the *Cx3cr1*$^{gfp/+}$ mouse is a well-accepted tool for microglia morphological and functional analysis in the community."

7. Sample size reporting is missing for Figure 5. The caption reports significance (e.g., $p < 0.001$), but we don't specify how many animals or cells were analyzed per group. Including n values (both animals and cells per region) would strengthen the statistical interpretation.

RESPONSE: This has been updated in the legend of Figure 5:
(n=5 mice; individual cells are plotted for volume and sphericity [≈n=500/region])

8. Image sampling for microglia morphology assessment. It's unclear how many images were collected per brain section and how many sections were analyzed per animal. This detail is important for assessing representativeness and should be added either in the figure legend or methods.

RESPONSE: Further clarification has been added to the methods section (P8 L25):

> "Three regions of brain tissue were identified per mouse in the ipsilateral tissue: infarct core, the peri-infarct region and a region distal to the infarct core. One FOV was acquired of the contralateral tissue, which was in a position mirroring the infarct location."

9. Was there an XY plane exclusion criterion used for analysis? In the methods, it says "cells were manually removed if they extended significantly beyond the XY plane." Was this determined manually or with a software-based cutoff? If manual, a brief explanation of the criteria used would improve transparency.

RESPONSE: We thank the reviewer for noticing this. Our criteria for removal of cells from analysis is if the cell **body** crosses the XY plane, which we manually remove on the IMARIS software. This has been adjusted in the text (P9 L10):

> "Cells were manually removed and discounted from analysis using IMARIS if the cell body extended beyond the XY plane"

10. Thresholding and normalization: There's currently no detail on how thresholding was performed during 3D reconstruction (e.g., global vs region-specific), or whether any normalization was applied across samples. This is especially relevant since intensity and tissue integrity can vary quite a bit post-stroke, and these differences could bias dendrite complexity or volume measurements.

RESPONSE: These details have now been added (P9 L14):

> "starting point 18 µm; 'Use surface as markers'; Seed point start 1.5 µm; seed threshold automatically defined; terminal ends 3 µm; cylinder 1.5 µm with detail 1 µm."

Reviewer #1 (Remarks to the Author):

I have carefully reviewed the revised manuscript by Bourne et al. and their detailed point-by-point rebuttal in response to the initial review. The study aims to dissect the temporal and cellular drivers of neuroinflammation in the hyperacute phase of ischemic stroke, a critical but under-investigated period with clinical relevance. Overall, the authors have provided substantial clarifications and experimental additions in response to the reviewers' comments.

Response: We thank the reviewer for their time, and their kind words.

Remaining Concerns:

- Interpretational Overreach: Despite rewording, the assertion that microglia are the primary cytokine producers still risks overgeneralization. While the methodology supports a dominant role, the limitations of ex vivo culture and intracellular staining merit a more cautious tone.
- Astrocytic Contribution: The field recognizes astrocytes as early responders. The data shown here are limited in scope (GLAST+ astrocytes, few cytokines), and alternative mechanisms (e.g., gliotransmission) remain unexplored.
- Microglial Heterogeneity: While the authors cite recent scRNA-seq studies (e.g., Patir et al. 2024, Paolicelli et al. 2022), they do not fully contextualize how hyperacute phenotypes may relate to known DAM, IFN-responsive, or PLX-resistant clusters.

Response: We agree with the reviewer that limitations of our study must be added to avoid interpretational overreach. As such, we have added the following to the discussion (P15 L2):

> "Our study has provided evidence that microglia are dominant producers of inflammatory cytokines in the hyperacute phase following ischemic stroke. We used an *ex vivo* culture approach to quantify the production of cytokines, but have not discriminated for microglia sub-type contribution. Furthermore, astrocytes have been shown to rapidly respond to ischemia, notably through reactive gliosis (and later glial scar formation) (Shen et al., *Front Cell Neuro*, 2021), which was not assessed with our experimental design. Future studies implementing a single cell 'omics' approach will delineate heterogenous cell populations and further characterise the roles glial cells play in the early response to ischemia."

Reviewer #2 (Remarks to the Author):

The authors have satisfied both my major and my minor comments. Thank you for detailed responses and collection of additional data to demonstrate and interpret your findings.

Response: We thank the reviewer for their time, and their kind words.

In the manuscript entitled "Microglia are prominent producers of inflammatory cytokines during the hyperacute phase of ischemic stroke", Bourne *et al.* provide the molecular mechanisms underlying ischemic stroke-induced neuroinflammation, clarifying that microglia, rather than the infiltration of peripheral immune cells, are the primary producers of inflammatory cytokines during the hyperacute phase of ischemic stroke. The comments are given as follows.

(1) The study utilizes 3 hours post-stroke (hyperacute phase) and 24 hours post-stroke (acute phase) as experimental time points. However, they did not provide a clear rationale for the selection of these specific time points. It remains unclear whether these time points were chosen based on clinical considerations or derived from prior experimental findings. The authors should clarify the reasoning behind the selection of these time points.

(2) A significant portion of the article addresses the role of peripheral immune cell-derived cytokines in neuroinflammation during the hyperacute phase, arguing that these cytokines are not the primary drivers of neuroinflammation at this stage. From Figure 5 onwards, the study illustrates morphological changes in microglia, and in Figure 7, the upregulation of cytokine secretion by microglia is demonstrated. In the discussion, the authors mention the potential for targeting microglia

removal to highlight their functional role. However, while the authors identify microglia as the source of neuroinflammation during the hyperacute phase of ischemic stroke, they do not propose any viable solutions or interventions to address this issue.

(3) In Figure 2I, the authors use CatchupIVM-red mice to observe neutrophil extravasation, but this experiment was conducted at 24 hours post-stroke, without a corresponding control experiment during the hyperacute phase. This creates some inconsistency with the claim in the manuscript that "peripheral immune cell infiltration occurs after 3 hours post-stroke, with inflammatory signals detected thereafter." Indeed, "the number of neutrophils in the ischemic tissue at 3 hours post-stroke was slightly increased compared to the contralateral or sham surgery control groups." Therefore, the authors should include complementary experiments conducted during the hyperacute phase to ensure the consistency and accuracy of the conclusion.

(4) In Figure 2, the authors report that there is no significant change in the number of microglia in the stroke-affected hemisphere during the hyperacute phase. However, in Figure S7, the authors note that there is little to no change in the expression of classical pro-inflammatory (CD80, CD86, MHC-II, iNOS) or anti-inflammatory (CD206, Arg1, EGR2) markers in

microglia on the ischemic side (IL) compared to the contralateral (CL) side or sham controls. Subsequently, the study investigates the morphological changes and cytokine secretion by microglia. Although these morphological and cytokine changes may reflect early responses to microglial activation, they may not immediately correspond to the upregulation of classical markers such as CD80 or MHC-II. The authors should refine the discussion and ensure clearer connections between these observations.