

Repair-Associated Macrophages Increase after Early-phase Microglia Attenuation to Promote Ischemic Stroke Recovery

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The study by Xiaotao Zhang et al. studies brain cells and infiltrating leukocytes after stroke using scRNAseq. The mice were transiently (5-7 days) exposed to a treatment in the diet with a CSF1R inhibitor (PLX5622) followed by restoration of normal chow for another week or up to 35 days post-stroke. The treatment can only transiently reduce the number of microglia since it is short duration and mice do not eat much after stroke. Mice that received this treatment showed reduced infarct volume and a better neurological function 35 days post-stroke. Moreover, at day 14 post-stroke, the mice showed improved vascular coverage and signs of more myelin. The study identified a subset of macrophages, rich in lipids, with a transcriptomic profile compatible with reparative functions. The presence of this subset of macrophages was more prominent when the mice were transiently exposed to a treatment in the diet that reduces the number of microglia followed by a recovery of microglial cell numbers. The actual cause for the enrichment in this potentially reparative macrophage subset in the stroked brain after treatment is not entirely clear. While the study touches points of interest and provides novel data, there are a number of issues that need careful attention.

1. Fig. 1B shows immunolabeling with several markers. From the images shown, it is interpreted that there is a decrease of Tmem119+ microglia, as expected. The reduction of the number of microglial cells after this treatment post-ischemia should be provided. Normally, treatment with PLX5622 requires at least two weeks of diet for a strong microglia depletion.

Moreover, microglia proliferate after stroke. Furthermore, the mice do not eat very much following stroke. Therefore, the efficiency of the depletion of microglia should be shown. It seems that this model may reduce but it is most unlikely to cause severe microglia depletion. Please provide absolute microglia numbers.

2. Fig. 1B. Please provide information of the regions sampled anatomically and in relation to the brain lesion. There is no quantification of the immunofluorescence. Therefore, it is not possible to figure out the microglia reduction from these images.

3. Fig. 1C. Values are expressed as number of cells per 1,000 single cells. Given that mice treated with PLX5622 are depleted of microglia, it is expected that after treatment the number of macrophages is higher per 1,000 given cells, since they do not contain microglia. Please provide the absolute numbers of cells counts.

4. How many microglial cells were analyzed in the single cell experiment (Fig. 1D and Fig. S2) after depletion of microglia with PLX5622?

5. Please revise in the text (page 6) the interpretation of results. Fig. 1E does not show increase in the number of macrophages, but a higher proportion of macrophages in the PLX5622.

6. Please provide plots as in Fig. S2D for proinflammatory cytokines, eg. Il1b and the inflammasome pathway.

7. Fig. 1F shows that infiltrating monocytes in PLX5622-treated mice upregulate brain-repair functions. However, it is not possible to know whether this phenomenon is due to microglia depletion or to depletion of peripheral myeloid cells.

8. In supplementary Fig. S2E, the MRI images show large cortico-striatal infarctions 1-day post-ischemia to illustrate that the lesions in the mice assigned to each group, prior to microglia depletion-repopulation, are similar. Fig. 1H shows the brain of mice (certainly not the same as shown in Fig. S2E), one of them with a cortico-striatal infarction and the other with a striatal infarction. These two mice are not comparable. Do the infarct volume measures shown in Fig. 1H correspond to the same mice as those shown in Fig. 2E, F? The progression of the lesion in the same mice should be shown. The PLX5622 mouse shown in Fig. 1H (35 days) does not have cortico-striatal infarction as the mouse of the corresponding group at day 1 shown in Fig. S2E. This is very confusing.

9. The quantification of the lesion volume shown in Fig. 1H, 35 days post-ischemia, should be accompanied by a measure of brain atrophy. The lesion is partly re-absorbed. Therefore, a smaller affected volume at this long time point after stroke onset may be due to a better resolution of the lesion rather than to reduced infarction.

10. Figure 11. The time to removal (s) appears to be expressed as an absolute value. Was there any normalization step performed? It is stated in the methods section that the maximum time to removal was 120 s. How could it be then that the error bar reaches nearly 150 s?
11. Were the behavioral tests and infarct volume measurements performed by researchers blinded to treatment?
12. Referring to the experiment where mice are treated for 5 days with PLX5622 and then returned to control diet until day 14 post-ischemia, regarding the sentence in page 7: 'there was an increase in the cell fractions of oligodendrocyte lineage cells (OLG and OPC) and endothelial cells (Figure 2B), suggesting a potential direct effect of early-phase microglia attenuation on white matter and vascular integrity'. There seems to be a reduction of infiltrating macrophages. Did the diet affect circulating myeloid cells? The study does not demonstrate whether the reduction of the microglia or the reduction of circulating monocytes, or rather the induction of repopulation, after discontinuation of the PLX5622 diet, is responsible for any increase in oligodendrocytes and endothelial cells at day 14.
13. The reduced SM32/MBP ratio in the PLX5622 group is interpreted as a sign of remyelination but it could also be a sign of reduced demyelination.
14. Please detail how was vascular coverage measured. How many sections per mouse and fields per section were counted? Following ischemia, the loss of endothelial cells depends on the volume of the lesion. Please provide the volume of the lesion of the mice shown in figure 2L.
15. Compared to resident microglia, more repopulating microglia remain in a resting state 14 days post-stroke (top of page 9). Fig. S4 refers to microglia subset in ischemic mice. How do they compare with the microglia of non-ischemic control mice?
16. Fig. S5A and C show blood monocytes from sham mice and brain macrophages from ischemic mice with and without PLX5622 treatment, at day 14 or 5 post-ischemia, respectively. Corresponding monocytes of PLX5622-treated mice should be shown to find out the impact of the treatment in the periphery. Moreover, the subsets of blood monocytes at the ischemic time points should be shown, since the populations of circulating myeloid cells are affected by brain ischemia. It will be interesting to see the UMAP projections of brain macrophages only.
17. I cannot find the absolute numbers of infiltrating macrophages at the different time points after ischemia and the effect of PLX5622.
18. Dendritic cells infiltrate the ischemic brain tissue. According to the markers, the cells shown in Fig. 3H could be infiltrating dendritic cells. Could the MF3 cells be infiltrating DCs?
19. Systemic clodronate injection after ischemia may directly affect the brain phagocytes, i.e. microglia and macrophages, due ischemia-induced blood-brain barrier breakdown. Therefore, it is difficult to attribute all effects to depletion of circulating cells.
20. In the experiment shown in Fig. 3L and M, the control for cell transplantation is PBS. Transplantation with macrophages other than GPNMB+CD63+ macrophages is necessary to extrapolate that these cells promote axonal remyelination.
21. Fig. 4A is an interpretation of the findings, not a direct result.
22. Fig. 4D shows an example of Iba1+ cells, some positive for GPNMB, at the vicinity of myelin. It is unclear whether these cells phagocytose myelin and whether other subsets of macrophages or microglia can also do it. The result does not demonstrate that those cells are the unique cell subset able to phagocytose myelin.
23. Lipid accumulation in myeloid cells of the brain after stroke has been demonstrated in previous works that are not cited, e.g. Arbaizar-Roviroso et al. (2023) doi: 10.15252/emmm.202217175; Zbesko JC, et al. (2023) doi: 10.1016/j.nbd.2023.106130.
24. Please notice that the images and quantification in Fig. 4F cannot distinguish macrophages from microglia. Why quantification is carried out in the periinfarct area where microglia accumulate rather than in the core of the lesion where macrophages accumulate?
25. Gating strategy in Fig. 4G, have CD45 CD11b and other markers been used before to separate macrophages?
26. The method used to quantify the % of CD31+ vessel area expressing ZO-1 should be explained in the Methods section. Which brain regions, how many sections per mice, how many areas per section and region? Was quantification performed in a blinded fashion?
27. Degradation of tight junction proteins may be the result of more severe ischemia and may lead to BBB breakdown. Was the integrity of the BBB poorer in mice under control diet?
28. For how long do the macrophages transplanted into the brain survive?
29. Please name the X-axis of graph in panel of Fig 5I. How was vascular coverage quantified?
30. The in vitro study is performed in BMDMs. How is the transcriptomic profile of these cells compared to that of the MF2 macrophage subset found in ischemic brain tissue?
31. The effect of Mafb lentivirus treatment versus control lentivirus on Mafb expression and cell survival prior to macrophage reconstitution of Ccr2-KO mice should be shown. Is the number of circulating and brain infiltrating macrophages similar for both groups of mice with lentiviral-treated macrophages (lenti-Mafb vs. lenti-control)?
32. Were any animals excluded in this study? Please report mortality, if any, in the different experiments.
33. I believe behavioral data, particularly the Fault/total test, shown in Fig. 11 do not correspond to continuous data and it is not possible to use parametric tests. Two-way ANOVA is the test currently used. Please revise.
34. I am not convinced about the title since I believe the study does not demonstrate that microglia attenuation provides a spatial niche for repair associated macrophages to promote ischemic stroke recovery. The reasons are as follows:
 - The niche is not depleted, but numbers of microglia are reduced during a few days (this is a point that has to be better shown provided actual cell numbers).
 - The benefit is shown in Fig. 11-H 35 days post stroke while the microglia numbers should have recovered after 5-7 days of discontinuation of the treatment.
 - It could be the repopulating microglia rather than the 'depleted niche' the one that is responsible to attract the macrophages or to promote the reparative phenotype. In any case, I believe this point is not demonstrated.
 - The demonstrations supporting that the reduced volume of the lesion and improved functional outcome is entirely attributable to the subset of phenotypically reparative macrophages and the key role of Mab are not very robust.

Reviewer #2

(Remarks to the Author)

This manuscript examines the role of microglia in ischemic stroke and used microglial depletion strategies (PLX5622) to determine if changing the spatial niche (for peripheral immune cells) once microglia were depleted had an impact on outcome. This has been done in prior studies, but this work used single cell sequencing, which adds novelty.

Depletion of microglia early after stroke enhanced long term recovery after experimental ischemic stroke. Monocyte derived macrophages (termed “reparative or RAMf) were found, that had increased debris clearance, lipid droplet formation and lipid efflux “thereby promoting remyelination through enhanced lipid recycling”. However, most of the data involves gene changes, and a major concern is whether the manipulations altered the actual ischemic injury. If so, changes (such as improved white matter, vessel coverage etc.) could just be related to the extent of ischemic injury. This is a difficult problem to address. There is some evidence of actual remyelination/angiogenesis by markers such as BRDU and Ki67, but these studies are pretty minimal.

The main findings are that with microglial depletion (at a relatively lower level than expected with PLX) led to repopulation of the niche with peripheral cells, as has been shown in other studies.

Five microglial subpopulations were identified 14 days after stroke (7 days after PLX withdrawal). They focused on “repair associated macrophages” as defined by their sequencing characteristics and describe a transcription factor, *Mafb* that governs the repair phenotype.

-A limitation is that all studies were performed in young male mice, and aging alters the transcriptional and functional role of microglia, and is translationally less relevant. There are no details on mortality, randomization or blinding except for the behavioral studies.

-Investigation of outcomes at more chronic phase (a month after stroke) is a strength

-62% depletion is relatively low

-Do the CCR2 mice used have a knock out allele (for the GFP)? Could that influence the results?

-PLEX diet has off target effects outside of the microglial niche, this is noted, and genetic models are proposed for future studies, but this is a limitation

-Comments like “suggesting that early-phase microglia attenuation could promote oligodendrogenesis after ischemic stroke” based on gene profiles is tenuous, without showing actual evidence of oligodendrogenesis.

Similarly, “we conducted scRNA-seq on enriched CD45^{high} infiltrating immune cells sorted from the ischemic mouse brain at 14 dpi to identify the cell types “contributing to remyelination and angiogenesis” without showing actual remyelination or angiogenesis is an overstatement, they are just describing genes that are present. Although the authors did examine the number of ZO-1+ vessels, PLX treatment led to smaller infarcts, so is this just an epiphenomenon due to the reduction in infarct damage?

The most mechanistic work involves the use of siRNA to suppress a target, *Mafb*, done in bone marrow cells in vitro (presumably also from young donors) and in vivo, but stroke size is not mentioned in many of the studies.

Are there off target effects of giving clodronate prior to stroke on infarct size?

The use of an acute intervention with PLX led to significant changes in infarct size, which does lead to multiple confounds. It is not surprising that behavior was improved in treated mice, or that it reduced the SMI/MBP ratio or increased vascular integrity (Fig 5, Ki67 not shown except graphically). Was there a difference in infarct in the RAMf transplanted mice?

Also this is needed for Figure 6, as if there are differences in infarct size, this needs to be mentioned.

Was there a difference in the infarct volume in CCR2 mice (Figure 1)?

Figure 2 showing BRDU could be improved, as this is really the only evidence of angiogenesis or remyelination shown histologically.

Was there a difference in infarct size in the mice transplanted with CD45^{high} CD11b⁺ CD63⁺ GPNMB⁺? Again, it is hard to know if this is “preservation” of myelin (not enhanced remyelination) otherwise.

Reviewer #3

(Remarks to the Author)

The study by Zhang et al. investigates the implication of peripheral immune cells in stroke recovery after partial depletion of microglia. Microglia number was attenuated in the stroke acute phase by feeding the mice with PLX5622 diet during the first 5 or 7 days after induction of focal cerebral ischemia (transient MCAO model). Single-cell transcriptomic analysis 5 and 14 days after injury and flow cytometry experiments, indicated expansion of a macrophage subpopulation (RAMf) with reparative characteristics in the PLX5622 group as compared to controls. Additionally, lipidomic, flow cytometry assays and immunohistochemistry studies showed that RAMf significantly contributed to remyelination and angiogenesis. Furthermore, both in vitro and in vivo approaches supported a key role for the transcription factor *Mafb* in regulating the reparative

phenotype of RAMf.

This is an interesting study investigating the contribution of infiltrating leukocytes in tissue repair in response to experimental stroke. The manuscript reads well, and it provides evidence of a role of macrophages in tissue remodeling. The employed approach of modulating the immune niche competition by decreasing the number of early-phase microglia, might be relevant to open new avenues for potential therapeutic approaches for stroke. My specific comments for consideration by the authors are as follows:

Major comments:

1. For the experiments of RAMf transplant into mice undergoing cerebral ischemia, PBS is not the right control to support a pro-reparative function of RAMf. The transplant of GPNMBNegCD63Neg macrophages would be the best approach to use as control group.
2. CCR2-knockout mice were reconstituted with BMDMs transduced with either *Mafb* or control lentivirus to assess the role of *Mafb* in fostering remyelination and angiogenesis after ischemic stroke (lines 405-409). These experiments are key to support the role of *Mafb* in regulating RAMf function and therefore, they should be described in more detail. The authors indicated that CCR2-KO mice were reconstituted with BMDM, but it is not clear whether transduced BMDM were adoptively transferred into mice. In that case, the presence of transferred BMDM in the brain should be shown. Also, were BMDMs transferred into mice before or after stroke?
3. Monocytes/macrophages scRNAseq data from sham mouse brain and/or from stroke mouse blood should be used to best infer pseudotime trajectory analysis of macrophages (Fig. 3C-D and Suppl. Fig. 5F).

Other comments:

4. Please indicate whether the *Ccr2*-GFP mice used in the study were homo- or heterozygous. The insertion of GFP in the mouse strain used in the study (RRID:IMSR_JAX:027619) abolishes the gene expression of CCR2, and therefore *Ccr2*gfp/gfp homozygous are CCR2-KO. A completely lack of infiltrating macrophages in the ischemic brain of CCR2-KO mice has been reported.
5. The data shown in Figures 1C and 1E do not match. Fig. 1C shows that the number of NK cells, B cells and T cells are higher in the ischemic brain of PLX5622 treated mice as compared with control diet, but the proportions over the total leukocyte infiltrate of these same cell types are lower in the PLX5622 group in Fig. 1E.
6. Does Fig. 1H show volume of tissue loss? Or does it show infarct volume (MAP2 lesion)?
7. Suppl. Fig. 1C: CD45hiCD11b+ gate is defined as macrophages, which is not correct since it also will include neutrophils, as well as some DC.
8. For a better understanding of the conclusions drawn from the comparison between the studies performed at 5- and 14-day time points, it would be helpful to report the amount of microglia depleted after 7 days of PLX5622 treatment as well as the levels of recovered microglia at 14 days. These results are only reported for the study performed at day 5 post-MCAO.
9. The authors observed upregulation of genes related to angiogenesis, as well as to proliferation (*Mki67* and *Pdgfrb*), in EC (lines 216-221). *Pdgfrb* is a gene marker for pericytes/fibroblast and smooth muscle cells, and it is very low expressed in EC even 14 days after MCAO (PMID: 38177281). Therefore, for clarification of the reported results, it would be helpful to show a heatmap or similar plot describing the top regulated genes in EC of control and PLX5622-treated mice. Also, the classification of arterial, venous or capillary endothelial cells based just on one marker gene is too simple (lines 375-376).
10. The authors pointed out that MG2 showed a signature of early activation state as this cluster was characterized by upregulation of immediate early genes (line 242-243). The upregulated genes shown in Suppl. Fig.4C seems to overlap with a DIM signature (PMID: 35931085; PMID: 38177281). As mentioned above, it would be helpful to report a more extensive list of the top genes obtained in the scRNAseq analysis for a better comprehension of the microglial signatures.
11. Line 257-259: "Among the infiltrated immune cells, macrophages exhibited the most significant elevation in remyelination and angiogenesis AUCCell scores in PLX5622-treated mice (Figure 3B), highlighting their critical roles in brain repair". However, the plot shows an angiogenesis AUCCell score higher in neutrophil than in macrophages.
12. The authors pointed out a potential dendritic cell differentiation of MF3 (lines 276-278). I think that MF3 could be described as a moDC cluster (PMID: 29545365).
13. In general, it would be nice to indicate in the figures the brain region and the exact localization relative to the ischemic lesion where immunofluorescent images were taken.

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Main comment

I believe the manuscript has improved; however, several points still require further attention.

Specific points

1. In the introduction (line 87), the following statement is made: "Multiple studies have demonstrated that CSF1R inhibitors can effectively ameliorate neurological deficits in neurodegenerative diseases¹⁴, demyelinating diseases¹⁵, CNS infections¹⁶, and brain injuries^{17, 18, 19}. CSF1R inhibitors have been reported to facilitate neurological recovery by inhibiting neuroinflammation, promoting myelin regeneration²⁰, and reducing both neuronal²¹ and vascular damage²²." It should be noted that reference 19 specifically pertains to the benefits of microglia repopulation, which is distinct from the benefits of microglia depletion discussed in the current manuscript. Furthermore, none of the cited references attributing beneficial effects to microglia depletion are related to stroke, except for reference 18. In contrast, there are studies suggesting detrimental effects of microglia depletion in stroke that are not mentioned in this manuscript, such as Szalay et al., 2016, *Nature Communications* (doi: 10.1038/ncomms11499); Otxoa-de-Amezaga et al., 2019; *Acta Neuropathologica* (doi: 10.1007/s00401-018-1954-4); Marino Lee S et al., 2021 (doi: 10.1016/j.expneurol.2020); and Hamner MA et al., 2022 (doi: 10.1002/glia.24132). I believe these studies should be cited, at least a few of them, to clarify that there are studies showing various detrimental effects of microglia depletion in experimental stroke.

2. Does the statement in the introduction (line 93), "Upon withdrawal of CSF1R inhibitors, the vacated microglial niche is promptly replenished by regenerating microglia and monocyte-derived macrophages (MDMs) from peripheral blood," refer to a non-injured brain? If so, please include a corresponding reference that demonstrates MDM infiltration into the brain following microglia depletion in the absence of injury.

3. Do the images shown in Supp. Fig. S1H and those shown in Fig. 1K (35 days later) correspond to the same mice? Please notice that showing infarction on the same side of the brain through the manuscript would help the readers to understand the model.

4. In the new scRNAseq experiments on blood cells, monocytes exhibit a limited number of differentially expressed genes (DEGs). It is suggested (line 217) that PLX5622 has a limited functional effect on monocytes. However, the data presented in Supplementary Figure S2G show an improvement in mitochondrial activity, which could have significant effects on cell activity and survival.

5. The new text (line 238) indicates that there was no significant variation in the number of APC⁺ BrdU⁺ cells in the infarcted hemisphere. This observation is interpreted as indicative of a comparable extent of white matter damage in both groups of mice, regardless of the PLX5622 diet. However, the connection between APC labeling and oligodendrocytes is not clarified at this point but is instead explained later in the text (line 276). To enhance clarity and facilitate understanding, please consider revising the text to introduce this association earlier.

6. The experiments where the microglia population was partially depleted are mixed with experiments (some of them are new experiments) where microglia partial depletion is followed by microglia repopulation after returning to control diet. From these experiments, it is not possible to conclude that the observed benefits are due to the elimination of microglia excluding any potential benefits of proliferative microglia (Lalancette-Hebert et al., 2007, *J. Neurosci.* (10.1523/JNEUROSCI.5360-06.2007)). In my view, Supplementary Fig. S6F-G is very important since it shows an increase in MF2 5 days following PLX5622 prior to return to control diet. This is a strong argument to support that microglial depletion drives this change. I suggest showing this result in a main figure.

7. In the experiments of stereotactic transplantation, please clarify whether RAMf cells used for transplantation were obtained from ischemic mice receiving control diet or PLX5266 diet followed by control diet. Please describe, at least in the figure legend, where in the brain were the RAMf cells transplanted.

8. Figure 3B shows the elevation in remyelination and angiogenesis AUC scores in infiltrating macrophages of PLX5622-treated mice followed by control diet for a week. How do these pro-remyelination and angiogenic features of macrophages compare with those of microglia subsets in the same mice?

9. Off-target effects of concern are related to the effects of PLX5622 on endothelial metabolism, which is not addressed here. The study of Profaci et al., 2024, *Neuron* (doi: 10.1016/j.neuron.2024.07.015) showed that PLX5622 alters the lipid metabolism of brain endothelial cells. This finding is highly relevant for the current study, particularly related to Fig. 5, and should be cited and discussed.

Minor points

* Fig 1F, shows different subsets of immune cells, including two populations of microglia (MG1 and MG2). However, the title of the graph is CD45 high immune cells.

*In line 144, the following text is repeated: 'PLX5622 treatment for 7 days led to approximately 70% depletion of microglia

(Figure S1D).'

*Please state the age of the mice newly included in Supplementary Fig. S11 figure legend.

All the experiments performed in male mice. This is not guarantee that the same mechanisms apply in female mice.

Reviewer #2

(Remarks to the Author)

This is a resubmission of a manuscript primarily focused on single cell analysis after stroke after transient depletion (5 days or sometimes 7 days?) of microglia by a CSF-R inhibitor. This was done to "open" the immune niche for peripheral (potentially reparative) immune cells. Some of the other studies used mice with 7 days of diet, so the results remain a little hard to follow and compare, why 5 days in some experiments, 7 days in others?

They have added many new studies, and repeated some of the studies in aged mice, with similar findings. Mortality and blinding are now mentioned.

Mice with transient and partial depletion of microglia (by PLEX) had an improvement in brain atrophy at 30 days, with no difference in BBB or infarct earlier after stroke. The authors note better vascular and myelin after depletion which they ascribe to enhances monocyte-derived macrophages (peripheral) that infiltrate the brain and have a reparative phenotype based on transcriptomics (RAMf). These cells "demonstrate a high ability in myelin debris clearance (although based on transcriptomics rather than functional assays), lipid droplet formation and lipid efflux and it is suggested that this "promotes remyelination through enhanced lipid recycling". In Figure 1, the authors interpret the increase in CCR2+ cells as an expansion of their proposed "reparative macrophages." However, CCR2 is also expressed on endothelial cells (PMID15927208, 10479649), so the observed increase may instead reflect post-stroke angiogenesis at the recovery stage? This might explain the improved long-term outcomes. Including a time course showing a gradual rise in CCR2+ cells would help clarify whether this increase truly reflects macrophage activity as angiogenesis is unlikely to be as acute. The new data showing the "reparative" transcriptomic profile was in brain infiltrating macrophages helps a bit (not in peripheral monocytes), but with the expected repopulation of MG the results are still very difficult to interpret. They also transferred Plex treated peripheral immune cells from 45.1 into 45.2 mice after stroke, and note no changes in expression of repair markers in infiltrating CD45.1+

Additionally, as previously pointed out, their depletion rate with PLX is low, only about 70% after 5 days in their revision, whereas studies using the same dosage (1200 ppm/kg) have shown >90% depletion by day 3. It is possible that some of the cells sequenced include repopulated microglia, which is supported by their sequencing data as well as previous studies reporting repopulated microglia exhibit a CD45 hi phenotype (PMID26163371, 26074918). Since repopulated microglia have been shown to confer protection, the observed benefits may not be solely due to "reparative macrophages" but could also involve these repopulated microglia. Moreover, the use of PLX likely depletes not only microglia but also peripheral Ly6C+ inflammatory myeloid cells (also CSF1R+), which could independently contribute to the improved outcomes. This possibility should be discussed as it may challenge their interpretation that the benefits largely arise from this new macrophage subpopulation. Discussing this possibility in the discussion is likely sufficient.

There seems to be no differences in infarct size at day 1 or 5 after Plex treatment with a 70% microglial depletion. The histology in Figure 1 does seem to show a decrease in infarct size in PLEX-treated mice, and the n is very low (5) and may be underpowered, but the MRI shown in supplement (day 1) with a n of 7 adds credence to the idea that the infarct volume is the same, at least early after stroke. There also was a difference on tissue loss at 14 days after 7 days of PLEX (and day 35). There were more macrophages at day 5, 7 and 14 in PLEX treated mice.

This is important to later findings (correlational changes based on infarct size with "remyelination etc).

It is unclear if the effects are due to the loss of MG (70%), the remaining MG (30%) that transition phenotypes, or the infiltrating monocytes. New results show that MG have repopulated by day 14 (when many of the assays were done), but the higher levels of macrophages that infiltrated at day 7 remains at day 14. The sc sequencing was done at day 5 post Plex in the setting of 70% MG depletion.

Overall some of the conclusions should be "softened" to account for these areas of uncertainty.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed my previous comments. The addition in the revised version of new scRNAseq data from peripheral blood of stroke mice and from ischemic brain tissue of aged mice, as well as the clodronate experiments and the inclusion of the time course of microglia numbers after PLX5622 treatment, has much improved the content and the clarity of the manuscript.

Regarding my second question of whether transferred BMDM are found in the ischemic brain, the authors have included additional flow cytometry analysis indicating the presence of infiltrating macrophages in the brain of CCR2KO mice undergoing stroke. However, to clearly demonstrate the homing of adoptively macrophages in the ischemic brain, it would have been better to conduct experiments that show specific infiltration of BMDM into the brain, maybe by using fluorescent labeled BMDM.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I have no further comments

Reviewer #2

(Remarks to the Author)

The authors have responded appropriately to my prior concerns. They have clarified the experimental timeline. They have added supplementary data on CCR2+ cells in S1A and S1C. They now at least mention endothelial cells and potential sex differences in the discussion, which is wise.

They have added additional animals to show no effect on infarct size in PLEX-treated mice (now a n of 7). They have also softened some of their statements in the discussion which is helpful. Overall, although there are still some lingering concerns regarding the “partial depletion” strategy, this is an important and comprehensive study.

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RESPONSES TO REVIEWERS' COMMENTS

Reviewer #1:

The study by Xiaotao Zhang et al. studies brain cells and infiltrating leukocytes after stroke using scRNAseq. The mice were transiently (5-7 days) exposed to a treatment in the diet with a CSFIR inhibitor (PLX5622) followed by restoration of normal chow for another week or up to 35 days post-stroke. The treatment can only transiently reduce the number of microglia since it is short duration and mice do not eat much after stroke. Mice that received this treatment showed reduced infarct volume and a better neurological function 35 days post-stroke. Moreover, at day 14 post-stroke, the mice showed improved vascular coverage and signs of more myelin. The study identified a subset of macrophages, rich in lipids, with a transcriptomic profile compatible with reparative functions. The presence of this subset of macrophages was more prominent when the mice were transiently exposed to a treatment in the diet that reduces the number of microglia followed by a recovery of microglial cell numbers. The actual cause for the enrichment in this potentially reparative macrophage subset in the stroked brain after treatment is not entirely clear. While the study touches points of interest and provides novel data, there are a number of issues that need careful attention.

1. Fig. 1B shows immunolabeling with several markers. From the images shown, it is interpreted that there is a decrease of Tmem119+ microglia, as expected. The reduction of the number of microglial cells after this treatment post-ischemia should be provided. Normally, treatment with PLX5622 requires at least two weeks of diet for a strong microglia depletion. Moreover, microglia proliferate after stroke. Furthermore, the mice do not eat very much following stroke. Therefore, the efficiency of the depletion of microglia should be shown. It seems that this model may reduce but it is most unlikely to cause severe microglia depletion. Please provide absolute microglia numbers.

Response: Thank you for this important suggestion. It is true that the depletion strategy we used in this study (transient PLX5622 diet for 7 days after MCAO) led to partial depletion of microglia. To accurately evaluate the efficiency of microglial depletion, we conducted a flow cytometry experiment to quantify the absolute number of microglia. The results indicated that, at 7 dpi, the number of microglia in the PLX5622 treatment group decreased by approximately 70% compared to the control group (**Figure S1D**). We opted for this treatment regimen because the following reasons: 1) It has been reported that elimination of microglia after 14 days of PLX5622 diet deprived the protective effects of microglia and exacerbated brain injury following ischemic stroke^{1,2}. 2) In contrast, we found that partial microglial depletion reshaped the immune microenvironment and protect the brain. 3) Initiating PLX5622 treatment after the stroke is clinically relevant.

2. Fig. 1B. Please provide information of the regions sampled anatomically and in relation to the brain lesion. There is no quantification of the immunofluorescence. Therefore, it is not possible to figure out the microglia reduction from these images.

Response: We have included low power images to clearly demonstrate the imaging areas and their relation to the brain infarct. The immunofluorescence staining in **Figure 1B-C** has been quantified as requested. In addition, new experiments have been performed to quantify the absolute numbers of microglia in PLX5622 diet and control diet groups using flow cytometry (**Figure S1C**). PLX5622 diet for 5 days resulted in 70% microglia depletion compared to the control group.

3. Fig. 1C. Values are expressed as number of cells per 1,000 single cells. Given that mice treated with PLX5622 are depleted of microglia, it is expected that after treatment the number of macrophages is higher per 1,000 given cells, since they do not contain microglia. Please provide the absolute numbers of cells counts.

Response: This is a valid concern. To address this concern, we conducted a flow cytometry experiment to quantify the absolute number of immune cells in the ischemic brain (**Figure S1C**).

4. How many microglial cells were analyzed in the single cell experiment (Fig. 1D and Fig. S2) after depletion of microglia with PLX5622?

Response: The single-cell analysis presented in **Figure 1F** and **Figure S1** examined a total of 2,804 microglia isolated from mice treated with PLX5622.

5. Please revise in the text (page 6) the interpretation of results. Fig. 1E does not show increase in the number of macrophages, but a higher proportion of macrophages in the PLX5622.

Response: Thank you for pointing this out. We have corrected this error in our manuscript (**Line 165, Page 6 in Manuscript file**).

6. Please provide plots as in Fig. S2D for proinflammatory cytokines, eg. Il1b and the inflammasome pathway.

Response: Thank you for this important point. We have included an AUCell analysis of the inflammasome pathway (**Figure 1H**), along with an examination of pro-inflammatory cytokines (**Figure 1I**). The results indicate that changes in functional terms and genes related to acute inflammation were inconsistent across different immune cell types.

7. Fig. 1F shows that infiltrating monocytes in PLX5622-treated mice upregulate brain-repair functions. However, it is not possible to know whether this phenomenon is due to microglia depletion or to depletion of peripheral myeloid cells.

Response: Thank you for this insightful and important comment. We performed two new experiments to evaluate if PLX5622-mediated depletion of peripheral myeloid cell can directly reprogram these cells into a reparative phenotype. And the data are presented in **Figure S2**.

- 1) We performed scRNA-seq on peripheral blood immune cells from both the PLX5622 and control groups at 5 days post-injury (dpi). The results revealed that although PLX5622 treatment reduced the number of monocytes in peripheral blood, it showed little impact on transcriptomic profiles of blood monocytes. Specifically, the brain repair genes that were upregulated in brain infiltrating macrophages remained at low levels in peripheral monocytes. This data suggests that monocytes in PLX5622-treated mice gain their brain-repair features after infiltrating into the microglia-depleted niches in the ischemic brain.
- 2) We collected peripheral immune cells from PLX5622-treated CD45.1 mice after MCAO and iv transferred them into CD45.2 wild-type mice (without microglia depletion) right after stroke. We did not observe changes in the expression of repair-related markers in infiltrating CD45.1⁺ macrophages in the recipient mice.

8. In supplementary Fig. S2E, the MRI images show large cortico-striatal infarctions 1-day post-ischemia to illustrate that the lesions in the mice assigned to each group, prior to microglia depletion-repopulation, are similar. Fig. 1H shows the brain of mice (certainly not the same as shown in Fig. S2E), one of them with a cortico-striatal infarction and the other with a striatal infarction. These two mice are not comparable. Do the infarct volume measures shown in Fig. 1H correspond to the same mice as those shown in Fig. 2E, F? The progression of the lesion in the same mice should be shown. The PLX5622 mouse shown in Fig. 1H (35 days) does not have cortico-striatal infarction as the mouse of the corresponding group at day 1 shown in Fig. S2E. This is very confusing.

Response: We apologize for the confusion because of the use of images from different mice in **Figure 1H** and **Figure S2E-F**. We have corrected this error and ensure that the representative images in **Figure 1H** and **Figure S2E-F** are from the same mouse in respective groups (**Figure 1K** and **Figure S1H** in the revised manuscript).

9. The quantification of the lesion volume shown in Fig. 1H, 35 dpi, should be accompanied by a measure of brain atrophy. The lesion is partly re-absorbed. Therefore, a smaller affected volume at this long time point after stroke onset may be due to a better resolution of the lesion rather than to reduced infarction.

Response: We are sorry for the confusion. We mislabeled the Y-axis of **Figure 1K**. **Figure 1K** indeed measured tissue loss by calculating the volume of the contralateral hemisphere minus the volume of the non-infarcted area of the ipsilateral hemisphere. At chronic phase, tissue loss reflects the combined effects of brain infarct volume (tissue damage) and brain atrophy. Tissue damage can be measured at early stage after stroke. We additionally provided the extent of tissue damage between groups by calculating infarct volume at 5 dpi (**Figure S3**). The results showed no difference in tissue damage between groups, indicating that PLX5622 or RAMf did not affect the extent of tissue damage. The difference of tissue loss we observed at the chronic phase after MCAO is primarily due to the extent of brain atrophy.

We used MRI to evaluate the brain infarct of the same groups of mice at 1d after MCAO by calculating the volume of the contralateral hemisphere minus the volume of non-infarcted area of

the ipsilateral hemisphere, which exclude the impact of brain edema on infarct size (**Figure S1H**). We also compared infarct volume at 5 dpi between PLX5622 and control groups (**Figure S3A**). The results indicated that the brain infarct volume was comparable between the PLX5622 and control groups early after stroke. Therefore, the smaller tissue loss in the PLX5622 group should be attributed to late-stage improvements rather than reduced infarct early after stroke.

10. Figure 1I. The time to removal (s) appears to be expressed as an absolute value. Was there any normalization step performed? It is stated in the methods section that the maximum time to removal was 120 s. How could it be then that the error bar reaches nearly 150 s?

Response: We presented the absolute values in **Figure 1I** based on a consistent baseline between two groups. The error bars represent the mean plus or minus the standard deviation (SD), which may result in values exceeding the maximum removal time of 120 seconds.

11. Were the behavioral tests and infarct volume measurements performed by researchers blinded to treatment?

Response: We have now clarified in the manuscript that the MCAO model, behavioral tests, and statistical analyses were performed by researchers who were blinded to experimental groups in the method section.

12. Referring to the experiment where mice are treated for 5 days with PLX5622 and then returned to control diet until day 14 post-ischemia, regarding the sentence in page 7: ‘there was an increase in the cell fractions of oligodendrocyte lineage cells (OLG and OPC) and endothelial cells (Figure 2B), suggesting a potential direct effect of early-phase microglia attenuation on white matter and vascular integrity’. There seems to be a reduction of infiltrating macrophages. Did the diet affect circulating myeloid cells? The study does not demonstrate whether the reduction of the microglia or the reduction of circulating monocytes, or rather the induction of repopulation, after discontinuation of the PLX5622 diet, is responsible for any increase in oligodendrocytes and endothelial cells at day 14.

Response: This is an insightful comment. We have corrected the sentence ‘..., suggesting a potential direct effect of PLX5622 treatment on white matter and vascular integrity’. As for your concern, we conducted the following experiments:

First, using the BrdU labeling method, we assessed the proliferation of oligodendrocytes and endothelial cells in the brain at 5 dpi and found no significant differences between the PLX5622 and control groups (**Figure S3**). Therefore, the reductions in microglia or circulating myeloid cells at 5 dpi do not increase the proliferation of oligodendrocytes or endothelial cells.

Second, scRNA-seq analysis of the 14 dpi microglia revealed reduced proportion of microglia expressing repair-related markers in the PLX5622 treatment group compared with control diet group (**Figure S5**). Thus, the repopulated microglia may not be the primary contributors to remyelination and angiogenesis.

In contrast, scRNA-seq and flow cytometry analyses confirmed that a higher proportion of brain infiltrating macrophages had transformed into repair-associated macrophages (RAMf) with enhanced repair functions. To further confirm that brain infiltrating RAMf contribute to the enhanced proliferation of oligodendrocytes and endothelial cells in PLX5622-treated stroke mice, we injected RAMf (icv, 30,000/mouse) into stroke mice and observed increased proliferation of oligodendrocytes (**Figure S7I**) and endothelial cells (**Figure 5J**) in the ischemic brain.

13. The reduced SM32/MBP ratio in the PLX5622 group is interpreted as a sign of remyelination but it could also be a sign of reduced demyelination.

Response: Thank you for this important comment. We assessed the SMI32/MBP ratio in the brain at 5 dpi and found no significant differences between the PLX5622 and control groups. This indicates that the extent of demyelination caused by ischemic stroke was similar in both groups of mice early after stroke (**Figure S3B-C**).

14. Please detail how was vascular coverage measured. How many sections per mouse and fields per section were counted? Following ischemia, the loss of endothelial cells depends on the volume of the lesion. Please provide the volume of the lesion of the mice shown in figure 2L.

Response: We have added these description in the methods section in our manuscript.

For the measurement of vascular coverage and ZO-1 expression in the vessel area, we selected three 25 μ m thick slices from each mouse brain and identified 4 regions around the infarct in each slice for imaging and quantification. The recorded images were loaded into Image J (NIH) and manually quantified by two observers who were blinded to the grouping. (Line 808, Page 27 – Line 812, Page 28 in Manuscript file)

We have calculated the brain tissue loss at 14 dpi in **Figure S4A**. Additionally, to evaluate the volume of lesion and their effect on the loss of endothelial cells, we assessed infarct volume, vascular coverage, ZO-1-positive vessels, and BrdU⁺CD31⁺ cells in both the PLX5622 and control groups at 5 dpi (**Figure S3**). We observed that the infarct volume and the loss of endothelial cells were similar between the two groups at 5 dpi. These data indicated that the comparable volume of lesion at 5 dpi between two groups allows for an accurate assessment of the effect of PLX5622 treatment on vascular coverage at 14 dpi.

15. Compared to resident microglia, more repopulating microglia remain in a resting state 14 days post-stroke (top of page 9). Fig. S4 refers to microglia subset in ischemic mice. How do they compare with the microglia of non-ischemic control mice?

Response: We compared the five microglia subsets to those from sham mice. The MG1 subset exhibited higher correlation coefficients in transcriptional profiles with sham microglia (**Figure S5B**). Furthermore, we observed increased expression of typical resting microglia markers, such as Tmem119 and P2ry12, in MG1 microglia (**Figures S5C-D**). These findings indicate that MG1 microglia are in a relatively more resting state compared to other subsets.

16. Fig. S5A and C show blood monocytes from sham mice and brain macrophages from ischemic mice with and without PLX5622 treatment, at day 14 or 5 post-ischemia, respectively. Corresponding monocytes of PLX5622-treated mice should be shown to find out the impact of the treatment in the periphery. Moreover, the subsets of blood monocytes at the ischemic time points should be shown, since the populations of circulating myeloid cells are affected by brain ischemia. It will be interesting to see the UMAP projections of brain macrophages only.

Response: Thank you for your comment.

- 1) To find out the impact of PLX5622 treatment on the blood monocytes, we have performed flow cytometry and scRNA-seq analysis on monocytes between two groups. PLX5622 reduced the number of peripheral monocytes at 5 dpi (**Figure S2 A-B**), but its effects on their functional role in the MCAO environment were limited (**Figure S2 F-I**).
- 2) We have showed the subsets of blood monocytes at 5 dpi (**Figure S6F**) and 14 dpi (**Figure S6C**) respectively. Only two subsets were observed in blood monocytes. The major subset was MF1 in a resting state. The other subset was MF3 highly expressing the antigen presenting related genes.
- 3) We also included UMAP projections of brain macrophages only in **Figure 3C**.

17. I cannot find the absolute numbers of infiltrating macrophages at the different time points after ischemia and the effect of PLX5622.

Response: Thank you for your comment. We have quantified the absolute numbers of infiltrating macrophages in the brains of both PLX5622-treated and control groups at days 3, 5, 7, and 14 post-MCAO. The new data are presented in **Figure 1E**.

18. Dendritic cells infiltrate the ischemic brain tissue. According to the markers, the cells shown in Fig. 3H could be infiltrating dendritic cells. Could the MF3 cells be infiltrating DCs?

Response: Thank you for pointing this out. The marker of MF3 is consistent with monocyte derived dendritic cells³. We have revised the manuscript accordingly. (**Line 349, Page 12 – Line 354, Page 12 in Manuscript file**)

19. Systemic clodronate injection after ischemia may directly affect the brain phagocytes, i.e. microglia and macrophages, due ischemia-induced blood-brain barrier breakdown. Therefore, it is difficult to attribute all effects to depletion of circulating cells.

Response: Thank you for pointing this out. We performed systemic clodronate injection to explore the origin of RAMf. To address your concern, we performed additional experiments to make our conclusion more reliable. We utilized CCR2-KO mice to demonstrate that the absence of infiltrating monocytes significantly reduced the number of RAMf in the brain (**Figure 3K**).

20. *In the experiment shown in Fig. 3L and M, the control for cell transplantation is PBS. Transplantation with macrophages other than GPNMB⁺CD63⁺ macrophages is necessary to extrapolate that these cells promote axonal remyelination.*

Response: Thanks for this important comment. We repeated the experiment and changed the control group to include transplantation of GPNMB⁺CD63⁺ macrophages. The results indicated that white matter repair was significantly enhanced in the GPNMB⁺CD63⁺ macrophage group compared to the GPNMB⁺CD63⁺ macrophage group (**Figure 3L-M**).

21. *Fig. 4A is an interpretation of the findings, not a direct result.*

Response: Thank you for your comment. We have removed Figure 4A from our manuscript.

22. *Fig. 4D shows an example of Iba1⁺ cells, some positive for GPNMB, at the vicinity of myelin. It is unclear whether these cells phagocytose myelin and whether other subsets of macrophages or microglia can also do it. The result does not demonstrate that those cells are the unique cell subset able to phagocytose myelin.*

Response: Thank you for your comment. We have added statistics on the co-localization of IBA1⁺ GPNMB⁺ cells and IBA1⁺ GPNMB⁺ cells with MBP. The results indicated that IBA1⁺ GPNMB⁺ cells co-localized more frequently with MBP (**Figure 4C**), and 3D reconstruction revealed the internalization of myelin by IBA1⁺ GPNMB⁺ cells. Additionally, a recent article has reported the phagocytosis of myelin by GPNMB^{high} macrophages, supporting our findings⁴.

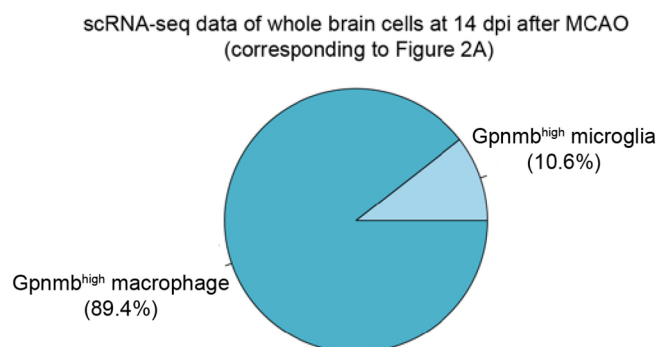
23. *Lipid accumulation in myeloid cells of the brain after stroke has been demonstrated in previous works that are not cited, e.g. Arbaizar-Rovirosa et al. (2023) doi: 10.15252/emmm.202217175; Zbesko JC, et al. (2023) doi: 10.1016/j.nbd.2023.106130.*

Response: Thanks for pointing this out. We have incorporated the relevant descriptions and cited the mentioned articles in the discussion section.

24. *Please notice that the images and quantification in Fig. 4F cannot distinguish macrophages from microglia. Why quantification is carried out in the periinfarct area where microglia accumulate rather than in the core of the lesion where macrophages accumulate?*

Response: It is true that distinguishing microglia from macrophages using immunofluorescent staining can be challenging. However, our analysis of the whole brain scRNA-seq data at 14 dpi revealed that the number of GPNMB^{high} macrophages was approximately 8.5 times greater than that of GPNMB^{high} microglia. Therefore, we used IBA1 and GPNMB double staining to define RAMf in our immunofluorescence analysis. This study explores the effect of RAMf on myelin and vascular repair after stroke. We therefore focused our analyses in the periinfarct areas where these repair

processes are active.



25. Gating strategy in Fig. 4G, have CD45⁺ CD11b⁺ and other markers been used before to separate macrophages?

Response: In this experiment, we compared the mean fluorescence intensity of BODIPY signal between CD45^{high} CD11b⁺ Ly6G⁻ GPNMB⁺ CD63⁺ cells and CD45^{high} CD11b⁺ Ly6G⁻ GPNMB⁻ CD63⁻ cells in **Figure 4F**. To avoid any potential misunderstanding, we have revised both the figure and the related descriptions in the manuscript.

26. The method used to quantify the % of CD31⁺ vessel area expressing ZO-1 should be explained in the Methods section. Which brain regions, how many sections per mice, how many areas per section and region? Was quantification performed in a blinded fashion?

Response: We have added this description to the methods section in our manuscript.

For the measurement of vascular coverage and ZO-1 expression in the vessel area, we selected three 25 μ m thick slices from each mouse brain and identified 4 regions around the infarct in each slice for imaging and quantification. The recorded images were loaded into Image J (NIH) and manually quantified by two observers who were blinded to the grouping. (Line 808, Page 27 – Line 812, Page 28 in Manuscript file)

27. Degradation of tight junction proteins may be the result of more severe ischemia and may lead to BBB breakdown. Was the integrity of the BBB poorer in mice under control diet?

Response: We have assessed infarct volume, vascular coverage, ZO-1-positive vessels, and BrdU⁺CD31⁺ cells in both the PLX5622 and control groups at 5 dpi. Our results indicated that the integrity of the blood-brain barrier was comparable between the two groups (**Figure S3E-F**).

28. For how long do the macrophages transplanted into the brain survive?

Response: We used FACS to isolate CCR2⁺ CD45⁺ CD11b⁺ GPNMB⁺ CD63⁺ cells from CCR2-

GFP mice at 14 dpi and transplanted them into wild type mouse brain at 5 dpi. At 14 dpi, we observed CCR2⁺ IBA1⁺ GPNMB⁺ cells (**Figure S7F**). Additionally, immunofluorescence studies showed a significant increase in IBA⁺GPNMB⁺CD63⁺ cells in the ischemic brain of mice that underwent RAMf transplantation at 14 dpi (**Figure S7G**). These data confirmed that these transplanted macrophages can survive for at least 14 days after MCAO.

29. Please name the X-axis of graph in panel of Fig 5I. How was vascular coverage quantified?

Response: Thank you for pointing this out. We have added the axis labels in **Figure 5J**. Additionally, we have included a detailed description of how vascular coverage was quantified in the methods section of our manuscript.

30. The in vitro study is performed in BMDMs. How is the transcriptomic profile of these cells compared to that of the MF2 macrophage subset found in ischemic brain tissue?

Response: Thank you for your question. We conducted RNA-seq of BMDMs in both the Mafb lentivirus and control lentivirus groups. The analysis revealed significant upregulation of specific genes associated with the RAMf phenotype, including Gpnmb, Cd63, Cd9, Syng1, and Fabp5 (**Figure S10B**). Gene Set Enrichment Analysis (GSEA) highlighted enriched pathways related to steroid biosynthesis and cholesterol metabolism (**Figure S10C**), indicating a transcriptomic profile in Mafb-overexpressing BMDMs that closely resembles that of RAMf. Additionally, flow cytometry experiments confirmed that the Mafb overexpressed BMDM expressed higher levels of GPNMB and CD63 (**Figure S10D**), which is similar to the MF2 macrophages.

31. The effect of Mafb lentivirus treatment versus control lentivirus on Mafb expression and cell survival prior to macrophage reconstitution of Ccr2-KO mice should be shown. Is the number of circulating and brain infiltrating macrophages similar for both groups of mice with lentiviral-treated macrophages (lenti-Mafb vs. lenti-control)?

Response: Thank you for your valuable comments. We have conducted additional experiments to address these concerns:

- 1) We confirmed the upregulation of Mafb following treatment with the Mafb lentivirus through qPCR (**Figure S10A**).
- 2) We assessed cell viability and apoptosis in both the Mafb lentivirus and control lentivirus groups using MTT assay and Annexin V staining, and the results indicated no significant differences between the two groups (**Figure S10E-F**).
- 3) We compared the numbers of circulating and brain-infiltrating macrophages in the Mafb lentivirus and control lentivirus groups, finding that the numbers were consistent across both groups (**Figure S10G-H**).

32. *Were any animals excluded in this study? Please report mortality, if any, in the different experiments.*

Response: We have added **Table S4** to report animal mortality in each group.

33. *I believe behavioral data, particularly the Fault/total test, shown in Fig. 11 do not correspond to continuous data and it is not possible to use parametric tests. Two-way ANOVA is the test currently used. Please revise.*

Response: Thank you for pointing this out. We have revised our analysis and now used a mixed-effects model based on the maximum likelihood method to reanalyze the foot fault test data.

34. *I am not convinced about the title since I believe the study does not demonstrate that microglia attenuation provides a spatial niche for repair associated macrophages to promote ischemic stroke recovery. The reasons are as follows:*

- *The niche is not depleted, but numbers of microglia are reduced during a few days (this is a point that has to be better shown provided actual cell numbers).*
- *The benefit is shown in Fig. 11-H 35 days post stroke while the microglia numbers should have recovered after 5-7 days of discontinuation of the treatment.*
- *It could be the repopulating microglia rather than the 'depleted niche' the one that is responsible to attract the macrophages or to promote the reparative phenotype. In any case, I believe this point is not demonstrated.*
- *The demonstrations supporting that the reduced volume of the lesion and improved functional outcome is entirely attributable to the subset of phenotypically reparative macrophages and the key role of Mab are not very robust.*

Response: Thanks for your important comment. We revised our title to 'Repair-Associated Macrophages Increase after Early-phase Microglia Attenuation to Promote Ischemic Stroke Recovery'.

Reviewer #2 (Remarks to the Author):

This manuscript examines the role of microglia in ischemic stroke and used microglial depletion strategies (PLX5622) to determine if changing the spatial niche (for peripheral immune cells) once microglia were depleted had an impact on outcome. This has been done in prior studies, but this work used single cell sequencing, which adds novelty.

Depletion of microglia early after stroke enhanced long term recovery after experimental ischemic stroke. Monocyte derived macrophages (termed “reparative or RAMf) were found, that had increased debris clearance, lipid droplet formation and lipid efflux “thereby promoting remyelination through enhanced lipid recycling”. However, most of the data involves gene changes, and a major concern is whether the manipulations altered the actual ischemic injury. If so, changes (such as improved white matter, vessel coverage etc.) could just be related to the extent of ischemic injury. This is a difficult problem to address. There is some evidence of actual remyelination/angiogenesis by markers such as BRDU and Ki67, but these studies are pretty minimal.

Response: Thanks for your important comment. We have conducted additional experiments to address these concerns:

Firstly, to confirm the comparable brain injury between two groups, we assessed infarct volume, vascular coverage, ZO-1⁺ CD31⁺ vessels, CD31⁺ BrdU⁺ cells, SMI32/MBP, and APC⁺ BrdU⁺ cells in both the PLX5622 and control groups at 5 days post-injury (dpi) (**Figure S3**). Our results indicated that the extent of ischemic injury, blood-brain barrier (BBB) integrity, and white matter integrity were similar between the two groups at 5 dpi.

Secondly, to further evaluate actual remyelination and angiogenesis, we evaluated CD31⁺ BrdU⁺ cells, PDGFRA⁺BrdU⁺ cells, and APC⁺ BrdU⁺ cells in both the PLX5622 and control groups. We observed that the numbers of CD31⁺ BrdU⁺ cells (**Figure 2L**), PDGFRA⁺BrdU⁺ cells (**Figure S4F**), and APC⁺ BrdU⁺ cells (**Figure 2J**) were significantly higher in the PLX5622 group, indicating enhanced remyelination and angiogenesis. Additionally, we transplanted GPNMB⁺ CD63⁺ macrophages into the mouse brain at 5 dpi. At 14 dpi, we found that the numbers of CD31⁺ BrdU⁺ (**Figure 5J**) cells and APC⁺ BrdU⁺ cells (**Figure S7I**) in the GPNMB⁺ CD63⁺ macrophage group were greater than those in the GPNMB⁻ CD63⁻ macrophage group, suggesting that GPNMB⁺ CD63⁺ macrophages promote remyelination and angiogenesis following ischemic stroke.

The main findings are that with microglial depletion (at a relatively lower level than expected with PLX) led to repopulation of the niche with peripheral cells, as has been shown in other studies.

Response: To evaluate the underlying value of clinical translation of PLX5622-based therapy in ischemic stroke, we administrated PLX5622 after the onset of ischemic stroke. We found a special subset of macrophages, RAMf, showing high myelin-related lipid metabolic activity to promote brain repair. This type of macrophage was recently reported to fuel brain cancer malignancy by myelin recycling in *Cell*, which highly expressed GPNMB [PMID: 39137777]. We believed this

unique type of macrophage may exert central roles in multiple diseases.

Five microglial subpopulations were identified 14 days after stroke (7 days after PLX withdrawal). They focused on “repair associated macrophages” as defined by their sequencing characteristics and describe a transcription factor, Mafk that governs the repair phenotype.

-A limitation is that all studies were performed in young male mice, and aging alters the transcriptional and functional role of microglia, and is translationally less relevant. There are no details on mortality, randomization or blinding except for the behavioral studies.

Response: Thanks for your important comment. To address the impact of aging on the conclusions of this study, we conducted the following experiments:

- 1) We conducted scRNA-seq on enriched CD45^{high} infiltrating immune cells sorted from the ischemic mouse brain at 14 dpi in aged mice. Our analysis revealed that macrophages in aged mice exhibited two differentiation pathways: mo-DC and RAMf. Notably, 36.2% of macrophages differentiated into RAMf, and the characteristic genes associated with RAMf cells in aged mice were consistent with those in young mice. Functional enrichment analysis indicated that these cells were also enriched for functions related to brain repair and lipid metabolism. **(Figure S11A-G)**
- 2) We utilized flow cytometry to demonstrate that PLX5622 treatment increased the proportion of GPNMB⁺ CD63⁺ CD45^{high} CD11b⁺ cells at 14 dpi in aged mice. **(Figure S11J-K)**

As for the details on mortality, we provide the information in **Table S4**. Randomization or blinding except for the behavioral studies were further described in the method section.

-Investigation of outcomes at more chronic phase (a month after stroke) is a strength

Response: Thank you for your positive comment.

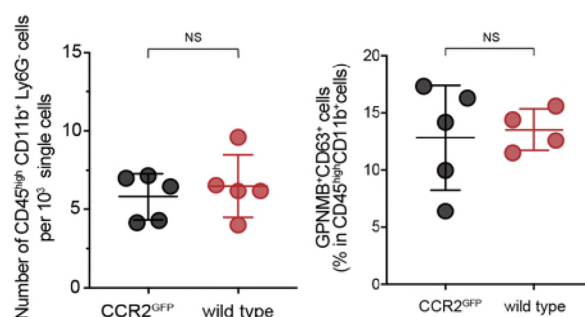
-62% depletion is relatively low

Response: Thanks for your important comment. In response to suggestions from other reviewers, we revised the statistical analysis in the flow cytometry experiment from the number of cells per 1,000 single cells to absolute values, providing a more accurate evaluation of microglial depletion efficiency. The results indicated that, at 5 dpi, the absolute number of microglia in the PLX5622 treatment group decreased by approximately 70% compared to the control group **(Figure S1C)**.

We opted for this treatment regimen because the following reasons: 1) It has been reported that elimination of microglia after 14 days of PLX5622 diet deprived the protective effects of microglia and exacerbated brain injury following ischemic stroke^{1, 2}. 2) In contrast, we found that partial microglial depletion reshaped the immune microenvironment and protect the brain. 3) Initiating PLX5622 treatment after the stroke is clinically relevant.

-Do the CCR2 mice used have a knock out allele (for the GFP)? Could that influence the results?

Response: Thank you for your question. The CCR2^{GFP/+} mice used in this study do carry a knockout allele for GFP. To evaluate the potential impact of the knockout-CCR2 allele on monocyte infiltration, we compared the number of macrophages and RAMf in the brains of CCR2^{GFP/+} mice and wild-type mice at 14 dpi. Our results indicated no significant differences in the number of macrophages or RAMf between the two groups.



-PLEX diet has off target effects outside of the microglial niche, this is noted, and genetic models are proposed for future studies, but this is a limitation.

Response: Thank you for this important comment. We have added this limitation in the discussion section.

In light of this limitation, we performed two new experiments to evaluate if PLX5622-mediated depletion of peripheral myeloid cell can directly reprogram these cells into a reparative phenotype. And the data are presented in **Figure S2**.

- 1) We performed scRNA-seq on peripheral blood immune cells from both the PLX5622 and control groups at 5 days post-injury (dpi). The results revealed that although PLX5622 treatment reduced the number of monocytes in peripheral blood, it showed little impact on transcriptomic profiles of blood monocytes. Specifically, the brain repair genes that were upregulated in brain infiltrating macrophages remained at low levels in peripheral monocytes. This data suggests that monocytes in PLX5622-treated mice gain their brain-repair features after infiltrating into the microglia-depleted niches in the ischemic brain.
- 2) We collected peripheral immune cells from PLX5622-treated CD45.1 mice after MCAO and iv transferred them into CD45.2 wild-type mice (without microglia depletion) right after stroke. We did not observe changes in the expression of repair-related markers in infiltrating CD45.1⁺ macrophages in the recipient mice.

-Comments like “suggesting that early-phase microglia attenuation could promote oligodendrogenesis after ischemic stroke” based on gene profiles is tenuous, without showing actual evidence of oligodendrogenesis.

Response: Thanks for your important comment. We evaluated SMI32/MBP (**Figure 2C**), PDGFRα⁺ BrdU⁺ cells (**Figure S4F**), and APC⁺ BrdU⁺ cells (**Figure 2J**) at 14 dpi, which indicated enhanced remyelination in the PLX5622 group. This provides evidence to support our suggestion that early-phase microglia attenuation may promote oligodendrogenesis after ischemic stroke.

Similarly, “we conducted scRNA-seq on enriched CD45^{high} infiltrating immune cells sorted from the ischemic mouse brain at 14 dpi to identify the cell types “contributing to remyelination and angiogenesis” without showing actual remyelination or angiogenesis is an overstatement, they are just describing genes that are present. Although the authors did examine the number of ZO-1⁺ vessels, PLX treatment led to smaller infarcts, so is this just an epiphenomenon due to the reduction in infarct damage?

Response:

We have conducted additional experiments to address these concerns, please refer to our responses to your first comment. Here we attach the responses as follows:

Firstly, to confirm the comparable brain injury between two groups, we assessed infarct volume, vascular coverage, ZO-1⁺ CD31⁺ vessels, CD31⁺ BrdU⁺ cells, SMI32/MBP, and APC⁺ BrdU⁺ cells in both the PLX5622 and control groups at 5 days post-injury (dpi) (**Figure S3**). Our results indicated that the extent of ischemic injury, blood-brain barrier (BBB) integrity, and white matter integrity were similar between the two groups at 5 dpi.

Secondly, to further evaluate actual remyelination and angiogenesis, we evaluated CD31⁺ BrdU⁺ cells, PDGFRA⁺BrdU⁺ cells, and APC⁺ BrdU⁺ cells in both the PLX5622 and control groups. We observed that the numbers of CD31⁺ BrdU⁺ cells (**Figure 2L**), PDGFRA⁺BrdU⁺ cells (**Figure S4F**), and APC⁺ BrdU⁺ cells (**Figure 2J**) were significantly higher in the PLX5622 group, indicating enhanced remyelination and angiogenesis. Additionally, we transplanted GPNMB⁺ CD63⁺ macrophages into the mouse brain at 5 dpi. At 14 dpi, we found that the numbers of CD31⁺ BrdU⁺ (**Figure 5J**) cells and APC⁺ BrdU⁺ cells (**Figure S7I**) in the GPNMB⁺ CD63⁺ macrophage group were greater than those in the GPNMB⁻ CD63⁻ macrophage group, suggesting that GPNMB⁺ CD63⁺ macrophages promote remyelination and angiogenesis following ischemic stroke.

The most mechanistic work involves the use of siRNA to suppress a target, Mafk, done in bone marrow cells in vitro (presumably also from young donors) and in vivo, but stroke size is not mentioned in many of the studies.

Are there off target effects of giving clodronate prior to stroke on infarct size?

Response: Thank you for pointing this out. We evaluated the infarct size between the two groups and found no significant differences. We have included these results in **Figure S7A**.

The use of an acute intervention with PLX led to significant changes in infarct size, which does lead to multiple confounds. It is not surprising that behavior was improved in treated mice, or that it reduced the SMI/MBP ratio or increased vascular integrity (Fig 5, Ki67 not shown except graphically). Was there a difference in infarct in the RAMf transplanted mice?

Response: Thank you for your comment. Reviewer 1 also brings forward the question regarding the difference between infarct size and tissue loss. We apologize that we mislabeled the Y-axis as infarct volume in **Figure 1K**, which we corrected it as tissue loss.

We measured tissue loss by calculating the volume of the contralateral hemisphere minus the volume of the non-infarcted area of the ipsilateral hemisphere. At chronic phase, tissue loss reflects the combined effects of brain tissue damage and tissue repair. Tissue damage can be measured at early stage after stroke. We additionally provided the extent of tissue damage between groups by calculating infarct volume at 5 dpi (**Figure S3**). The results showed no difference in tissue damage between groups, indicating that PLX5622 or RAMf did not affect the extent of tissue damage. The difference of tissue loss we observed at the chronic phase after MCAO is primarily due to the extent of tissue repair.

The RAMf or GPNMB⁻ CD63⁻ macrophages were transplanted at 5 dpi with comparable tissue damage between groups. We found an increased number of APC⁺ BrdU⁺ cells and CD31⁺ BrdU⁺ cells, and decreased tissue loss in the RAMf-transplanted group compared to the GPNMB⁻ CD63⁻ macrophage group at 14 dpi, suggesting that RAMf promote tissue repair following ischemic stroke.

Also this is needed for Figure 6, as if there are differences in infarct size, this needs to be mentioned.

Response: Thank you for your comment. We compared the infarct volumes at 14dpi between the two groups of mice in **Figure 6F**. The results indicated no significant difference in infarct volume between the CCR2-KO mice reconstructed with Mafb-overexpressing BMDMs and those reconstructed with control BMDMs (**Figure S10I**).

Was there a difference in the infarct volume in CCR2 mice (Figure 1)?

Response: Thank you for your comment. We compared the infarct volumes of CCR2 mice shown in **Figure 1C**. The results indicated no significant difference in infarct volume between CCR2^{GFP} mice treated with PLX5622 and those on a control diet.

Figure 2 showing BRDU could be improved, as this is really the only evidence of angiogenesis or remyelination shown histologically.

Response: Thank you for your comment. We have conducted additional experiments to evaluate PDGFRα⁺ BrdU⁺ cells (**Figure S4F**), APC⁺ BrdU⁺ cells (**Figure 2J**), vascular coverage (**Figure 2L**), and CD31⁺ BrdU⁺ cells (**Figure 2L**) in both the PLX5622 and control groups.

Was there a difference in infarct size in the mice transplanted with CD45^{high} CD11b⁺ CD63⁺ GPNMB⁺? Again, it is hard to know if this is “preservation” of myelin (not enhanced remyelination) otherwise.

Response: Thank you for your comment. We have provided additional data in our responses above, as follows:

We measured tissue loss by calculating the volume of the contralateral hemisphere minus the volume of the non-infarcted area of the ipsilateral hemisphere. At chronic phase, tissue loss

reflects the combined effects of brain tissue damage and tissue repair. Tissue damage can be measured at early stage after stroke. We additionally provided the extent of tissue damage between groups by calculating infarct volume at 5 dpi (**Figure S3**). The results showed no difference in tissue damage between groups, indicating that PLX5622 or RAMf did not affect the extent of tissue damage. The difference of tissue loss we observed at the chronic phase after MCAO is primarily due to the extent of tissue repair.

The RAMf or GPNMB⁻ CD63⁻ macrophages were transplanted at 5 dpi with comparable tissue damage between groups. We found that the increased numbers of APC⁺ BrdU⁺ cells and CD31⁺ BrdU⁺ cells, and decreased tissue loss in the RAMf-transplanted group compared to the GPNMB⁻ CD63⁻ macrophage group at 14 dpi, suggesting that RAMf promote tissue repair following ischemic stroke.

Reviewer #3 (Remarks to the Author):

The study by Zhang et al. investigates the implication of peripheral immune cells in stroke recovery after partial depletion of microglia. Microglia number was attenuated in the stroke acute phase by feeding the mice with PLX5622 diet during the first 5 or 7 days after induction of focal cerebral ischemia (transient MCAO model). Single-cell transcriptomic analysis 5 and 14 days after injury and flow cytometry experiments, indicated expansion of a macrophage subpopulation (RAMf) with reparative characteristics in the PLX5622 group as compared to controls. Additionally, lipidomic, flow cytometry assays and immunohistochemistry studies showed that RAMf significantly contributed to remyelination and angiogenesis. Furthermore, both in vitro and in vivo approaches supported a key role for the transcription factor Mafk in regulating the reparative phenotype of RAMf.

This is an interesting study investigating the contribution of infiltrating leukocytes in tissue repair in response to experimental stroke. The manuscript reads well, and it provides evidence of a role of macrophages in tissue remodeling. The employed approach of modulating the immune niche competition by decreasing the number of early-phase microglia, might be relevant to open new avenues for potential therapeutic approaches for stroke. My specific comments for consideration by the authors are as follows:

Major comments:

1. For the experiments of RAMf transplant into mice undergoing cerebral ischemia, PBS is not the right control to support a pro-reparative function of RAMf. The transplant of GPNMB⁺CD63⁻ macrophages would be the best approach to use as control group.

Response: Thanks for this important comment. We repeated the experiment and changed the control group to include transplantation of GPNMB⁺CD63⁻ macrophages. The results indicated that white matter repair was significantly enhanced in the GPNMB⁺CD63⁻ macrophage group compared to the GPNMB⁺CD63⁺ macrophage group (**Figure 3L-M**).

2. CCR2-knockout mice were reconstituted with BMDMs transduced with either Mafk or control lentivirus to assess the role of Mafk in fostering remyelination and angiogenesis after ischemic stroke (lines 405-409). These experiments are key to support the role of Mafk in regulating RAMf function and therefore, they should be described in more detail. The authors indicated that CCR2-KO mice were reconstituted with BMDM, but it is not clear whether transduced BMDM were adoptively transferred into mice. In that case, the presence of transferred BMDM in the brain should be shown. Also, were BMDMs transferred into mice before or after stroke?

Response: Thank you for your valuable comments. We conducted additional experiments to address these concerns. We compared the numbers of circulating and brain-infiltrating macrophages in the Mafk lentivirus and control lentivirus groups, finding that the numbers were consistent between both groups (**Figure S10G-H**). In this study, two million BMDMs were transferred into mice 6h after ischemic stroke.

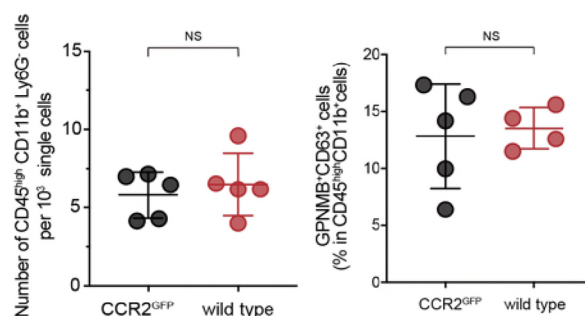
3. Monocytes/macrophages scRNAseq data from sham mouse brain and/or from stroke mouse blood should be used to best infer pseudotime trajectory analysis of macrophages (Fig. 3C-D and Suppl. Fig. 5F).

Response: Thank you for your valuable comments. We performed scRNA-seq on peripheral blood immune cells from both the PLX5622 and control diet groups at 5 dpi and 14 dpi. Our findings revealed that while PLX5622 treatment resulted in a reduction of monocytes in the peripheral blood (Figure S2 A-B), the changes in transcriptomic profiles were minimal (Figure S2 F-I). Subsequently, we conducted pseudotime trajectory analysis of macrophages using monocytes from stroke mouse blood, which yielded similar results (Figure S6D, Figure S6I).

Other comments:

4. Please indicate whether the *Ccr2*-GFP mice used in the study were homo- or heterozygous. The insertion of GFP in the mouse strain used in the study (RRID:IMSR_JAX:027619) abolishes the gene expression of *CCR2*, and therefore *Ccr2*gfp/gfp homozygous are *CCR2*-KO. A completely lack of infiltrating macrophages in the ischemic brain of *CCR2*-KO mice has been reported.

Response: Thank you for your question. The *CCR2*-GFP mice used in this study were *CCR2*^{GFP/+}, which carry a knockout allele for GFP. To evaluate the potential impact of the knockout-*CCR2* allele on monocyte infiltration, we compared the number of macrophages in the brains of *CCR2*^{GFP/+} mice and wild-type mice at 14 dpi. Our results showed no significant difference in the number of macrophages between the two groups.



5. The data shown in Figures 1C and 1E do not match. Fig. 1C shows that the number of NK cells, B cells and T cells are higher in the ischemic brain of PLX5622 treated mice as compared with control diet, but the proportions over the total leukocyte infiltrate of these same cell types are lower in the PLX5622 group in Fig. 1E.

Response: Thanks for your important comment. In response to suggestions from Reviewer 1, we revised the statistical analysis in the flow cytometry experiment, shifting from cell counts per 1,000 single cells to absolute values for a more accurate evaluation of immune cell numbers. The results indicated comparable levels of NK cells, B cells and T cells between two groups (Figure S1C). The

quantitative changes observed in scRNA-seq primarily reflect proportional changes.

6. Does Fig. 1H show volume of tissue loss? Or does it show infarct volume (MAP2 lesion)?

Response: Thanks for pointing this out.

We apologize that we mislabeled the Y-axis as infarct volume in **Figure 1K**, which we corrected it as tissue loss.

We measured tissue loss by calculating the volume of the contralateral hemisphere minus the volume of the non-infarcted area of the ipsilateral hemisphere. At chronic phase, tissue loss reflects the combined effects of brain tissue damage and tissue repair. Tissue damage can be measured at early stage after stroke. We additionally provided the extent of tissue damage between groups by calculating infarct volume at 5 dpi (**Figure S3**). The results showed no difference in tissue damage between groups, indicating that PLX5622 or RAMf did not affect the extent of tissue damage. The difference of tissue loss we observed at the chronic phase after MCAO is primarily due to the extent of tissue repair.

7. Suppl. Fig. 1C: CD45^{hi}CD11b⁺ gate is defined as macrophages, which is not correct since it also will include neutrophils, as well as some DC.

Response: Thank you for pointing this out. We have revised our gating strategy to define macrophages using CD45^{high}CD11b⁺Ly6G⁻. The updated data are presented in **Figure S1B**.

8. For a better understanding of the conclusions drawn from the comparison between the studies performed at 5- and 14-day time points, it would be helpful to report the amount of microglia depleted after 7 days of PLX5622 treatment as well as the levels of recovered microglia at 14 days. These results are only reported for the study performed at day 5 post-MCAO.

Response: Thank you for pointing this out. We added statistics on the number of microglia at 3, 5, 7 and 14 dpi. The updated data are presented in **Figure S1D**.

9. The authors observed upregulation of genes related to angiogenesis, as well as to proliferation (Mki67 and Pdgfrb), in EC (lines 216-221). Pdgfrb is a gene marker for pericytes/fibroblast and smooth muscle cells, and it is very low expressed in EC even 14 days after MCAO (PMID: 38177281). Therefore, for clarification of the reported results, it would be helpful to show a heatmap or similar plot describing the top regulated genes in EC of control and PLX5622-treated mice. Also, the classification of arterial, venous or capillary endothelial cells based just on one marker gene is too simple (lines 375-376).

Response: Thank you for pointing this out.

- 1) We have removed the description of Pdgfrb and included a volcano plot that illustrates the top regulated genes in EC of control and PLX5622-treated mice (**Figure S4I**).
- 2) We have provided a heatmap of marker genes for each EC subset and utilized classical markers⁵ to accurately classify the EC subsets: artery (EC2, Gkn3⁺, Tgfb2⁺, Bmx⁺), capillary (EC1, Tfrc⁺, Car4⁺, Slc16a1⁺), and vein (EC3, Nr2f2⁺, Lrg1⁺, Lcn2⁺), thereby enhancing the credibility of

the clustering analysis. (**Figure 5F, Figure S9C**)

10. The authors pointed out that MG2 showed a signature of early activation state as this cluster was characterized by upregulation of immediate early genes (line 242-243). The upregulated genes shown in Suppl. Fig.4C seems to overlap with a DIM signature (PMID: 35931085; PMID: 38177281). As mentioned above, it would be helpful to report a more extensive list of the top genes obtained in the scRNAseq analysis for a better comprehension of the microglial signatures.

Response: Thank you for your suggestion. We have listed the differentially expressed genes in microglia and included them as **Table S3** in our manuscript. The MG2 shares similarities with the DIM signature, and we have added relevant descriptions to the manuscript. (**Line 310, Page 11 – Line 312, Page 11 in Manuscript file**)

11. Line 257-259: “Among the infiltrated immune cells, macrophages exhibited the most significant elevation in remyelination and angiogenesis AUCell scores in PLX5622-treated mice (Figure 3B), highlighting their critical roles in brain repair”. However, the plot shows an angiogenesis AUCell score higher in neutrophil than in macrophages.

Response: Thank you for your comment. In **Figure 1**, we observed that at 5 dpi, the number of macrophages among the brain immune cells in the PLX5622 group increased significantly, with their repair functions notably enhanced. On the 14 dpi, while neutrophils demonstrated a significant role in promoting angiogenesis, their overall numbers remained considerably smaller than those of macrophages. Considering these points, we focused on macrophages and confirmed their critical role in supporting white matter and vascular repair. The angiogenic effect of neutrophils also arise our interest. We have added the potential reparative function of neutrophils in the discussion section.

12. The authors pointed out a potential dendritic cell differentiation of MF3 (lines 276-278). I think that MF3 could be described as a moDC cluster (PMID: 29545365).

Response: Thank you for pointing this out. The marker of MF3 is consistent with monocyte derived dendritic cells³. We have revised the manuscript accordingly. (**Line 349, Page 12 – Line 354, Page 12 in Manuscript file**)

13. In general, it would be nice to indicate in the figures the brain region and the exact localization relative to the ischemic lesion where immunofluorescent images were taken.

Response: Thank you for your valuable feedback. We have added low-power images to **Figure 1B** to clearly illustrate the imaging areas and their relationship to the brain infarct. In this study, we specifically focused on the effects of RAMf on myelin and vascular repair after stroke, concentrating our analyses on the periinfarct regions where these repair processes are most active. However, due to space limitations in the manuscript, we were unable to include localization information for every plot.

1. Marino Lee S, Hudobenko J, McCullough LD, Chauhan A. Microglia depletion increase brain injury after acute ischemic stroke in aged mice. *Exp Neurol* **336**, 113530 (2021).
2. Szalay G, *et al.* Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. *Nat Commun* **7**, 11499 (2016).
3. Cochain C, *et al.* Single-Cell RNA-Seq Reveals the Transcriptional Landscape and Heterogeneity of Aortic Macrophages in Murine Atherosclerosis. *Circ Res* **122**, 1661-1674 (2018).
4. Kloosterman DJ, *et al.* Macrophage-mediated myelin recycling fuels brain cancer malignancy. *Cell* **187**, 5336-5356.e5330 (2024).
5. Vanlandewijck M, *et al.* A molecular atlas of cell types and zonation in the brain vasculature. *Nature* **554**, 475-480 (2018).

RESPONSES TO REVIEWERS' COMMENTS

Reviewer #1:

Main comment

I believe the manuscript has improved; however, several points still require further attention.

Specific points

1. In the introduction (line 87), the following statement is made: "Multiple studies have demonstrated that CSF1R inhibitors can effectively ameliorate neurological deficits in neurodegenerative diseases¹⁴, demyelinating diseases¹⁵, CNS infections¹⁶, and brain injuries^{17, 18, 19}. CSF1R inhibitors have been reported to facilitate neurological recovery by inhibiting neuroinflammation, promoting myelin regeneration²⁰, and reducing both neuronal²¹ and vascular damage²²." It should be noted that reference 19 specifically pertains to the benefits of microglia repopulation, which is distinct from the benefits of microglia depletion discussed in the current manuscript. Furthermore, none of the cited references attributing beneficial effects to microglia depletion are related to stroke, except for reference 18. In contrast, there are studies suggesting detrimental effects of microglia depletion in stroke that are not mentioned in this manuscript, such as Szalay et al., 2016, Nature Communications (doi: 10.1038/ncomms11499); Otxoa-de-Amezaga et al., 2019; Acta Neuropathologica (doi: 10.1007/s00401-018-1954-4); Marino Lee S et al., 2021 (doi: 10.1016/j.expneurol.2020); and Hamner MA et al., 2022 (doi: 10.1002/glia.24132). I believe these studies should be cited, at least a few of them, to clarify that there are studies showing various detrimental effects of microglia depletion in experimental stroke.

Response: Thank you for this important point. We have revised the references and included a description of the detrimental effects of microglia depletion in experimental stroke in the introduction. **(Line 87, Page 3 – Line 94, Page 4 in Manuscript file)**

2. Does the statement in the introduction (line 93), "Upon withdrawal of CSF1R inhibitors, the vacated microglial niche is promptly replenished by regenerating microglia and monocyte-derived macrophages (MDMs) from peripheral blood," refer to a non-injured brain? If so, please include a corresponding reference that demonstrates MDM infiltration into the brain following microglia depletion in the absence of injury.

Response: We have revised the introduction and added a reference. **(Line 95, Page 4 – Line 97, Page 4 in Manuscript file)**

3. Do the images shown in Supp. Fig. S1H and those shown in Fig. 1K (35 days later) correspond to the same mice? Please notice that showing infarction on the same side of the brain through the manuscript would help the readers to understand the model.

Response: The images in **Figure S1H** and **Figure 1K** are from the same mouse within their respective groups. We have revised the representative images to ensure clarity and prevent any potential misunderstanding for the readers.

4. In the new scRNAseq experiments on blood cells, monocytes exhibit a limited number of differentially expressed genes (DEGs). It is suggested (line 217) that PLX5622 has a limited functional effect on monocytes. However, the data presented in Supplementary Figure S2G show an improvement in mitochondrial activity, which could have significant effects on cell activity and survival.

Response: Thank you for your valuable comment. We have conducted additional analyses to address these concerns:

- 1) We have added **Table S2**, which lists the enriched GO terms for monocytes from the PLX5622 group compared to those from the control diet group at 5 dpi. Notably, we found no significant enriched terms related to myeloid cell activity or survival.
- 2) We assessed cell survival of monocytes in both the PLX5622 and control diet groups using Annexin V/PI staining. The results indicated no significant differences between the two groups. These data are presented in **Figure S2H**.

5. The new text (line 238) indicates that there was no significant variation in the number of APC+ BrdU+ cells in the infarcted hemisphere. This observation is interpreted as indicative of a comparable extent of white matter damage in both groups of mice, regardless of the PLX5622 diet. However, the connection between APC labeling and oligodendrocytes is not clarified at this point but is instead explained later in the text (line 276). To enhance clarity and facilitate understanding, please consider revising the text to introduce this association earlier.

Response: Thank you for pointing this out. We have corrected this error in our manuscript (**Line 241, Page 9 – Line 243, Page 9 in Manuscript file**)

6. The experiments where the microglia population was partially depleted are mixed with experiments (some of them are new experiments) where microglia partial depletion is followed by microglia repopulation after returning to control diet. From these experiments, it is not possible to conclude that the observed benefits are due to the elimination of microglia excluding any potential benefits of proliferative microglia (Lalancette-Hebert et al., 2007, J. Neurosci. 10.1523/JNEUROSCI.5360-06.2007). In my view, Supplementary Fig. S6F-G is very important since it shows an increase in MF2 5 days following PLX5622 prior to return to control diet. This is a strong argument to support that microglial depletion drives this change. I suggest showing this result in a main figure.

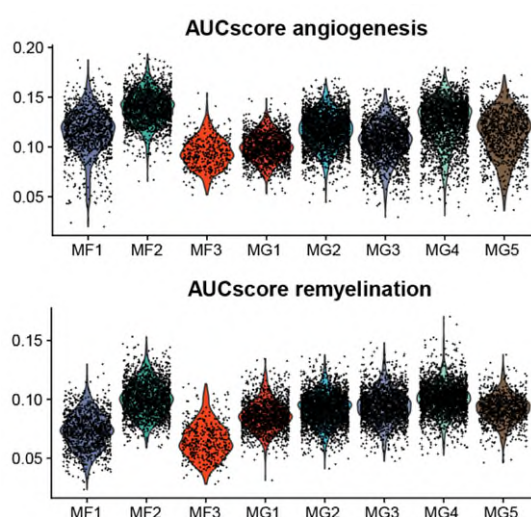
Response: Thank you for this important comment. We have moved the data from Supplementary Fig. S6F-G to the main **Figure 3I-J** to emphasize the increase in MF2 during the PLX5622 treatment.

7. In the experiments of stereotactic transplantation, please clarify whether RAMf cells used for transplantation were obtained from ischemic mice receiving control diet or PLX5622 diet followed by control diet. Please describe, at least in the figure legend, where in the brain were the RAMf cells transplanted.

Response: We apologize for not including this important information. We have clarified in the manuscript and in the figure legend for **Figure 3N** that the donor mice were treated with PLX5622 for the first 7 days, followed by a switch to a control diet until 14 dpi. Recipient mice received approximately 30,000 RAMf or GPNMB- CD63- macrophages via intraventricular transplantation at 5 dpi. **(Line 383, Page 13 – Line 390, Page 14 in Manuscript file)**

8. Figure 3B shows the elevation in remyelination and angiogenesis AUCell scores in infiltrating macrophages of PLX5622-treated mice followed by control diet for a week. How do these pro-remyelination and angiogenic features of macrophages compare with those of microglia subsets in the same mice?

Response: Thank you for this insightful and important comment. We utilized scRNA-seq data from whole brain cells at 14 dpi to compare the AUCell scores of macrophage subpopulations with those of various microglial subpopulations in the same mice. As illustrated in the figure below, the MF2 cluster (RAMf) demonstrates higher angiogenesis AUCell scores compared to the different microglial subpopulations. Furthermore, the remyelination AUCell scores of the MF2 cluster are comparable to those of the MG4 cluster, which we identified as exhibiting potential reparative functions.



9. Off-target effects of concern are related to the effects of PLX5622 on endothelial metabolism, which is not addressed here. The study of Profaci et al., 2024, Neuron (doi:

10.1016/j.neuron.2024.07.015) showed that PLX5622 alters the lipid metabolism of brain endothelial cells. This finding is highly relevant for the current study, particularly related to Fig. 5, and should be cited and discussed.

Response: Thanks for your comment. We have cited the article and included a discussion of the effects of PLX5622 on endothelial metabolism in the revised discussion section. **(Line 615, Page 21 – Line 619, Page 21 in Manuscript file)**

Minor points

** Fig 1F, shows different subsets of immune cells, including two populations of microglia (MG1 and MG2). However, the title of the graph is CD45 high immune cells.*

Response: Thank you for pointing this out. We have corrected this error in our manuscript.

**In line 144, the following text is repeated: 'PLX5622 treatment for 7 days led to approximately 70% depletion of microglia (Figure S1D).'*

Response: We have corrected this error in our manuscript.

**Please state the age of the mice newly included in Supplementary Fig. S11 figure legend.*

Response: We have included the age of the mice in the legend for **Figure S11**.

All the experiments performed in male mice. This is not guarantee that the same mechanisms apply in female mice.

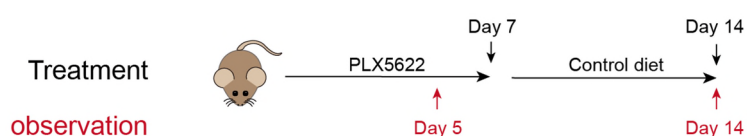
Response: Thank you for this important comment. We have added this limitation in the discussion section. **(Line 655, Page 22 – Line 658, Page 22 in Manuscript file)**

Reviewer #2:

This is a resubmission of a manuscript primarily focused on single cell analysis after stroke after transient depletion (5 days or sometimes 7 days?) of microglia by a CSF-R inhibitor. This was done to “open” the immune niche for peripheral (potentially reparative) immune cells. Some of the other studies used mice with 7 days of diet, so the results remain a little hard to follow and compare, why 5 days in some experiments, 7 days in others?

Response: We apologize that we failed to clarify the experimental time points in our original submission. To clarify, we utilized a 7-day PLX5622 diet in all experiments to induce partial and transient microglia depletion. And we selected the 5-day and 14-day observation time points for the following reasons:

- 1) This study primarily focuses on the effects of early-phase microglia attenuation on neural repair in mice. Consequently, we chose 14 days post-ischemia (dpi) as an observation point to capture the changes occurring in the brain during the recovery phase following ischemic stroke.
- 2) As shown in **Figure S1D**, the number of microglia was significantly reduced following 5 days of PLX5622 treatment in MCAO mice. Therefore, the 5-day time point was selected to represent the subacute stage, which is characterized by pronounced inflammatory responses before the onset of significant brain repair processes. This time point is crucial for identifying changes in the brain during CSF1R treatment.



They have added many new studies, and repeated some of the studies in aged mice, with similar findings. Mortality and blinding are now mentioned.

Mice with transient and partial depletion of microglia (by PLEX) had an improvement in brain atrophy at 30 days, with no difference in BBB or infarct earlier after stroke. The authors note better vascular and myelin after depletion which they ascribe to enhances monocyte-derived macrophages (peripheral) that infiltrate the brain and have a reparative phenotype based on transcriptomics (RAMf). These cells “demonstrate a high ability in myelin debris clearance (although based on transcriptomics rather than functional assays), lipid droplet formation and lipid efflux and it is suggested that this “promotes remyelination through enhanced lipid recycling”. In Figure 1, the authors interpret the increase in CCR2⁺ cells as an expansion of their proposed “reparative macrophages.” However, CCR2 is also expressed on endothelial cells (PMID15927208, 10479649), so the observed increase may instead reflect post-stroke angiogenesis at the recovery stage? This might explain the improved long-term outcomes. Including a time course showing a gradual rise in CCR2⁺ cells would help clarify whether this increase truly reflects macrophage activity as angiogenesis is unlikely to be as acute.

Response: Thank you for this valuable comment. 1) We have quantified the CCR2⁺ cells in both PLX5622 and control diet groups at 3, 5 and 7 dpi. The results indicated a gradual increase in CCR2⁺ cells, and the new data are presented in **Figure S1A**. 2) As shown in **Figure S1C**, our flow

cytometry analysis further confirmed the increased infiltration of macrophages.

The new data showing the “reparative” transcriptomic profile was in brain infiltrating macrophages helps a bit (not in peripheral monocytes), but with the expected repopulation of MG the results are still very difficult to interpret. They also transferred Plex treated peripheral immune cells from 45.1 into 45.2 mice after stroke, and note no changes in expression of repair markers in infiltrating CD45.1+

Additionally, as previously pointed out, their depletion rate with PLX is low, only about 70% after 5 days in their revision, whereas studies using the same dosage (1200 ppm/kg) have shown >90% depletion by day 3. It is possible that some of the cells sequenced include repopulated microglia, which is supported by their sequencing data as well as previous studies reporting repopulated microglia exhibit a CD45 hi phenotype (PMID26163371, 26074918). Since repopulated microglia have been shown to confer protection, the observed benefits may not be solely due to “reparative macrophages” but could also involve these repopulated microglia. Moreover, the use of PLX likely depletes not only microglia but also peripheral Ly6C+ inflammatory myeloid cells (also CSF1R+), which could independently contribute to the improved outcomes. This possibility should be discussed as it may challenge their interpretation that the benefits largely arise from this new macrophage subpopulation. Discussing this possibility in the discussion is likely sufficient.

Response: Thank you for this valuable comment. We have included a discussion of these possibility in the revised discussion section to soften our conclusions.

Repopulated microglia have been shown to exert neuroprotective effects in several studies^{1, 2, 3}. In this study, we identified 5 microglial subpopulations at 14 dpi, 7 days after the withdrawal of PLX5622. Among them, the MG4 subpopulation exhibited upregulation of neural repair-related genes, suggesting its potential role in brain repair. However, we observed a decrease in the proportion of MG4 cells within the PLX5622 group. While myelination-related functions were upregulated in this subset, its angiogenic capacity was diminished. These results suggest that repopulated microglia play a role in post-stroke repair, but their effects may be limited within the context of our treatment strategy. This limitation could be attributed to a narrow therapeutic window, as repopulated microglia are known to promote neural repair only when coinciding with brain injury in the TBI mouse model¹. Additionally, it has been reported that PLX5622 reduces the production and proliferation of peripheral monocytes.⁴ Our study corroborated this finding, revealing that PLX5622 decreased the number of peripheral monocytes, however, their reparative function remained largely unaltered. Previous studies have indicated that the number of Ly6C⁺ inflammatory myeloid cells in peripheral blood influences the injury and repair processes following ischemic stroke^{5, 6}. Therefore, future research should incorporate cell-specific genetic models to achieve a deeper understanding of

the effects of early-phase PLX5622 treatment on ischemic stroke, while minimizing the confounding influences of repopulated microglia and peripheral monocytes. (Line 569, Page 20 – Line 586, Page 20)

There seems to be no differences in infarct size at day 1 or 5 after Plex treatment with a 70% microglial depletion. The histology in Figure 1 does seem to show a decrease in infarct size in PLEX-treated mice, and the n is very low (5) and may be underpowered, but the MRI shown in supplement (day 1) with a n of 7 adds credence to the idea that the infarct volume is the same, at least early after stroke. There also was a difference on tissue loss at 14 days after 7 days of PLEX (and day 35). There were more macrophages at day 5, 7 and 14 in PLEX treated mice. This is important to later findings (correlational changes based on infarct size with “remyelination etc).

Response: We have conducted additional experiments to increase the number of biological replicates in both **Figure 1C** and **Figure S3A** (with n = 7). These results further confirm that brain injury is comparable between the PLX5622 and control diet groups at 5 dpi.

It is unclear if the effects are due to the loss of MG (70%), the remaining MG (30%) that transition phenotypes, or the infiltrating monocytes. New results show that MG have repopulated by day 14 (when many of the assays were done), but the higher levels of macrophages that infiltrated at day 7 remains at day 14. The sc sequencing was done at day 5 post Plex in the setting of 70% MG depletion.

Response: Thank you for this insightful and important comment. In this study, we provided the following evidence to address these concerns:

First, using the BrdU labeling method, we assessed the proliferation of oligodendrocytes and endothelial cells in the brain at 5 dpi and found no significant differences between the PLX5622 and control groups (**Figure S3**). Therefore, the reductions in microglia or circulating myeloid cells at 5 dpi do not increase the proliferation of oligodendrocytes or endothelial cells.

Second, scRNA-seq analysis of the 14 dpi microglia revealed reduced proportion of microglia expressing repair-related markers in the PLX5622 treatment group compared with control diet group (**Figure S5**). Thus, the repopulated microglia may not be the primary contributors to remyelination and angiogenesis.

In contrast, scRNA-seq and flow cytometry analyses confirmed that a higher proportion of brain infiltrating macrophages had transformed into repair-associated macrophages (RAMf) with enhanced reparative functions at 5 and 14 dpi. To further confirm that brain infiltrating RAMf contribute to the enhanced proliferation of oligodendrocytes and endothelial cells, we injected RAMf (icv, 30,000/mouse) into stroke mice and observed increased proliferation of oligodendrocytes (**Figure S7I**) and endothelial cells (**Figure 5J**) in the ischemic brain. Additionally, we have included a discussion addressing these concerns to remind readers of the confounding effects of microglia and monocytes.

Additionally, we have included a discussion that addresses these concerns and highlights the

confounding effects of microglia and monocytes, as previously mentioned. **(Line 569, Page 20 – Line 586, Page 20)**

Overall some of the conclusions should be “softened” to account for these areas of uncertainty.

Response: Thanks for your comment.

- 1) We have included a discussion addressing these concerns to highlight the potential confounding factors. **(Line 569, Page 20 – Line 586, Page 20)**
- 2) We have revised some of the phrasing in our conclusions to soften our assertions. **(Line 43, Page 1 – Line 46, Page 1; Line 297, Page 10; Line 403, Page 14)**

Reviewer #3

The authors have satisfactorily addressed my previous comments. The addition in the revised version of new scRNAseq data from peripheral blood of stroke mice and from ischemic brain tissue of aged mice, as well as the clodronate experiments and the inclusion of the time course of microglia numbers after PLX5622 treatment, has much improved the content and the clarity of the manuscript.

Regarding my second question of whether transferred BMDM are found in the ischemic brain, the authors have included additional flow cytometry analysis indicating the presence of infiltrating macrophages in the brain of CCR2KO mice undergoing stroke. However, to clearly demonstrate the homing of adoptively macrophages in the ischemic brain, it would have been better to conduct experiments that show specific infiltration of BMDM into the brain, maybe by using fluorescent labeled BMDM.

Response: Thank you for this important point. The lentivirus we utilized carries the mCherry fluorescent tag, which allowed us to detect mCherry⁺ CD45^{high} CD11b⁺ Ly6G⁻ macrophages in the ischemic brains of recipient mice that received lentivirus-treated BMDMs. Our analysis revealed that the number of this macrophage subset was similar in both the Mafb-lentivirus group and the control group. These data are presented in **Figure S10H**.

1. Willis EF, *et al.* Repopulating Microglia Promote Brain Repair in an IL-6-Dependent Manner. *Cell* **180**, 833-846.e816 (2020).
2. Wang W, *et al.* Microglial repopulation reverses cognitive and synaptic deficits in an Alzheimer's disease model by restoring BDNF signaling. *Brain Behav Immun* **113**, 275-288 (2023).
3. Elmore MRP, *et al.* Replacement of microglia in the aged brain reverses cognitive, synaptic, and neuronal deficits in mice. *Aging Cell* **17**, e12832 (2018).
4. Lei F, Cui N, Zhou C, Chodosh J, Vavvas DG, Paschalis EI. CSF1R inhibition by a small-molecule inhibitor is not microglia specific; affecting hematopoiesis and the function of macrophages. *Proc Natl Acad Sci U S A* **117**, 23336-23338 (2020).
5. Fang W, *et al.* CCR2-dependent monocytes/macrophages exacerbate acute brain injury but promote functional recovery after ischemic stroke in mice. *Theranostics* **8**, 3530-3543 (2018).
6. Pedragosa J, *et al.* CCR2 deficiency in monocytes impairs angiogenesis and functional recovery after ischemic stroke in mice. *J Cereb Blood Flow Metab* **40**, S98-s116 (2020).

RESPONSES TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I have no further comments

Response: Thank you very much for your thoughtful and constructive feedback on our manuscript. Your suggestions have been invaluable in enhancing the clarity and quality of our work.

Reviewer #2 (Remarks to the Author):

The authors have responded appropriately to my prior concerns. They have clarified the experimental timeline. They have added supplementary data on CCR2+ cells in S1A and S1C. They now at least mention endothelial cells and potential sex differences in the discussion, which is wise.

They have added additional animals to show no effect on infarct size in PLEX-treated mice (now a n of 7). They have also softened some of their statements in the discussion which is helpful. Overall, although there are still some lingering concerns regarding the “partial depletion” strategy, this is an important and comprehensive study.

Louise McCullough

Response: We greatly appreciate your recognition of the improvements made in response to your prior concerns. Your suggestions have been invaluable in enhancing the clarity and quality of our work.