Title: CD84 Links T Cell and Platelet Activity in Cerebral Thrombo-Inflammation in Acute Stroke

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Circulation Research Decision on Ms # CIRCRES/2020/316655
February 4, 2020

Dr. David Stegner
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Würzburg
Germany

RE: CIRCRES/2020/316655: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

Dear Dr. Stegner:

Your manuscript has been carefully evaluated by 3 external reviewers and the editors as a Regular Article. Although of potential interest, the paper is not acceptable for publication in Circulation Research in its present form.

As you will gather from the reviews, the referees identified a number of substantive conceptual and methodological problems. The editors concur. Major issues include the lack of a clear demonstration of CD84 being a soluble protein in platelet releasates and more information related to the pathogenic lymphocytes involved in the model.

Given the extensive new data that would be required for a responsive revision, we would understand if you were to decide to submit the paper elsewhere. Nevertheless, the editors see this manuscript as potentially important and would be willing to evaluate a revised version if you feel that you can effectively address the reviewers' concerns and are willing to perform the new experiments required. The paper would be reviewed again, with no assurance of acceptance.

As detailed in the reviewers' critiques, a responsive revision would require a substantial amount of new data and experiments. In particular, the editors feel that additional data would be necessary to address many important concerns raised by reviewer #1. This reviewer raised detailed concerns such as a lack of mechanistic insights into how platelet-derived CD84 promotes cerebral thrombosis, but not arterial thrombosis and the lack of functional recovery data.

NEW REQUIREMENTS:

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

Please note that revised and resubmitted manuscripts are not assured of publication, and that fewer than 15% of all papers submitted to Circulation Research are eventually published.

Our policy is to allow only one revision. Furthermore, our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted. If you need more than 90 days to submit a revised paper, please notify the editorial office.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Each page of the revised manuscript should be numbered in the top right corner, using your manuscript number followed by /R1 to denote a first revision.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Revisions that do not conform to the current limits on numbers of words (8000 total) and display items (maximum of 8 tables and/or figures) may be returned to the authors for abbreviation. If you cannot reduce the overall word count, the editors may deem an extended print version appropriate; the authors should provide written assurance that they will cover the costs of the pages that are in excess of these limits. Note that paying for excess display items is not an option. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

As of January 1, 2020, all corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To avoid potential processing delays in future, we recommend that you link an ORCID iD to your profile when you submit your revision. To register with ORCID or link your profile, please go to "Modify Profile/Password" on the submission site homepage (insert journal homepage link), and click the link in the "ORCID" section.

We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,
Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

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REVIEWER COMMENTS TO AUTHORS:

Reviewer #1:

The manuscript by Schulmann et al. defines the role of CD84, a homophilic immunoreceptor of the SLAM family in acute stroke. The authors conclude that platelet-derived CD84 worsens stroke outcomes by enhancing the motility of CD4+T cells. Furthermore, the authors found local shedding of CD84 in the ischemic circulation from the samples of AIS patients. High platelet CD84 expression was associated with poor outcome in stroke patients. Following points which need to be addressed to substantially improve that manuscript:
1. Figure 1. Are the representative TTC stained brain images from the same mouse? The infarct area generally develops around the ischemic core, which is not the case with CD84-/- mice. Please clarify.
2. Why only two females/group was used in some experiments? Need to evaluate sex-based differences.
3. T-cell numbers increase in the ischemic brain within the first 24 hours and can persist for days. The study by Gelderblom et al. and others suggest that CD4+ T cells are recruited within 24 hours after stroke and accumulation of these cells in the early inflammatory phase peaks 3 to 4 days after injury (Stroke. 2009;40:1849-1857). Whether T cells present in the chronic period following stroke are beneficial or detrimental to functional recovery is still debatable, long-term functional outcomes following stroke are essential to establish the role of CD84 in stroke outcome. The authors should evaluate long-term sensorimotor deficit (such as corner test, cylinder test, hanging wire test, accelerated rota-rod test) to determine the detrimental effect of CD84 on platelets or T cells or both.
4. Figure 2C. CD84 is highly expressed in other immune cells, including neutrophils and monocytes. Neutrophils are known to contribute to I/R injury following acute stroke. Surprisingly, the recruitment of neutrophils to the postischemic brain was comparable between control and CD84 KO mice. The potential role of CD84 deficiency in neutrophil/monocyte activation and cytokine secretion should be ruled out experimentally. The IHC results in Fig-2B are not convincing. Need to counterstain with DAPI and in parallel confirmed by flow-cytometry.
5. Figure 2D. Since CD84-deficiency did not affect the hemostatic or thrombotic function of platelets in mice, the observation that CD84 KO mice exhibit a reduced number of occluded vessels in the ipsilateral hemisphere by ~35% (Fig 2D), needs to be confirmed further by Western. Importantly, the data in current form lacks mechanistic insights how platelet-derived CD84 promotes cerebral thrombosis, but not arterial thrombosis. It is not clear from the method of whether the mice were perfused with saline before the brain sampling for the IHC studies.
6. Figure 3. Comparisons between the different groups are not rigorously tested and unreliable. It is extremely complex and hard to interpret due to the absence of several key control groups and a lack of detailed experimental protocol. It seems the experiments were not done in parallel, and importantly not all groups have received the adoptive transfer. There is no WT control for Rag1-/- mice, no WT AT of WT T-cells control mice to evaluate the relative role of WT vs. CD84-/- T-cells in Rag1-/- mice. Importantly, the lack of functional recovery data limits the interpretation of the overall stroke outcome.
7. Other parameters in Figures 1, 2, and 3, including edema, BBB, and neuronal injury, should be quantified and included.
8. It is not clear from the methods of whether CD84fl/flPF4Cre+ve mice and control CD84fl/fl PF4Cre-ve mice are littermate controls or not. Moreover, recent studies demonstrate transgene expression outside the megakaryocyte lineage, including leukocytes and macrophages (Pertuy F et al., J Thromb Haemost. 2015;13(1):115-125, Calaminus SD et al., PLoS One. 2012;7(12):e51361). To rule out this non-specificity of PF4 Cre-recombinase on stroke outcome, the authors should experimentally evaluate CD84 levels in other immune cells in CD84fl/flPF4Cre+ve mice and should also evaluate stroke outcome in CD84+/+PF4Cre+ve versus CD84fl/fl PF4Cre-ve mice.
9. Not sure whether the authors can conclude that platelet CD84 is required for the development of CD4+ T cell-dependent cerebral I/R injury. Is it not possible that platelet CD84 deficiency may protect from cerebral I/R injury

independent of CD4+ T cells? The authors should perform bone-marrow transplant experiments by reconstituting BM cells of CD84fl/flPF4Cre mice to the irradiated WT and CD84 KO mice, with appropriate controls.
10. The authors report that platelet surface abundance of CD84 was reduced locally within the ischemic circulation as compared with non-ischemic intra-individual systemic platelet CD84 surface abundance. The quantification of CD84 levels in the plasma from these samples will provide valuable information regarding the role of soluble CD84 on stroke outcome.
11. The role of CD84 should be tested in another stroke model.

Reviewer #2:

In this original article by Shuhmann et al., the authors investigated the role of a signaling lymphocyte activation molecule, CD84, in I/R injury in the classical mouse model of ischemic stroke induced by transient mechanical occlusion of the MCA. They show that mice with a total genetic deficiency in CD84 have reduced infarct volumes, as assessed by TTC staining 23h after a 1-hour-long transient occlusion of the MCA. The authors provide evidence that the decreased volume infarct in CD84-/- mice is not the consequence of improved perfusion during the ischemic period or after monofilament removal. This result indirectly suggests that the improved outcome in CD84-/- is not due to reduced microvascular thrombosis. Instead, they show that the protection is associated with a marked decrease in post-I/R accumulation of CD4+ T cells and monocytes, and no modification of neutrophil recruitment. Using adoptive transfer experiments, they show that both platelet and T cell CD84 is necessary for T cell-dependent I/R injury. Finally, they show in in vitro experiments that CD84 might be a regulator of T cell motility, and provide clinical evidence that CD84 levels are reduced on the platelet surface in the ischemic circulation.
The results are convincing and support the idea of a major contribution of CD84-dependent recruitment of CD4+ T cells to I/R injury in this model of ischemic stroke. These results are in line with a series of previous studies pointing to a central role for platelet/CD4+ T cell interactions in I/R injury. The main novelty here is thus the identification of a surface molecule expressed by both types of cells as a central player in this process.
Overall, the study is technically sound and the conclusions are well supported by the data and controls provided. The results bear interesting translational potential as they identify a pharmacological target for specific inhibition of the platelet deleterious action during I/R injury, without impairing hemostasis.
I have only a few comments.
- Do plasma samples from stroke patients stimulate T cell migration in in vitro assays? Is there any correlation between sCD84 levels in plasma from stroke patients and T cell migration in vitro?
- Do CD84 levels on platelets correlate with other markers of platelet activation in patients?
- In vitro studies: does CD84 need to be shed to exert its action toward T cells? Do CD84-dependent physical interactions between platelets and T cells have the same pro-motility effects?
- What about CD84 levels on patient T cells? Was it investigated?
- The authors could discuss further, even briefly, how CD4+ T cells cause neurotoxicity in this model? Is there any evidence of a cytokine release syndrome in the tMCAO?
- Besides their results, how important is CD84 to T cell function and/or activation in general? Is it recognized as a predominant player in CD4+ T cell migration or activation as compared to other SLAMs?
Minor
There seems to be a mistake in the sample size calculation on page 5 I assume one should read 0<0.05 instead of 0.5

Reviewer #3:

Stroke is one of the major causes of death and disability worldwide with limited treatment options. Despite recent advances in mechanical thrombectomy with high recanalization rates, the therapeutic efficacy of vessel reopening remains limited. The progression of cerebral infarction is frequently attributed to ischemia/reperfusion (I/R)

injury. Previous work by the authors and others had revealed that T lymphocytes (using Rag-1 mice and CD4 cells adoptive transfers) and platelets (through GPIb and GPVI, and platelet depletion in Rag-1 mice) both contribute to injury in a process of thrombo-inflammation.

In this manuscript by Schuhmann and colleagues, the authors define how platelets and lymphocytes promote thrombo-inflammation in this context. They elegantly demonstrate that a homophilic receptor, CD84, links platelets and lymphocytes in a mouse model of transient middle cerebral artery occlusion. Further in vitro experiments and analyses of human biospecimens also point to the implication of CD84. The manuscript is very well written, clear, and the conclusions are supported by convincing experiments. The inclusion of clinical data in another strength of the study.

There are however concerns that should be easily addressed:

In AT experiments presented in Figure 3; there is a wide variation of infract volume in the rag1 null AT CD84 null T-cell arm. Have the authors confirmed successful AT for each mouse?

Data suggesting that sCD84 present in platelet releasates stimulate T-cell migration are interesting and intriguing. Previous work had demonstrated cleavage of CD84 on platelet activation. The protein can be cleaved at its ectodomain and intracellularly. Another possibility is that CD84 is released by platelets but remains associated with extracellular vesicles. Given the experimental procedure used, vesicles are most likely present in platelet releasates. It is important to ensure the protein is indeed soluble if it is identified as soluble CD84 (sCD84). The authors could assess whether active CD84 is soluble in the supernatant, by performing high speed centrifugation for instance

Although in vitro experiments point to a role of sCD84 in lymphocyte migratory activities, it is unclear whether soluble or platelet bound CD84 stimulate thrombo-inflammation in the in vivo model.

More information and discussion regarding the pathogenic role of lymphocytes would substantiate the study. What do we know of the pathogenic T cells in the model, and of the CD4 T-cells co-activated through platelet-derived CD84? Do they present a Th1 or Th2 phenotype? How is the CD84 stimulation on T-cell promoting T-cell migration, and inflammation? Is this mechanism implicating check-point protein, such as PD-1 suggested by Lewinsky et al. JCI 2018, or IFN as suggested in citations17 and 19? As check-point molecules are already blocked in certain cancers in humans, determining whether it is related to their findings has high clinical value. Moreover, NKT cells are an important source of CD84 and are deficient in Rag mice. In addition to CD4 markers, the identification (or absence of) of CD1 in postischemic brain would help confirm the role of T cell subtypes, if any, in CD84-mediated recruitment.

RE: CIRCRES/2020/316655: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

REVIEWER COMMENTS TO AUTHORS:

We thank the reviewers for the appreciation of our work. We have done our best to address the reviewers' comment in the current situation, which helped us to improve our manuscript substantially. However, we could not perform some experiments requiring to patient material, as non-Covid19 related clinical research is put on hold. We hope that the reviewers can acknowledge this situation.

We would also like to mention that two patients of our prospective cohort study with ongoing follow-up, SICFAIL, dropped out during follow-up. Consequently, we have updated Table 1 and Supplemental Table 5.

Reviewer #1:

The manuscript by Schulmann et al. defines the role of CD84, a homophilic immunoreceptor of the SLAM family in acute stroke. The authors conclude that platelet-derived CD84 worsens stroke outcomes by enhancing the motility of CD4+T cells. Furthermore, the authors found local shedding of CD84 in the ischemic circulation from the samples of AIS patients. High platelet CD84 expression was associated with poor outcome in stroke patients.

We thank the reviewer for the appreciation of our work and the relevant comments and suggestions, which helped us to improve our manuscript substantially.

Following points which need to be addressed to substantially improve that manuscript:

1. Figure 1. Are the representative TTC stained brain images from the same mouse? The infarct area generally develops around the ischemic core, which is not the case with CD84-/- mice. Please clarify.

We thank the reviewer for this question. Indeed, the TTC-stained brain images are from the same mouse. We now specifically included this information in the legend of figure 1. Regarding infarct development, we apologize that the dotted line to highlight the infarcted brain areas in the TTC-stained brain sections was not placed correctly at the middle slice of the representative *Cd84*$^{-/-}$ mouse. These images of one *WT* and one *Cd84*$^{-/-}$ mouse are representative of significantly reduced infarct volumes for the *Cd84*$^{-/-}$ group. In the *Cd84*$^{-/-}$ group, as can be appreciated on the displayed single *Cd84*$^{-/-}$ mouse (grey dotted line), ischemic infarction involves the typical anatomical extent of the MCA cortical and subcortical territory. Indeed, also in *Cd84*$^{-/-}$ mice, parts of the cortex (those supplied by the MCA) are infarcted, however, on average, the volume of cortex infarction was clearly less extensive than for the control group. We changed the figure accordingly.

2. Why only two females/group was used in some experiments? Need to evaluate sex-based differences.

Like most research groups in the field, we predominantly use male mice for experiments. As requested by the reviewer, we have performed the relevant experiments to evaluate sex-based differences and included the data of additional female mice in Figure 1. The separated analysis of females and males is added as the new Supplemental Figure 2. Sex-specific evaluation revealed significantly smaller infarctions in both, male and female *Cd84*$^{-/-}$ mice compared to the respective *WT* controls.

3. T-cell numbers increase in the ischemic brain within the first 24 hours and can persist for days. The study by Gelderblom et al. and others suggest that CD4+ T cells are recruited within 24 hours after stroke and accumulation of these cells in the early inflammatory phase peaks 3 to 4 days after injury (Stroke. 2009;40:1849-1857). Whether T cells present in the chronic period following stroke are beneficial or detrimental to functional recovery is still debatable, long-term functional outcomes following stroke are essential to establish the role of CD84 in stroke outcome. The authors should evaluate long-term sensorimotor deficit (such as corner test, cylinder test, hanging wire test, accelerated rota-rod test) to determine the detrimental effect of CD84 on platelets or T cells or both.

The focus of our study is on the interplay of platelets and T cells in the (hyper-)acute phase of ischemic stroke as T cells have been shown to contribute to infarct growth within the first 24 h (Yilmaz *et al*. 2006; Kleinschnitz *et al*. 2010; Kleinschnitz *et al*. 2013). However, the reviewer's point regarding the long-term effects of CD84-deficiency in the setting of ischemic stroke is well taken. In order to monitor neurological outcome for several days, we had to reduce the occlusion time from 60 min to 30 min to ensure that mice survive and to be in accordance with the guidelines for animal experiments approved by our local authorities. Mice were monitored in several neurological tests on a daily basis. Of note, none of the operated *Cd84*$^{-/-}$ mice, but 3 of the 8 *WT* mice died or had to be taken out of the experiments as they were in a bad shape and reached the stop criteria of our animal license. In line with the results following 60 min tMCAO, *Cd84*$^{-/-}$ mice displayed less severe functional defects on day 1 following 30 min tMCAO as compared to *WT* controls. This difference between the two genotypes persisted for the 7 d observation period, but were less pronounced at the later time points. However, this can be explained by the fact that the three worst performing *WT* mice did not survive. We have included the new data in our manuscript (Figure 2, Supplementary Figure 3) and would like to thank the reviewer for this suggestion which clearly helped to strengthen our manuscript.

Moreover, we dissected the brains from these animals on day 7 and analyzed the infiltrating immune cells by flow cytometry. At this time point after tMCAO we did not observe any significant differences between *WT* and *Cd84*$^{-/-}$ mice. These results indicate that the beneficial effect of CD84-deficiency in the early phase of stroke development is either outweighing a beneficial defect of CD84 during recovery, or that CD84 is not contributing to the protective effect of T cells at later time points (see figure to the reviewers 1). This will need to be addressed in future studies, since it is clearly beyond the scope of this manuscript.

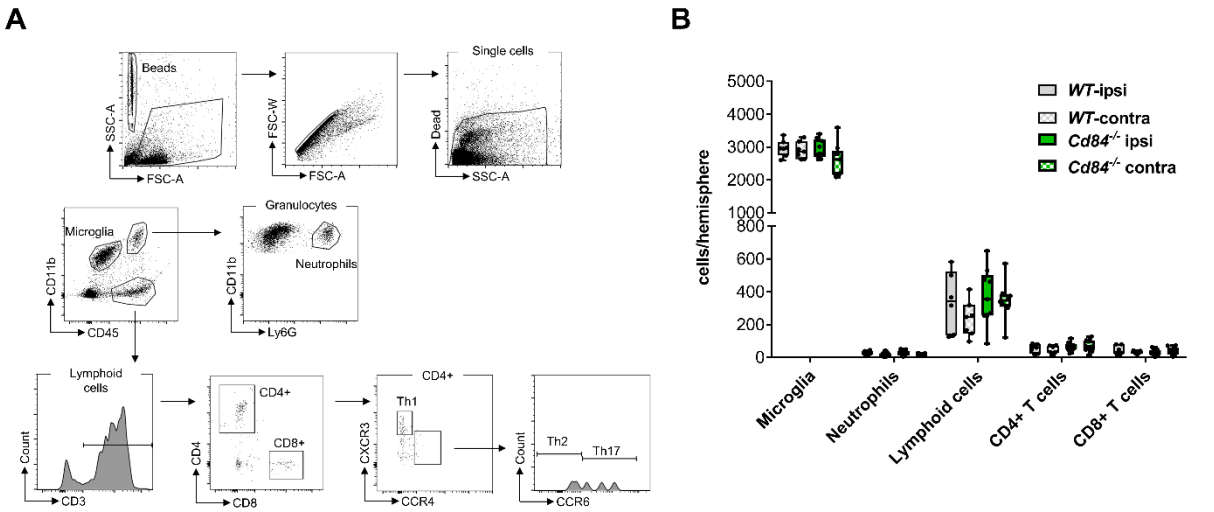

Figure to the reviewers 1. Analysis of immune cells in the brain on day 7 following 30 min tMCAO. Immune cell infiltration into the brain of *WT* and *Cd84*$^{-/-}$ mice was analyzed by flow cytometry. (**A**) Gating strategy for the flow cytometric analysis of immune cells. (**B**) Indicated immune cells per hemisphere (ipsi = ipsilateral, contra = contralateral hemisphere) 7 d after 30 min tMCAO of *WT* and *Cd84*$^{-/-}$ mice (n = 7-9 mice per group).

4. Figure 2C. CD84 is highly expressed in other immune cells, including neutrophils and monocytes. Neutrophils are known to contribute to I/R injury following acute stroke. Surprisingly, the recruitment of neutrophils to the postischemic brain was comparable between control and CD84 KO mice. The potential role of CD84 deficiency in neutrophil/monocyte activation and cytokine secretion should be ruled out experimentally. The IHC results in Fig-2B are not convincing. Need to counterstain with DAPI and in parallel confirmed by flow-cytometry.

As requested by the reviewer, we changed Figure 2B applying immunofluorescence staining of CD11b and counterstaining with DAPI. Furthermore, we confirmed our IHC results by flow-cytometry (new Supplemental Figure 4). These data confirmed the reduced number of infiltrating T cells in *Cd84*$^{-/-}$ mice, but the numbers of lymphoid cells and neutrophils were indistinguishable between both genotypes.

With regard to activation and cytokine secretion, we performed RT-PCR analysis of the ischemic basal ganglia and cortices of *WT* and *Cd84*$^{-/-}$ mice at day 1 after tMCAO and did not observe a difference in the gene expression of IL-1beta and TNF-alpha between the groups (new Supplemental Figure 5D,E). With regard to the contribution of neutrophils to I/R injury it is important to note that, in our hands, anti-Ly6G-antibody-mediated depletion prior to 60 min tMCAO did not affect infarct volumes at day 1 compared to isotype-treated control mice. (Figure for the reviewer).

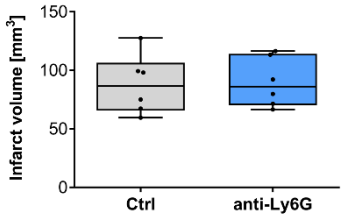

Figure to the reviewers 2. Depletion of neutrophils using an anti-Ly6G antibody does not affect infarct sizes on d 1 post 60 min tMCAO. C57Bl/6J mice received vehicle or 20 µg/g body weight anti-Ly6G antibody i.p.. Three days later mice were subjected to 60 min tMCAO and 24 h later infarct volumes were determined by TTC staining. Infarct volumes are depicted, each symbol represents one individual (n = 6-7 mice per group).

5. Figure 2D. Since CD84-deficiency did not affect the hemostatic or thrombotic function of platelets in mice, the observation that CD84 KO mice exhibit a reduced number of occluded vessels in the ipsilateral hemisphere by ~35% (Fig 2D), needs to be confirmed further by Western. Importantly, the data in current form lacks mechanistic insights how platelet-derived CD84 promotes cerebral thrombosis, but not arterial thrombosis. It is not clear from the method of whether the mice were perfused with saline before the brain sampling for the IHC studies.

Mice were not perfused before brain sampling for our IHC studies.

The reviewer's question with regard to the differences in cerebral thrombosis and arterial thrombosis is a valid one and to the best of our knowledge, the factors that trigger cerebral thrombosis are not understood. However, we are currently finalizing a separate study that investigates the kinetics of infarct growth following 1 h tMCAO and thrombotic activity in the ischemic brain using light-sheet fluorescence microscopy. These data (see figure to the reviewers 3 below) clearly show that infarct growth *precedes* thrombus formation in the cerebral vasculature in this setting. Despite the fact that the occurrence of cerebral thrombi correlates well with the infarct size on day 1 after tMCAO thrombi are observed only at time points at which the neuronal damage is already established and is thus appears to be a secondary event that is not contributing to the growth of cerebral infarcts.

In this context we would like to stress that *Rag1*$^{-/-}$ mice lacking T and B cells display reduced cerebral thrombi after tMCAO despite having completely unaffected platelet function and develop arterial thrombosis with the same kinetics and to the same extent as *WT* mice (Kleinschnitz *et al*., Blood 2010 see figure to the reviewers 3 below). Likewise, we analyzed the number of occluded vessels in *Rag1*$^{-/-}$ mice that received *WT* T cells with those receiving *Cd84*$^{-/-}$ T cells. Paralleling the differences in infarct sizes, *Rag1*$^{-/-}$ mice receiving *Cd84*$^{-/-}$ T cells displayed less occluded vessels as compared to those receiving *WT* T cells (see figure to the reviewers 4). Also here, the platelets in both groups are indistinguishable underscoring the notion that cerebral thrombosis might be a secondary phenomenon in this disease setting. Moreover, GPIIb/IIIa blockers, which abolish platelet aggregation and thrombus formation in mice, did not reduce infarct sizes following tMCAO (Kleinschnitz *et al*., Circulation 2007). Likewise, GPIIb/IIIa blockers did not improve the outcome in patients with acute ischemic stroke (Adams *et al.*, Stroke 2008; Kellert *et al.*, Stroke 2013).

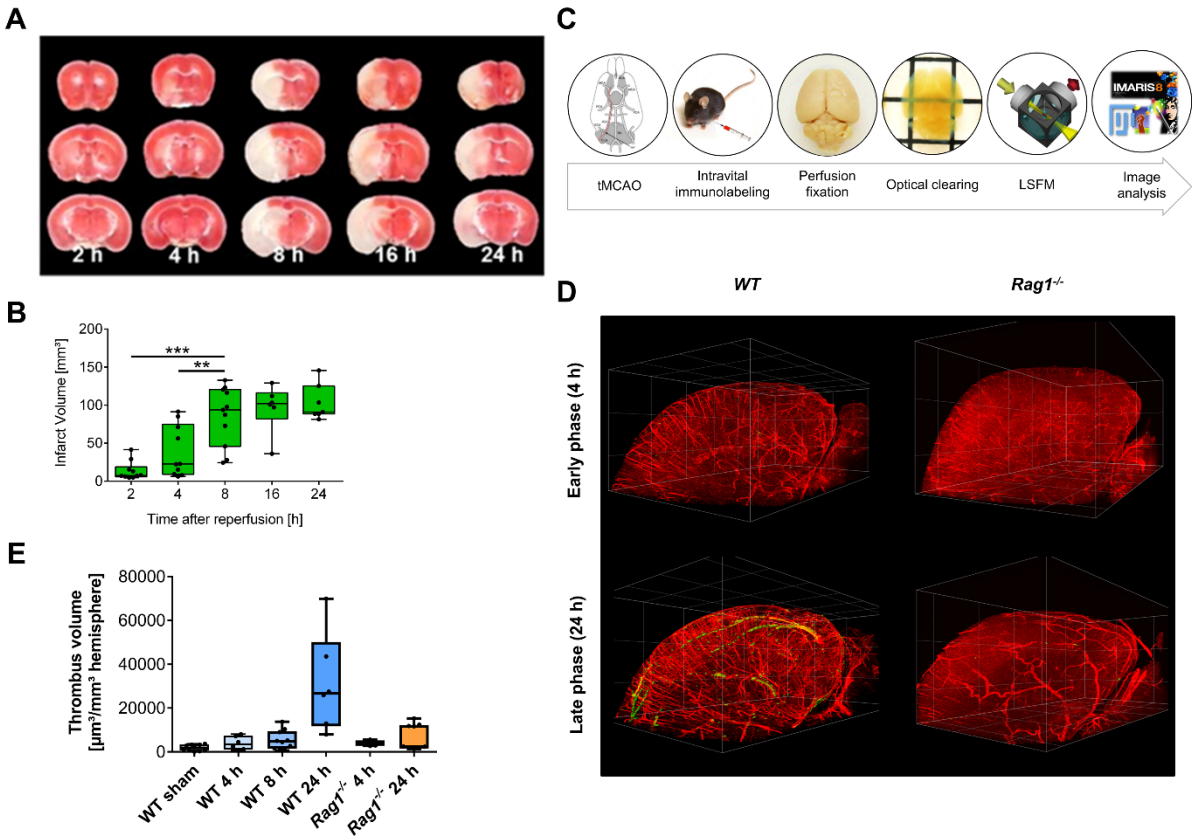

Figure to the reviewers 3. Cerebral thrombus volume correlates with infarct sizes on day 1 after tMCAO, but infarct growth precedes thrombosis. (**A**) Representative images of coronal sections stained with TTC, 2-, 4-, 8-, 16- and 24 hours after tMCAO (60 min) in C57Bl/6J mice. Infarcted areas are shown in white. (**B**) Planimetric analysis was used to quantify the infarct volume. (**C**) Work flow for light sheet fluorescence microscopy of large ischemic mouse brain samples: 8-12 weeks old mice were subjected to the transient middle cerebral artery occlusion (60 min tMCAO) model of ischemic stroke. The animals were immunolabeled by intravenous injection of fluorophore-conjugated antibody derivatives. Subsequently, the animals were sacrificed by cardiac perfusion fixation and the brains were harvested. The organs were optically cleared and imaged with a LSFM microscope. Acquired 3D image stacks were processed using deconvolution, segmentation and object recognition software. (**D**) Representative 3D reconstructions of LSFM image stack containing the vasculature (anti-CD31, red) and platelet channels (anti-GPIX antibody derivative, p0p6, green) reveal considerable cerebral thrombi in brain of *WT* mice 24 h after tMCAO. After 4 h of reperfusion, however, or in *Rag1*$^{-/-}$ mice no cerebral thrombi could be observed. (**E**) Quantitative analysis of total thrombus volume sham-operated *WT* mice (24 h post OP), *WT* and *Rag1*$^{-/-}$ mice at the indicated time after reperfusion following 1 h tMCAO. The values are normalized for imaged brain volume. Data from Gorelashvili, Klaus *et al*., manuscript in preparation.

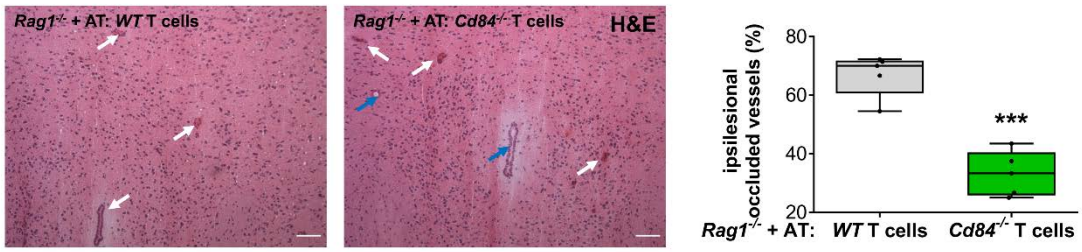

Figure to the reviewers 4. Reduced cerebral thrombosis in *Rag1*$^{-/-}$ mice receiving *Cd84*$^{-/-}$ T cells as compared to *Rag1*$^{-/-}$ mice receiving *WT* T cells. Representative hematoxylin and eosin staining and quantification of the percentage of occluded vessels in the infarcted hemispheres of *Rag1*$^{-/-}$ mice with adoptive transfer (AT) of CD4^{+} T cells from *WT* and *Cd84*$^{-/-}$ mice (n = 5 mice per group). White arrows indicate occluded vessels, blue arrows indicate patent vessels. Bar, 100 µm. Statistical significances analyzed by Student *t* test. ***P<0.001.

6. Figure 3. Comparisons between the different groups are not rigorously tested and unreliable. It is extremely complex and hard to interpret due to the absence of several key control groups and a lack of detailed experimental protocol. It seems the experiments were not done in parallel, and importantly not all groups have received the adoptive transfer. There is no WT control for Rag1-/- mice, no WT AT of WT T-cells control mice to evaluate the relative role of WT vs. CD84-/- T-cells in Rag1-/- mice. Importantly, the lack of functional recovery data limits the interpretation of the overall stroke outcome.

We disagree with the reviewer on this point of critique. The numbers of animals needed for such an extensive study makes it impossible to analyze all mice at once. However, we do of course always compare our transgenic mice to the appropriate controls. Like all laboratories that do animal studies we are bound to the 3R principles. As *Rag1*$^{-/-}$ mice (originally received from Jackson laboratories and kept in our animal house in the same room as the *Cd84*$^{-/-}$ mice) and *Cd84*$^{-/-}$ mice (generated in our lab) are both on C57Bl/6J background, with regard to animal welfare our authorities required us to use the same control group (as both would require C57Bl/6J *WT* mice as controls) for these studies to reduce the number of mice needed for animal experiments. Thus, we chose to work with *Cd84*$^{+/+}$ litter mate control mice as control mice for both, *Cd84*$^{-/-}$ mice and *Rag1*$^{-/-}$ mice. Naturally, we always operate *WT* control mice in the same experimental setting and in double-blinded manner along with *Cd84*$^{-/-}$ mice or *Rag1*$^{-/-}$ mice. We are convinced that this control group is superior to *WT* mice that are directly purchased and used in experiments as control for transgenic mice that are kept in-house.

Regarding the question of adoptive transfer of *WT* T cells in *WT* mice, we have of course performed these experiments initially when we started to use *Rag1*$^{-/-}$ mice for adoptive transfer experiments (Kleinschnitz *et al*., Blood 2010 and Kleinschnitz *et al*., Blood 2013). The “extra number” of *WT* T cells in *WT* mice did not affect the outcome (figure to the reviewers 5). As the adoptive transfer of T cells into *Rag1*$^{-/-}$ mice is a widely use model, our ethical board excluded such studies to be performed, and we do agree with their view that this control group should not be essential to be included once again in this study.

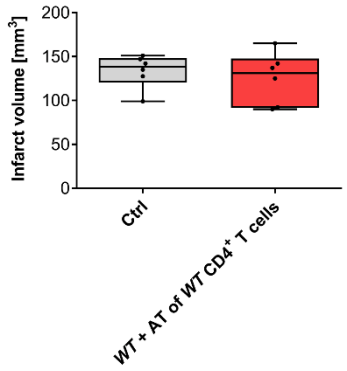

Figure to the reviewers 5. Adoptive transfer of *WT* CD4⁺ T cells into C57Bl/6J mice does not affect infarct sizes on d 1 post 60 min tMCAO. C57Bl/6J mice received vehicle or 750,000 CD4⁺ T cells. 24 h later mice were subjected to 60 min tMCAO and 24 h later infarct volumes were determined by TTC staining. Infarct volumes are depicted, each symbol represents one individual (n = 6 mice per group).

7. Other parameters in Figures 1, 2, and 3, including edema, BBB, and neuronal injury, should be quantified and included.

We thank the reviewer for this suggestion. We performed extensive additional experiments addressing the raised points and indeed observed reduced neuronal injury in *Cd84*$^{-/-}$ mice at day 1 after tMCAO (new Supplemental Figure 5A).

In addition, BBB function in the mutant mice was improved as assessed by Western blot analysis of Albumin extravasation (new Supplemental Figure 5B). This data is supported by confocal microscopy of brain sections (new Supplemental Figure 5C). Of note, we observed a similar trend in *Rag1*$^{-/-}$ mice receiving *Cd84*$^{-/-}$ CD4⁺ T cells as compared to *Rag1*$^{-/-}$ mice receiving *WT* T cells (see figure to the reviewers 6).

Notably, we did not observe differences in MMP2 or MMP9 expression levels in the brain tissue of *WT* and *Cd84*$^{-/-}$ mice (new Supplemental Figure 5F,G).

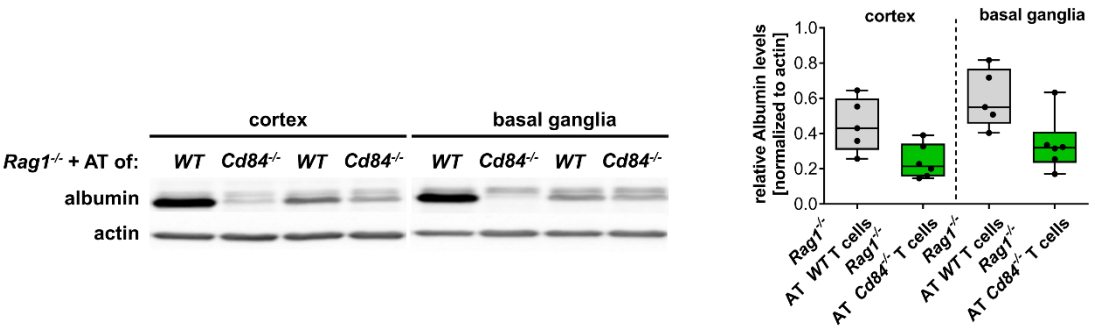

Figure to the reviewers 6. *WT*, but not *Cd84*$^{-/-}$ CD4⁺ T cells contribute to blood brain barrier damage in *Rag1*$^{-/-}$ following tMCAO. Representative anti-albumin Western blot analysis and densiometric quantification of ipsilesional albumin protein expression in the basal ganglial as well as cortical regions (n= 5-6 mice per group) after tMCAO.

8. It is not clear from the methods of whether CD84fl/flPF4Cre+ve mice and control CD84fl/fl PF4Cre-ve mice are littermate controls or not. Moreover, recent studies demonstrate transgene expression outside the megakaryocyte lineage, including leukocytes and macrophages (Pertuy F et al., J Thromb Haemost. 2015;13(1):115-125, Calaminus SD et al., PLoS One. 2012;7(12):e51361). To rule out this non-specificity of PF4 Cre-recombinase on stroke outcome, the authors should experimentally evaluate CD84 levels in other immune cells in CD84fl/flPF4Cre+ve mice and should also evaluate stroke outcome in CD84+/+PF4Cre+ve versus CD84fl/fl PF4Cre-ve mice.

We thank reviewer for raising these important points. Yes, *Cd84*$^{fl/fl;\ PF4Cre+ve}$ mice and control *Cd84*$^{fl/fl;\ PF4Cre-ve}$ mice are littermates. As requested, we performed flow cytometry and quantified CD84 levels in other immune cells (new Supplemental Figure 7) to exclude potential expression of cre-recombinase outside the megakaryocyte lineage.

In addition, we evaluated stroke outcome in *Cd84*$^{+/+;\ PF4Cre+ve}$ versus *CD84*$^{fl/fl;\ PF4Cre-ve}$ (*CD84*$^{fl/fl,\ Cre-neg.}$) mice, and could not detect any differences in between the groups (see Supplemental Figure 8).

9. Not sure whether the authors can conclude that platelet CD84 is required for the development of CD4+ T cell-dependent cerebral I/R injury. Is it not possible that platelet CD84 deficiency may protect from cerebral I/R injury independent of CD4+ T cells? The authors should perform bone-marrow transplant experiments by reconstituting BM cells of CD84fl/flPF4Cre mice to the irradiated WT and CD84 KO mice, with appropriate controls.

A bone marrow transfer of *Cd84*$^{fl/fl;\ PF4Cre+ve}$ would mean that megakaryocytes and platelets would lack CD84 while all other blood cells, including T cells, would express CD84 (see our response to comment #8 and new Supplemental Figure 7). Thus, the bone marrow chimera mice would simply recapitulate the 'ordinary' *Cd84*$^{fl/fl;\ PF4Cre+ve}$ mice, but with the additional suffering due to the irradiation before transplantation. Therefore, this experiment would not add any new information and we chose not to perform it. We are certain that such an experiment would not be approved by our local ethics committee.

Concerning the reviewer's question with regard to platelet CD84 acting on T cell CD84, we would not be aware of any approach that would allow us to exclude all other options. However, as *Cd84*$^{-/-}$ mice and *Cd84*$^{fl/fl;\ PF4Cre+ve}$ have a comparably reduced infarct size and in light of the fact that the transfer of *WT* T cells in *Rag1*$^{-/-}$ mice but not in *Cd84*$^{-/-}$ mice restores infarct sizes to *WT* levels we consider the CD84 platelet – CD84 T cell interaction as the most likely explanation. Moreover, our in vitro data with CD4^{+} T cells and platelet supernatant from activated *WT* and *Cd84*$^{-/-}$ platelets strongly support this concept.

10. The authors report that platelet surface abundance of CD84 was reduced locally within the ischemic circulation as compared with non-ischemic intra-individual systemic platelet CD84 surface abundance. The quantification of CD84 levels in the plasma from these samples will provide valuable information regarding the role of soluble CD84 on stroke outcome.

We thank the reviewer for raising this interesting point. Unfortunately, no intra-individual plasma is available from these samples as the samples originate from an initial small cohort study in which no plasma samples were collected. We agree with the reviewer that plasma

levels, in particular obtained from the ischemic core would be very informative and would have liked to address this experimentally. However, in the current situation all non-Covid19 related clinical research is put on hold so that we could not enroll more patients into our study to investigate the role of sCD84 in ischemic stroke.

11. The role of CD84 should be tested in another stroke model.

In acute ischemic stroke, there is an alarming discrepancy between recanalization rates of up to 80-90%, and no clinical benefit in at least every second stroke patient. Experimental stroke research strongly has implied for decades that ischemia/reperfusion injury may deliver one important explanation for progressive infarction after / despite recanalization.

The specific goal of our study was to **unravel the molecular mechanism** underlying T cell/platelet interactions that have been delineated as a dominant causative driving force of **I/R-injury**. The findings that constitute a T cell/platelet centered model of I/R have been replicated by independent research groups over many years using the exact same tMCAO model that was applied here. At least an equally important argument in favor of this tMCAO model is that translation with regard to the specific cerebral arterial occlusion location is straightforward. Our human prospective study with ischemic arterial endovascular sampling observed precisely the exact same occlusion locations in patients that are simulated in tMCAO mice (MCA M1 and ICA-T occlusions).

Our study is a **mechanistic study**, defining in CD84 as one missing link of platelet and T cell interactions during I/R-injury. We do not raise the claim of a **preclinical drug study**. The two clinical strategies that we used in this study were observational and included the distinct and novel innovation of direct sampling of ischemic cerebral arterial blood (Kollikowski *et al*., 2020 Ann Neurol). Of course we are aware that according to the STAIR recommendations, a positive result achieved from a **drug study** in a species should always be verified in another species and should be replicable in a further stroke model. However, as the aim of our study was assessing the relevance of CD84 for cerebral infarct growth, we feel that it is far beyond the scope of our current work to ask for additional stroke models. Many other recent mechanistic stroke studies published in high profile journals (e.g. Ito *et al*., Nature 2019; Hayakawa *et al*., Nature 2016; Roth *et al*., Sci Transl Med 2018; Alawieh *et al*., Sci Transl Med 2018; Sauter *et al*., Circulation 2018; Choe *et al*., Circulation 2013; Dhanesha *et al*., Circulation 2015; Courties *et al*., Circ Res 2015; Senchenkova *et al*., Circulation 2019; Benakis *et al*., Nat Med 2016) were based only on the tMCAO model as experimental stroke model. Given the extensive and detailed analyses we did in this widely used stroke model, we are convinced that our data provides strong evidence for a major pathogenic function of CD84 in the course (experimental) ischemic stroke development. Once a CD84-blocking agent becomes available, we will of course adhere to the STAIR recommendations; this is, however, beyond the scope of our current study.

To address the reviewer's concern we have included a sentence in the discussion section to underscore the need for further studies.

Reviewer #2:

In this original article by Shuhmann et al., the authors investigated the role of a signaling lymphocyte activation molecule, CD84, in I/R injury in the classical mouse model of ischemic stroke induced by transient mechanical occlusion of the MCA. They show that mice with a total genetic deficiency in CD84 have reduced infarct volumes, as assessed by TTC staining 23h after a 1-hour-long transient occlusion of the MCA. The authors provide evidence that the decreased volume infarct in CD84-/- mice is not the consequence of improved perfusion during the ischemic period or after monofilament removal. This result indirectly suggests that the improved outcome in CD84-/- is not due to reduced microvascular thrombosis. Instead, they show that the protection is associated with a marked decrease in post-I/R accumulation of CD4+ T cells and monocytes, and no modification of neutrophil recruitment. Using adoptive transfer experiments, they show that both platelet and T cell CD84 is necessary for T cell-dependent I/R injury. Finally, they show in in vitro experiments that CD84 might be a regulator of T cell motility, and provide clinical evidence that CD84 levels are reduced on the platelet surface in the ischemic circulation.

The results are convincing and support the idea of a major contribution of CD84-dependent recruitment of CD4+ T cells to I/R injury in this model of ischemic stroke. These results are in line with a series of previous studies pointing to a central role for platelet/CD4+ T cell interactions in I/R injury. The main novelty here is thus the identification of a surface molecule expressed by both types of cells as a central player in this process.

Overall, the study is technically sound and the conclusions are well supported by the data and controls provided. The results bear interesting translational potential as they identify a pharmacological target for specific inhibition of the platelet deleterious action during I/R injury, without impairing hemostasis.

We would like to thank the reviewer for the positive evaluation of our work and the constructive comments and suggestions, which helped us to improve our manuscript.

I have only a few comments.

Do plasma samples from stroke patients stimulate T cell migration in in vitro assays? Is there any correlation between sCD84 levels in plasma from stroke patients and T cell migration in vitro?

We agree with the reviewer that this information would be highly interesting. We tried to stimulate T cell migration with human stroke plasma samples *in vitro*. Unfortunately, using plasma concentrations of up to 10% were not sufficient to induce T cell migration when compared to citrate/PBS control. Therefore, even higher plasma concentrations (and consequently plasma volumes beyond our capacity) would have been needed to induce T cell migration *in vitro*, which were currently not available to us. The expansion of our clinical cohort was unfortunately so far not possible due to the pandemic that put all non-Covid19-related clinical research on hold. Therefore, we are not able to answer the reviewer's question.

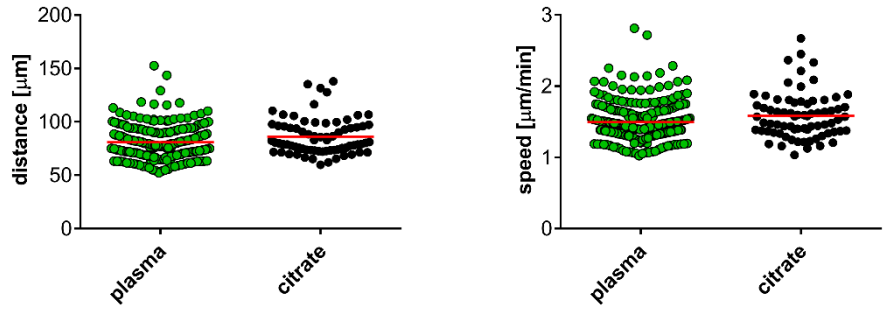

Figure for the reviewers 7. Diluted plasma samples from human stroke patients do not promote CD4⁺ T cell migration *in vitro*. Migration distance and velocity of healthy donor CD4⁺ T cells was analyzed in response to treatment with 10 % (V/V) plasma from stroke patients compared to citrate/phosphate buffered saline control. Each dot represents the migrated distance over 30 min of one CD4⁺ T cell (n = 73-272 cells per group of 3 independent experiments). Horizontal lines correspond to the mean.

Do CD84 levels on platelets correlate with other markers of platelet activation in patients?

We agree with the reviewer that this information would be highly interesting. However, we did unfortunately not assess platelet activation systematically in our initial clinical cohort. A second study was planned to investigate platelet activation in stroke patients (using unstimulated and platelets that are stimulated with different agonists) and expression levels of relevant platelet glycoproteins. Due to the pandemic all non-Covid19-related clinical research is put on hold so that we cannot perform this study at the moment. Therefore, we are unfortunately not able to answer the reviewer's question.

In vitro studies: does CD84 need to be shed to exert its action toward T cells? Do CD84-dependent physical interactions between platelets and T cells have the same pro-motility effects?

As requested by the reviewer, we have performed the relevant experiments and did not observe an effect of physical interactions between platelets and T cells (see Supplemental Figure 9E,F).

What about CD84 levels on patient T cells? Was it investigated?

The reviewer raises an interesting question. Unfortunately, we so far did not have the chance to analyze patient T cells with regard to CD84 levels. The sampled blood volume per patient has been very small and follow-up studies are currently on hold.

The authors could discuss further, even briefly, how CD4+ T cells cause neurotoxicity in this model? Is there any evidence of a cytokine release syndrome in the tMCAO?

The detrimental role of T cells during ischemia-reperfusion injury is antigen-independent (Kleinschnitz, C. Blood 2010). The effects of T cells during ischemia-reperfusion injury could be mediated by the release of cytokines, but the typical pro-inflammatory cytokines, such as

IFNγ and IL-23, seem to originate from other cells, such as dendritic cells (Yilmaz, G. Circulation 2006; Shichita, T. Nat med 2009; Stoll, G. 2019 Nat rev Neurol). In our current study, we did not observe differences in the gene expression of cytokines after tMCAO (Supplemental Figure 5D,E) nor cytokine release of in vitro activated T cells (Supplemental Table 2; Figure to the reviewers 8). Importantly, when platelets are depleted in *Rag1*$^{-/-}$ mice before tMCAO, infarcts remained small in these mice, even if adoptive transfer of T cells was performed (Kleinschnitz, C. Blood 2013), demonstrating that the T-cell effects in ischemia-reperfusion injury depend on platelets, but the effector mechanisms of T cells so far remain elusive.

Besides their results, how important is CD84 to T cell function and/or activation in general? Is it recognized as a predominant player in CD4+ T cell migration or activation as compared to other SLAMs?

To the best of our knowledge, the literature on the role of CD84 on T cell migration is very limited as this aspect has not yet been studied. With regard to a potential role on T cell activation we have assessed the effect of sCD84 on T cell cytokine production in the absence or presence of a plate-bound anti-CD3 antibody. In this experimental setup, the presence of CD84-Fc did not trigger a detectable T cell response (not shown) and did not affect anti-CD3 induced cytokine expression (see figure to the reviewers 8).

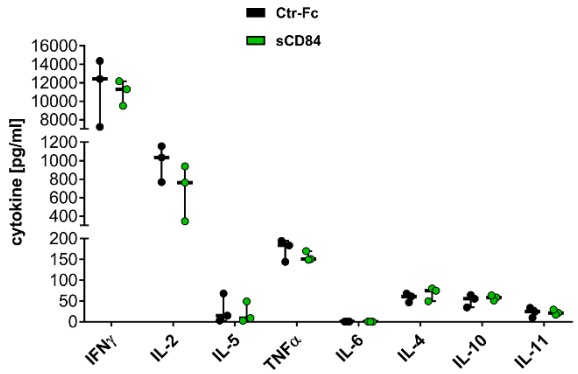

Figure for the reviewers 8. sCD84 does not affect anti-CD3 induced T cell cytokine release. Purified *WT* CD4$^+$ T cells were adjusted to a concentration of 1x10^6/ml in RPMI supplemented with 2 mM L-Glutamine, 10% FCS, sodium-pyruvate, 30 µM ß-mercaptoethanol and penicllilin/streptomycin (100 U/ml each). The GPVI-Fc fusion protein control-Fc or CD84-Fc were added at a final concentration of 10 µg/ml and 1 ml of the cell suspension was transferred to 24 well plates, coated with 300 µl of hamster anti-mouse CD3ε antibody (clone: 145-2C11, 10 µg/ml in PBS; produced in house) or PBS. After 48 h incubation, the cell supernatants were yielded by centrifugation of cell suspensions at 5,000 rpm for 5 min and frozen at -20°C for later analysis. Cytokine levels in the supernatants were determined using the LEGENDplex™ Mouse Th1/Th2 Panel (Cat# 740751, Biolegend) according to the manufacturer's instructions.

Minor

There seems to be a mistake in the sample size calculation on page 5 I assume one should read 0<0.05 instead of 0.5

We apologize for this typographic error that is now corrected.

Reviewer #3:

Stroke is one of the major causes of death and disability worldwide with limited treatment options. Despite recent advances in mechanical thrombectomy with high recanalization rates, the therapeutic efficacy of vessel reopening remains limited. The progression of cerebral infarction is frequently attributed to ischemia/reperfusion (I/R) injury. Previous work by the authors and others had revealed that T lymphocytes (using Rag-1 mice and CD4 cells adoptive transfers) and platelets (through GPIb and GPVI, and platelet depletion in Rag-1 mice) both contribute to injury in a process of thrombo-inflammation.

In this manuscript by Schuhmann and colleagues, the authors define how platelets and lymphocytes promote thrombo-inflammation in this context. They elegantly demonstrate that a homophilic receptor, CD84, links platelets and lymphocytes in a mouse model of transient middle cerebral artery occlusion. Further in vitro experiments and analyses of human biospecimens also point to the implication of CD84. The manuscript is very well written, clear, and the conclusions are supported by convincing experiments. The inclusion of clinical data in another strength of the study.

There are however concerns that should be easily addressed:

In AT experiments presented in Figure 3; there is a wide variation of infract volume in the rag1 null AT CD84 null T-cell arm. Have the authors confirmed successful AT for each mouse?

Yes, we always control the adoptive T cell transfer by flow cytometry of peripheral blood 1 h after T cell transfer. This information has now been added to the material and methods section.

Data suggesting that sCD84 present in platelet releasates stimulate T-cell migration are interesting and intriguing. Previous work had demonstrated cleavage of CD84 on platelet activation. The protein can be cleaved at its ectodomain and intracellularly. Another possibility is that CD84 is released by platelets but remains associated with extracellular vesicles. Given the experimental procedure used, vesicles are most likely present in platelet releasates. It is important to ensure the protein is indeed soluble if it is identified as soluble CD84 (sCD84). The authors could assess whether active CD84 is soluble in the supernatant, by performing high speed centrifugation for instance

Following the reviewer's suggestion, we have performed high-speed centrifugation of platelet releasate. Using this approach in combination with a CD84-ELISA we detected CD84 in the majority of supernatants indicating that is indeed predominantly sCD84 that is released from activated platelets (Figure for the reviewers 9) or present in the serum (not shown). However, we did also find a smaller proportion of CD84 in the 100,000 x*g* pellet indicating that a fraction of CD84 might indeed be bound to extracellular vesicles. Of note, platelet-bound CD84 did not enhance T cell motility (Supplemental figure 9E,F) and mice lacking ADAM10 in megakaryocytes and platelets have been shown to be unable in generating sCD84

(Hofmann *et al*., J Thromb Haemost 2012). Thus, we would assume that the dominant fraction of sCD84 is indeed soluble and not associated to microvesicles.

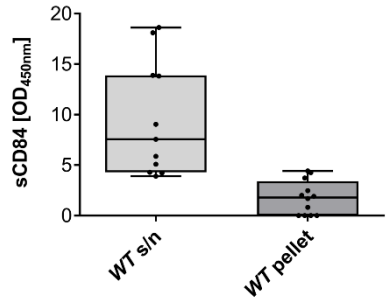

Figure for the reviewers 9. CD84 found in platelet releasate is found predominantly in the membrane-free fraction. *WT* platelets (500,000/µl) were stimulated with 10 µg/ml collagen-related peptide for 15 min at 37°C in Tyrode's buffer with calcium. Platelets were removed by centrifugation with 800 x*g* and platelet releasate was collected after another centrifugation step at 22,000 *xg* for 5 min at 4°C. 200 µl of the platelet-free supernatant (= PLT-R) were centrifuged 1 h with 100,000 x*g* at 4°C. Subsequently, the upper 100 µl of the centrifuged supernatant (*WT* s/n) and the lower 100 µl, resuspended pellet fraction (*WT* pellet) were analyzed separately in our home-made sCD84-ELISA as described in Hofmann *et al*., J Thromb Haemost 2012.

Although in vitro experiments point to a role of sCD84 in lymphocyte migratory activities, it is unclear whether soluble or platelet bound CD84 stimulate thrombo-inflammation in the in vivo model.

The reviewer's point is well taken, but we are not able to address this *in vivo*. Thus, we performed an *in vitro* experiment as requested by reviewer 2 and included the results in Supplemental figure 9E,F. These data indicate that platelet-bound CD84 did not enhance T cell motility, therefore, sCD84 appears to be the predominant driving mode of action of platelet-derived CD84 on T cell motility.

More information and discussion regarding the pathogenic role of lymphocytes would substantiate the study. What do we know of the pathogenic T cells in the model, and of the CD4 T-cells co-activated through platelet-derived CD84? Do they present a Th1 or Th2 phenotype? How is the CD84 stimulation on T-cell promoting T-cell migration, and inflammation? Is this mechanism implicating check-point protein, such as PD-1 suggested by Lewinsky et al. JCI 2018, or IFN as suggested in citations17 and 19? As check-point molecules are already blocked in certain cancers in humans, determining whether it is related to their findings has high clinical value.

T cell responses in adaptive immunity are triggered by defined antigens that are presented to T cells by antigen-presenting cells, whereas the detrimental role of T cells during ischemia-reperfusion injury is antigen-independent (Kleinschnitz, C. Blood 2010). Consequently, mice lacking essential costimulatory molecules such as CD28, programmed death 1 (PD1) or B7 homolog 1 (B7-H1) still developed complete infarctions after tMCAO that were indistinguishable from those in wild-type mice. The effects of T cells during ischemia-reperfusion injury could be mediated by the release of cytokines, but the typical pro-

inflammatory cytokines, such as IFNγ and IL-23, seem to originate from other cells, such as dendritic cells (Yilmaz, G. Circulation 2006; Shichita, T. Nat med 2009; Stoll, G. 2019 Nat rev Neurol). In our current study we did not observe differences in the gene expression of cytokines after tMCAO (Supplemental Figure 5D,E) nor cytokine release of in vitro activated T cells (Supplemental Table 2; Figure to the reviewers 8). Importantly, when platelets are depleted in *Rag1*$^{-/-}$ mice before tMCAO, infarcts remained small in these mice, even if adoptive transfer of T cells was performed (Kleinschnitz, C. Blood 2013), demonstrating that the T-cell effects in ischemia-reperfusion injury depend on platelets, but the effector mechanisms of T cells so far remain elusive.

We tried to address the reviewer's point by analyzing the immune cells that infiltrated the ischemic brain. In line with our histological analyses, we observed a reduced number of CD4$^+$ T cells in *Cd84*$^{-/-}$ brains as compared to *WT* brains on day 1 after tMCAO. There was a trend towards less Th1 or Th17 cells in *Cd84*$^{-/-}$ mice. However, due to the low overall number of T cells in the brain at day 1 we would be reluctant to over-interpret this data. We have included the flow cytometric data on CD4$^+$ and CD8$^+$ T cells in Supplementary Figure 4, but would prefer to display the Th1 and Th2 data only as figure to the reviewers (nr. 10, see below), since the total cell count was quite low.

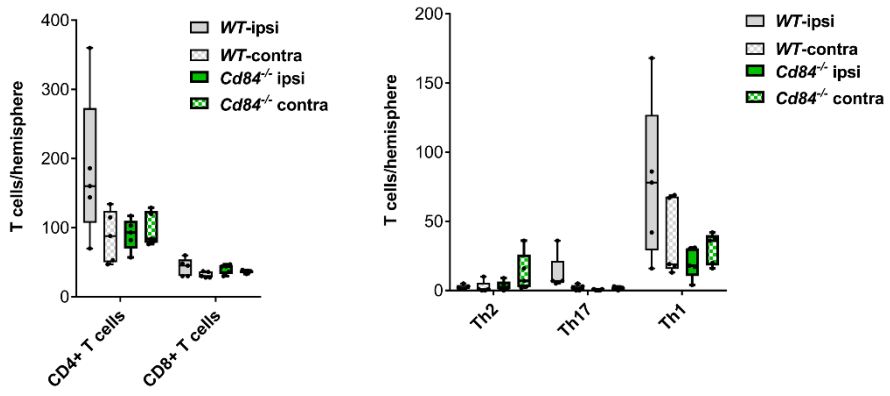

Figure to the reviewers 10. T cell recruitment to the brain on day 1 following 60 min tMCAO. T cell infiltration into the brain of *WT* and *Cd84*$^{-/-}$ mice was analyzed by flow cytometry 24 h after tMCAO of *WT* and *Cd84*$^{-/-}$ mice (n = 5 mice per group).

In addition, we assessed the effect of sCD84 on T cell cytokine production in the presence or absence of anti-CD3 antibodies as additional stimulus. CD84 alone did not trigger any detectable cytokine production and ìt did not modulate anti-CD3 triggered cytokine production (see figure to the reviewers 8 above as response to reviewer 2).

As T cell cytokine production was not altered by sCD84 we did not follow up on the checkpoint molecules suggested by this reviewer. We agree with her/him that more knowledge on CD84 affecting T cell biology in the context of ischemic stroke would be of interest, but considered more experiments in this setting being beyond the scope of that study.

Moreover, NKT cells are an important source of CD84 and are deficient in Rag mice. In addition to CD4 markers, the identification (or absence of) of CD1 in postischemic brain would help confirm the role of T cell subtypes, if any, in CD84-mediated recruitment.

To unravel the relevance of different T cell subtypes for stroke outcome is an important question. Regarding the role of natural killer [NK] T cells, we have previously induced stroke in mice deficient in NKT cells by transient middle cerebral artery occlusion (tMCAO) and assessed stroke outcome at day 1. We showed that, similar to wild-type controls, mice lacking NKT cells were fully susceptible to tMCAO (Kleinschnitz *et al*., Blood 2010). These data indicate that NKT cells do not critically contribute to I/R injury in the mouse tMCAO model.

Nevertheless, we tried to stain our brain sections with an anti-CD1 antibody (clone PK136, BioLegend) that worked nicely on spleen sections, but did not stain any cells on brain sections (not shown). We would assume that the number of NKT cells is below our detection limit, as previous reports have shown that only very few NKT cells can be detected in the ischemic brain (Chu *et al*., JCBFM 2014; Gelderblom *et al*., Stroke 2009).

Circulation Research Decision on Ms #CIRCRES/2020/316655R1
June 22, 2020

Dr. David Stegner
University of Würzburg
Institute of Experimental Biomedicine
Josef-Schneider-Str. 2, D15
Würzburg
Germany

RE: CIRCRES/2020/316655R1: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

Dear Dr. Stegner:

Your manuscript has been carefully evaluated by 5 external reviewers and the editors as a Regular Article. We regret to inform you that the paper is not acceptable for publication in its present form.

As you will gather from the reviews, the referees identified a number of methodological problems. The editors concur. Major issues include some additional confirmatory studies and issues related to formatting and statistical tests.

Despite these concerns, the editors see this paper as potentially important and wish to encourage revision. If you would like to revise the manuscript in accordance with the suggestions of the reviewers and editors, we would be willing to evaluate a new version. The manuscript would be reviewed again, with no assurance of acceptance.

Among the concerns cited by the reviewers, the editors feel that the most important issues that need to be addressed are some minor concerns raised by Reviewer #1. Please address the question regarding why platlelet-derived CD84 promotes cerebral thrombosis, but not arterial thrombosis in your discussion, confirm that mice were indeed neutrophil deficient at the time of the stroke procedure, and provide more definitive/specific antibody staining. Please also pay close attention to concerns raised by the statistical reviewer.

NEW REQUIREMENTS:
Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are required to upload the Table at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

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PLEASE READ: During this unprecedented and challenging time, the health and safety of you, your family, and your community is of utmost importance. We appreciate that one aspect of this current situation is the inability to continue research work and that some measures will lead to a significant hindering of research progress. Please know that we are flexible regarding turnaround times for revisions and other tasks during this stressful time and deadlines will be extended as needed. If you are able to, please contact us if you need any extensions or if you experience any challenges around manuscript preparation; we will work with you.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Number each page in the top right corner, using your manuscript number followed by /R2 to denote a second revision.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Revisions that do not conform to the current limits on numbers of words (8000 total) and display items (maximum of 8 tables and/or figures) may be returned to the authors for abbreviation. If you cannot reduce the overall word count, the editors may deem an extended print version appropriate; the authors should provide written assurance that they will cover the costs of the pages that are in excess of these limits. Note that paying for excess display items is not an option. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

NEW: As of January 1, 2020, all corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To avoid potential processing delays in future, we recommend that you link an ORCID iD to your profile when you submit your revision. To register with ORCID or link your profile, please go to "Modify Profile/Password" on the submission site homepage, and click the link in the "ORCID" section.

We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****

Reviewer comments to the Authors:

Reviewer #1:

1. Still not convinced why platlelet-derived CD84 promotes cerebral thrombosis but not arterial thrombosis.
2. Not clear why tMCAO was done 3 days after neutrophil depletion. Did authors made sure that mice were indeed neutrophils-deficient.
3. The staining for neutrophils and thrombi in figure 3C and D is not convincing. Need to stain thrombi with platelet-specific antibody. For neutrophils staining, need to counter-stain nuclei.

Reviewer #2:

The authors have addressed my comments to the best of their possibilities considering the current COVID crisis context.
I have no further comments.

Reviewer #3:

All my concerns are addressed.

Statistical Reviewer:

Please report exact p-values and sample sizes throughout the manuscript and supplement, rather than reporting range values. For very small p-values, I strongly suggest using scientific notation with two significant digits.

Please include race/ethnicity information for the SICFAIL study

It's not always clear how p-values were calculated in the tables?

It's not always clear how many tests were run or when multiple testing corrections were applied? Eg, fig 4B, Sfig3, others.

Claims of "no difference" should be checked to see if it would be more accurate to claim "no significant difference". Eg "Of note, no difference in T cell motility was observed in the presence of WT or Cd84-/- platelets (Supplemental Figure 9E,F)"

How were representative images/figures chosen? Please note the approach used to select representative images in the main text.

If no corrections for multiple testing were made across tests (only within-test corrections were made), please note this in the statistical section of the manuscript.

Tests to establish normality are not powered to detect deviation from normality when n is small (eg, n{less than or equal to}5 as in figure 3). Please check if a non-parametric test would be more appropriate to use in cases where you have analyzed small samples.

Technical Reviewer:

Comments to Authors on Rigor Checklists:
The current study was evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The study closely complied with said guidelines, however, the reviewer has identified a few minor items that will need to be addressed in a revision:

In vitro checklist:
1) To maintain consistency, please provide the number of biological replicates (n numbers) in the figure legend accompanying supplemental figure 1.
2) Please ensure that all presented immunoblots contain markers denoting the location of molecular weight standards electrophoretically resolved with experimental protein lysates (e.g., Supplemental figure 5B).
3) Where immunostaining procedures are described, please provide a brief description of controls employed to validate antibody specificity and/or distinguish genuine target staining from background.
4) Ensure that all presented micrographs contain a scale with reported units of measure (e.g., Supplemental Figure 1B).

Other:
1) Checklists indicate the use of blinding procedures; however, this was not evidenced in text. Please address this disparity.
2) Please indicate why animals were not randomized to experimental groups.

Comments to the Authors on Research Guidelines and Reporting:
None.

RE: CIRCRES/2020/316655R1: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

REVIEWER COMMENTS TO AUTHORS:

Reviewer #1:

1. Still not convinced why platelet-derived CD84 promotes cerebral thrombosis but not arterial thrombosis.

The mechanisms underlying cerebral thrombosis following ischemia/reperfusion injury are only partly understood. What is known, however, is that the occurrence of cerebral thrombi correlates well with the infarct size on day 1 after tMCAO. Therefore, less thrombi are observed in mice lacking CD84, as CD84-defiency results in less T cell recruitment and subsequently less cerebral damage (as we could show in this manuscript). Consequently, less cerebral thrombi are observed in *Cd84*$^{-/-}$ mice, simply due to the fact that the infarct sizes are smaller. Thus, we do not see a discrepancy to the fact that arterial thrombosis is not affected by CD84-deficiency. In the case of cerebral thrombi post tMCAO the contribution of CD84 is most likely an indirect one via T cells, while in arterial thrombosis the damage is caused by the experimenter (via application of $FeCl_3$ or mechanical injury) and therefore CD84-independent. As CD84 does not contribute to classical platelet activation pathways no difference between WT and *Cd84*$^{-/-}$ mice is observed in arterial thrombosis.

We have now expanded the discussion section to address the reviewer's comment (see line 425 following in our revised manuscript).

2. Not clear why tMCAO was done 3 days after neutrophil depletion. Did authors made sure that mice were indeed neutrophils-deficient.

Yes, of course we have confirmed that mice were indeed neutrophil deficient by flow cytometry and IF of bone sections (see figure to the reviewers 1). We have chosen day 3 in line with our previous study (Volz *et al*., Blood 2019;133:2696-2706), for which we performed a time course experiment that revealed that day 3 is the 'peak' day of depletion.

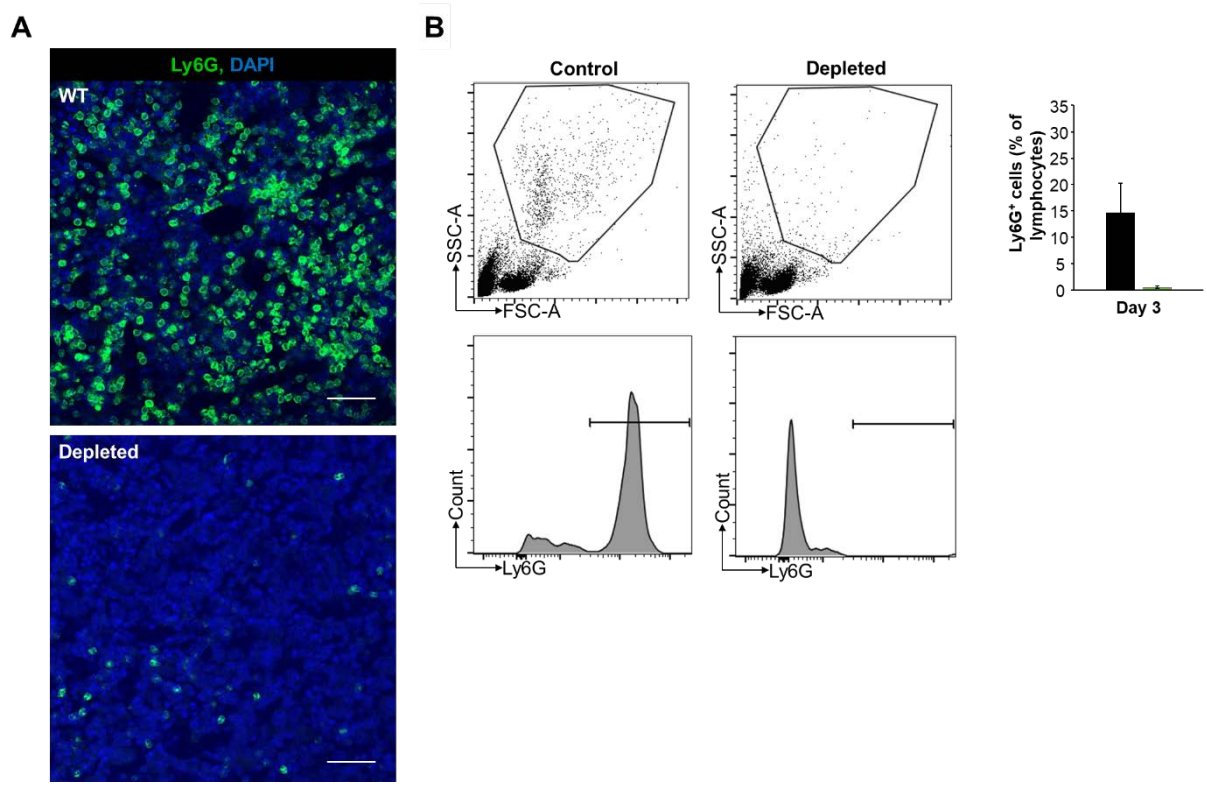

Figure to the reviewers 1. Neutrophil-depletion on day 3 after anti-Ly6G-treatment was confirmed by flow cytometry and immune fluorescence of bone sections. (**A**) Representative images of cryo-sections of femora from C57Bl/6J mice on day 3 after anti-Ly6G treatment stained with for neutrophils (anti-Ly6G, green; counterstained with DAPI, blue); scale 25 µm. (**B**) The efficacy of the PMN-depletion on day 3 after anti-Ly6G-treatment (green bar) or control-treatment (black bar) was analyzed by flow cytometry of peripheral blood cells (n = 5 mice per group). The bars depict mean + SD.

3. The staining for neutrophils and thrombi in figure 3C and D is not convincing. Need to stain thrombi with platelet-specific antibody. For neutrophils staining, need to counter-stain nuclei.

As requested by the reviewer we have replaced figure 3C with IF images including a counter-stain for nuclei. Regarding the thrombi we would like to keep the H&E stainings in the manuscript as it is easier to see whether the vessel is occluded or still open in H&E stainings as compared to immunofluorescence stainings and as H&E stainings are a standard approach to address this question. However, we stained platelets using an anti-GPIX antibody (p0p6) and did find considerably more thrombi in *Cd84*$^{-/-}$ mice on day 1 after tMCAO as compared to WT mice (see figure to the reviewers 2), which we now mention in the revised version of the manuscript.

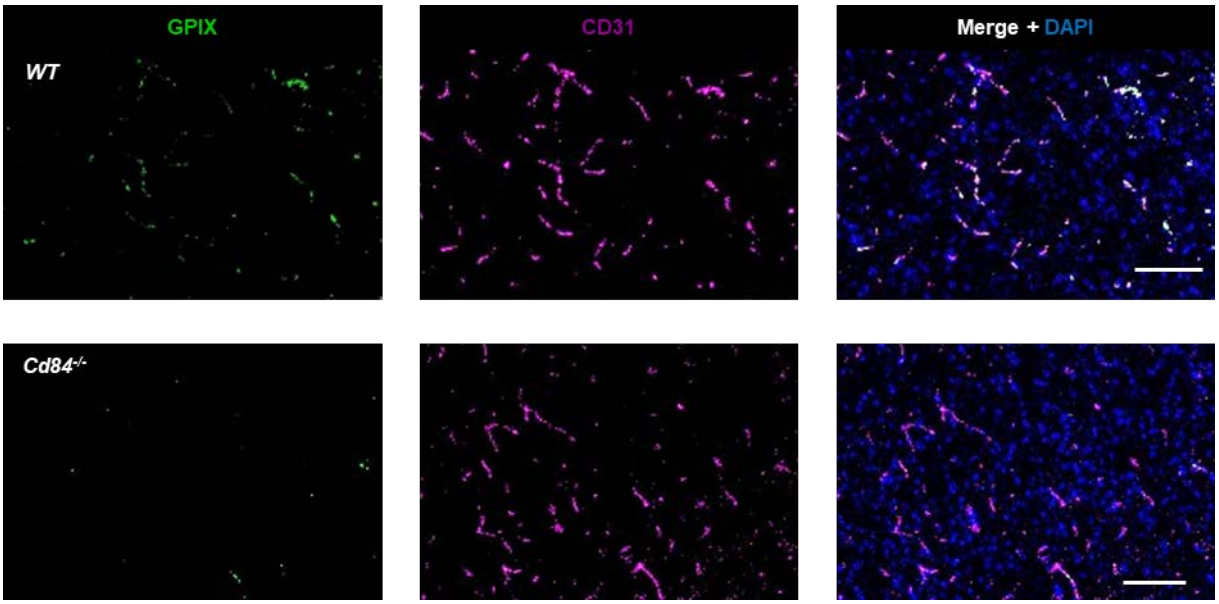

Figure to the reviewers 2. Less cerebral thrombi in *Cd84*$^{-/-}$ mice on day 1 after tMCAO as compared to control animals. Representative images of platelet accumulation (p0p6, green) in cerebral vessels (anti-CD31, magenta) in the ipsilateral hemisphere 24 h after tMCAO of WT and *Cd84*$^{-/-}$ mice (n = 5 mice per group). Bar, 100 µm.

Reviewer #2:

The authors have addressed my comments to the best of their possibilities considering the current COVID crisis context.

I have no further comments.

We thank the reviewer for the appreciation of our work.

Reviewer #3:

All my concerns are addressed.

We thank the reviewer for the appreciation of our work.

Statistical Reviewer:

Please report exact p-values and sample sizes throughout the manuscript and supplement, rather than reporting range values. For very small p-values, I strongly suggest using scientific notation with two significant digits.

As recommended by the reviewer we now report the exact p-values in the manuscript. As our symbols in the graphs represent one animal each we did not state the exact sample sizes within the text and kept the range values instead.

Please include race/ethnicity information for the SICFAIL study

Information on race/ethnicity was not collected within the SICFAIL study. However, we have information on migration background: A total of 15 patients reported at least one parent born outside Germany. Countries of origin were (former) European countries (Ukraine, Yugoslavia, Austria, Greece, Poland, Czech), USA and Russia, suggesting that differences in race/ethnicity were rather not present in the studied population.

It's not always clear how p-values were calculated in the tables?

This information was added in the revised version of this manuscript.

It's not always clear how many tests were run or when multiple testing corrections were applied? Eg, fig 4B, Sfig3, others.

We have now specified this information in the corresponding figure legends.

Claims of "no difference" should be checked to see if it would be more accurate to claim "no significant difference". Eg "Of note, no difference in T cell motility was observed in the presence of WT or Cd84-/- platelets (Supplemental Figure 9E,F)"

As recommended we have rephrased this sentence.

How were representative images/figures chosen? Please note the approach used to select representative images in the main text.

Representative images were chosen among the images of one genotype to best reflect the mean values of this genotype. This is now stated in the methods section.

If no corrections for multiple testing were made across tests (only within-test corrections were made), please note this in the statistical section of the manuscript.

To the best of our knowledge this is not applicable to our study and all corrections are mentioned in the statistical section.

Tests to establish normality are not powered to detect deviation from normality when n is small (eg, n{less than or equal to}5 as in figure 3). Please check if a non-parametric test would be more appropriate to use in cases where you have analyzed small samples.

As recommended by this comment, we have re-analyzed all data that has previously been assessed using the Student's t-test using the Mann-Whitney test and replaced the p-values. This led to bigger p-values in some cases (e.g. Figure 1B, S2B, 3D), however, significant differences persisted.

Technical Reviewer:

Comments to Authors on Rigor Checklists:

The current study was evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The study closely complied with said guidelines, however, the reviewer has identified a few minor items that will need to be addressed in a revision:

We thank the reviewer for making us aware of the points mentioned below.

In vitro checklist:

1) To maintain consistency, please provide the number of biological replicates (n numbers) in the figure legend accompanying supplemental figure 1.

This information was added in the revised version of this manuscript.

2) Please ensure that all presented immunoblots contain markers denoting the location of molecular weight standards electrophoretically resolved with experimental protein lysates (e.g., Supplemental figure 5B).

This information was added in the revised version of this manuscript.

3) Where immunostaining procedures are described, please provide a brief description of controls employed to validate antibody specificity and/or distinguish genuine target staining from background.

We have tested all antibodies using for immune fluorescence stainings in parallel to isotype controls and this information is now provided in the methods section.

4) Ensure that all presented micrographs contain a scale with reported units of measure (e.g., Supplemental Figure 1B).

This information was added in the revised version of this manuscript.

Other:

1) Checklists indicate the use of blinding procedures; however, this was not evidenced in text. Please address this disparity.

This information was added in the revised version of this manuscript.

2) Please indicate why animals were not randomized to experimental groups.

We did not have treatment groups in our study and compared *WT* and knockout mice. Consequently, we could not randomize our experimental groups.

Comments to the Authors on Research Guidelines and Reporting:

None.

Circulation Research Decision on Ms # CIRCRES/2020/316655R2
July 6, 2020

Dr. David Stegner
University of Würzburg
Institute of Experimental Biomedicine
Josef-Schneider-Str. 2, D15
Würzburg
Germany

RE: CIRCRES/2020/316655R2: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

Dear Dr. Stegner:

Your manuscript has been carefully evaluated by 1 external reviewers, a Statistical reviewer, a Technical reviewer, and the editors as a Regular Article. Although of potential interest, the paper is not acceptable for publication in Circulation Research in its present form.

As you will gather from the reviews, the statistical referee identified a number of substantive conceptual and methodological problems. The editors concur.

Given the extensive new data that would be required for a responsive revision, we would understand if you were to decide to submit the paper elsewhere. Nevertheless, the editors see this manuscript as potentially important and would be willing to evaluate a revised version if you feel that you can effectively address the reviewers' concerns and are willing to perform the new experiments required. The paper would be reviewed again, with no assurance of acceptance.

As detailed in the reviewers' critiques, a responsive revision would require a substantial amount of new data. In particular, the editors feel that additional data analysis would be necessary to address each concern raised by the statistical reviewer. The journal takes the statistical review very seriously and these comments must each be addressed if this manuscript is to move forward.

NEW REQUIREMENTS:

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

Please note that revised and resubmitted manuscripts are not assured of publication, and that fewer than 15% of all papers submitted to Circulation Research are eventually published.

Our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted. If you need more than 90 days to submit a revised paper, please notify the editorial office.

PLEASE READ: During this unprecedented and challenging time, the health and safety of you, your family, and your community is of utmost importance. We appreciate that one aspect of this current situation is the inability to continue research work and that some measures will lead to a significant hindering of research progress. Please know that we are flexible regarding turnaround times for revisions and other tasks during this stressful time and deadlines will be extended as needed. If you are able to, please contact us if you need any extensions or if you experience any challenges around manuscript preparation; we will work with you.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Each page of the revised manuscript should be numbered in the top right corner, using your manuscript number followed by /R1 to denote a first revision.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Revisions that do not conform to the current limits on numbers of words (8000 total) and display items (maximum of 8 tables and/or figures) may be returned to the authors for abbreviation. If you cannot reduce the overall word count, the editors may deem an extended print version appropriate; the authors should provide written assurance that they will cover the costs of the pages that are in excess of these limits. Note that paying for excess display items is not an option. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

As of January 1, 2020, all corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To avoid potential processing delays in future, we recommend that you link an ORCID iD to your profile when you submit your revision. To register with ORCID or link your profile, please go to "Modify Profile/Password" on the submission site homepage (insert journal homepage link), and click the link in the "ORCID" section.

We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****
REVIEWER COMMENTS TO AUTHORS:

Reviewer #1:

No further comments.

Statistical Reviewer:

P-values are still being given as range values in the manuscript (eg see many figures). Please provide precise p-values with two significant digits (rather than P<0.0x). For small values, I suggest using scientific notation.

In several figures it is difficult to count the number of data points (not to mention cumbersome) because the datapoints overlap. Please give exact sample sizes for all statistical tests. If you prefer, you may include this information in a supplemental file rather than in the figures themselves.

Normality can not be established (as there is no power to detect deviations from normality) in small sample sizes. Please use non-parametric tests and present medians and IQR in cases where sample sizes are small (eg Supplemental Table 2, Supplemental Figure 7).

In several cases, it is not clear what groups were compared to result in the highlighted p-values (eg fig 3B, Supplemental Figure 2, Supplemental Figure 6).

In fig 3B, and I'm also not sure why the authors used K-W followed by M-W in this panel? Can the authors provide explanation/justification for the two tests (I would have expected a single test followed by a post hoc test correction)

When many hypotheses are tested, the risk of type 1 error increases. If no corrections for multiple testing were made across tests (e.g. only within-test corrections were made), please note this in the statistical section of the manuscript.

In fig 6, (and Supplemental Figure 2, etc.), I suggest normalizing to the mean so that the variation in F can be observed.

Vague language is still used in the manuscript, eg "No substantial changes of the effect of CD84..." what constitutes a 'substantial' change? If the authors mean that there is no statistically significant difference, I suggest they use this language.

Please show +/-SEM or SD in addition to mean for normally distributed data. Consider showing medians and IQR for data that does not pass normality tests... eg the statistical section describes "...Neuroscore and the migration assays, which are depicted as scatter plots including the mean and standard error of the mean" except SEMs are not shown in neuroscore and migration figures (eg fig 1C, Supplemental Figure 2, figure 5, Supplemental Figure 6, etc).

What multiple test corrections were applied in Supplemental Figure 3? No measure of variance of the data is given in this figure, and it's not clear what the red lines indicate.

Was any adjustment made for multiple test correction in figure 2?

It's not clear when statistical tests were performed and found to be not significant, and when no tests were performed. There are many examples, but for example, Supplemental Figure 4 B. It would be helpful if the authors could note the comparisons that were made and the number of tests that were corrected for, in addition to noting if the presented p-values are raw or corrected.

Technical Reviewer:
Comments to Authors on Rigor Checklists:
The authors have appropriately addressed the technical reviewer's queries. No further suggestions.

Comments to the Authors on Research Guidelines and Reporting:
None.

RE: CIRCRES/2020/316655R2: CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

REVIEWER COMMENTS TO AUTHORS:

Reviewer #1:

No further comments.

We thank the reviewer for the appreciation of our work.

Statistical Reviewer:

P-values are still being given as range values in the manuscript (eg see many figures). Please provide precise p-values with two significant digits (rather than P<0.0x). For small values, I suggest using scientific notation.

As requested, we have provided all n numbers and exact p-values either in the corresponding figure legends or as new supplemental tables 5 and 6.

In several figures it is difficult to count the number of data points (not to mention cumbersome) because the datapoints overlap. Please give exact sample sizes for all statistical tests. If you prefer, you may include this information in a supplemental file rather than in the figures themselves.

As suggested, we have provided all n numbers and exact p-values either in the corresponding figure legends or as new supplemental tables 5 and 6.

Normality can not be established (as there is no power to detect deviations from normality) in small sample sizes. Please use non-parametric tests and present medians and IQR in cases where sample sizes are small (eg Supplemental Table 2, Supplemental Figure 7).

We re-analyzed the data displayed in Supplemental Figure 7 using non-parametric Mann-Whitney tests and updated the figure legend accordingly.

Supplemental Table 2 and 3 data distribution is now displayed by giving median and IQR values as requested. Likewise, we have changed the heart rate display in the text accordingly.

In several cases, it is not clear what groups were compared to result in the highlighted p-values (eg fig 3B, Supplemental Figure 2, Supplemental Figure 6).

Apparently our delineation markers were not clear enough. Thus, we have now included horizontal lines in addition, to address this point and highlight which groups have been compared in these figures.

In fig 3B, and I'm also not sure why the authors used K-W followed by M-W in this panel? Can the authors provide explanation/justification for the two tests (I would have expected a single test followed by a post hoc test correction)

We thank the reviewer for this comment. As the groups have been assessed in separate assays (as it is not possible to operate so many mice at once), which is indicated by the vertical delineation lines, we have used the Mann-Whitney test for the comparison of two groups. Of note, we do not make comparisons of groups between different test settings (e.g. no comparison of *Rag1*$^{-/-}$ mice and *Cd84*$^{fl/fl}$ mice), they are just grouped together to save space. However, all groups of mice are discussed separately within the manuscript.

The use of the Mann-Whitney test is explicitly stated in the figure legend and the exact p-values for the assays that yielded significant results are now stated in the legend as well.

When many hypotheses are tested, the risk of type 1 error increases. If no corrections for multiple testing were made across tests (e.g. only within-test corrections were made), please note this in the statistical section of the manuscript.

As requested, we now state in the method section that we did not correct for multiple testing across tests.

In fig 6, (and Supplemental Figure 2, etc.), I suggest normalizing to the mean so that the variation in F can be observed.

We agree with the reviewer that the variation of the CD84 surface expression of both sampling sites is important. Therefore, we have now replaced figure 6 with table 1 (previously supplemental table 4) to provide the MFI (median and IQR) within the main manuscript.

Vague language is still used in the manuscript, eg "No substantial changes of the effect of CD84..." what constitutes a 'substantial' change? If the authors mean that there is no statistically significant difference, I suggest they use this language.

We rephrased this sentence as requested.

Please show +/-SEM or SD in addition to mean for normally distributed data. Consider showing medians and IQR for data that does not pass normality tests... eg the statistical section describes "...Neuroscore and the migration assays, which are depicted as scatter plots including the mean and standard error of the mean" except SEMs are not shown in neuroscore and migration figures (eg fig 1C, Supplemental Figure 2, figure 5, Supplemental Figure 6, etc).

As suggested we have changed the display of our data accordingly and updated our statistical section to be in line with the revised data display.

What multiple test corrections were applied in Supplemental Figure 3? No measure of variance of the data is given in this figure, and it's not clear what the red lines indicate.

As elaborated above, we did not correct for multiple testing across tests, however, we have analyzed statistical significance using the Holm-Sidak method, to include a multiple test correction within the test. Supplemental table 5 now displays all n numbers and p-values and we have changed the figure display to a box plot display including median IQR, so that the red lines (that indicated the mean values) are now replaced by box whisker blots.

Was any adjustment made for multiple test correction in figure 2?

See previous response.

It's not clear when statistical tests were performed and found to be not significant, and when no tests were performed. There are many examples, but for example, Supplemental Figure 4 B. It would be helpful if the authors could note the comparisons that were made and the number of tests that were corrected for, in addition to noting if the presented p-values are raw or corrected.

All our data was analyzed for statistical significance and we now display the corrected exact p-values throughout the manuscript. This is now specified in the revised version of the statistical section.

Technical Reviewer:

Comments to Authors on Rigor Checklists:

The authors have appropriately addressed the technical reviewer's queries. No further suggestions.

Comments to the Authors on Research Guidelines and Reporting: None.

We thank the reviewer for the appreciation of our work.