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Cross-talk between monocyte invasion and astrocyte proliferation regulates scarring in brain injury

Jesica Frik, Juliane Merl-Pham, Nikolaus Plesnila, Nico Mattugini, Jacob Kjell, Jonas Kraska, Ricardo M Gomez, Stefanie M Hauck, Swetlana Sirko, Magdalena Götz

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 November 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees highlight the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here, as we think all points need to be addressed. However, we feel that it is of peculiar importance to address the major concern of referee #1 experimentally, thereby proving a cause-effect relationship between proliferating juxtavascular astrocytes, inflammation and scarring, and to analyse BBB permeability (point 1 of referee #3).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section

called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors: http://embor.embopress.org/authorguide#manuscriptpreparation

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. Please also add scale bars to all microscopical images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Please also format the references according to EMBO reports style. See: http://embor.embopress.org/authorguide#referencesformat

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Frik et al. explore the relationship between astrocyte proliferation, monocyte invasion, and glial scar formation in experimental traumatic brain injury and ischemic brain injury. The authors show that increasing astrocyte proliferation by conditional genetic ablation of cdc42 or smo attenuates leukocyte invasion. A similar effect was observed when astrocyte proliferation was enhanced via transgenic overexpression of a constitutively active form of smoothened (caSMOP-YFP;GLASTCreERT2 mice) in adult astrocytes. Conversely, reducing leukocyte infiltration via genetic deletion of CCR2 also increased astrocyte proliferation.

Overall, the authors do a beautiful job of documenting the gliotic and inflammatory responses to a stab wound injury using the varied genetic mouse models described above; the anatomical presentation is top-notch and the images are compelling. Where the manuscript needs improvement is in proving that the inverse relationship between juxtsavascular astrocyte proliferation and leukocyte influx also influences microglia proliferation/activation and gliosis/scarring. Early in the paper, the data prove an inverse relationship between juxtavascular astrocyte proliferation and leukocyte infiltration but later the focus shifts to correlative studies showing the effects of reducing infiltrating monocytes on gliosis and scarring. There is no attempt to prove that juxtavascular astrocytes are affected in CCR2rfp/rfp mice or that gliosis or scarring (e.g., Figs. 5-7) are differentially affected in the cdc42, smo or SMOM2-YFP transgenic mice (as used in Figs. 2&3). The immunohistochemical and proteomic analysis also are correlative and indicate that extracellular matrix (ECM) proteins synthesized by juxtavascular astrocytes may initiate scar formation and limit monocyte invasion.

Additional comments are provided below and should be addressed during revision:

(1) The introduction should more clearly state the aim or hypothesis that guides studies in this report. The importance of understanding glial-monocyte interactions should be clarified, particularly

since CCR2 deficiency has already been investigated in both middle cerebral artery occlusion (e.g. Dimitrijevic et al., 2006, Stroke) and traumatic brain injury (e.g. Hsieh et al., 2014, J Neurotrauma). The statement that 'virtually nothing is known about the effects of invading immune cells on macroglia reactivity' should be tempered, given progress in this field in recent years.

Page 5:

(2) Fig. S1A-D: Are there differences in proportion of GFAP vs. S100b+ astrocytes at the justavascular position (or as a function of distance from the lesion margins)? Presumably, the combined use of both markers is meant to capture a larger population of astrocytes, which are phenotypically heterogeneous cells.

(3) In the last sentence of the first paragraph, please add the word "all" before "astrocytes", or, specify that of proliferating astrocytes, which make up ~1/3 of all astrocytes, most (~2/3) are juxtavascular.

Page 9

(4) Fig. S3A: There is no WT comparison shown in this figure. However, microglia morphology does appear to be different between the WT and SMOM2-YFP images provided in Figs. 3G&H. Were attempts made to quantify microglia morphology (e.g., proportional area differences) between experimental and control groups?

Page 10:

(5) (first paragraph) The authors refer to NG2 glia in Fig S3 but there are no NG2 data in Fig. S3.

(6) (second paragraph) CD11b is not an ideal activation marker for microglia. Other markers should also be tested (and combined with Ki67) to more rigorously confirm this observation. Similarly, although the identification of infiltrating leukocytes as CD45+Iba1- cells is correct, some CD45+Iba1+ cells, which are exclusively considered in this report at resident microglia, may also be infiltrating Iba1+ macrophages (Jeong et al., 2013, Exp Neurobiol). The authors should address the limitations of the antibody combinations used or provide references that unequivocally prove that CD45+Iba1+ cells are microglia that are distinct from early arriving monocyte-derived macrophages. Finally, identification of reactive microglia as Iba1+CD11b+ cells (Fig. 5) could also include monocyte-derived macrophages.

(7) CD45+Iba1- cells could be infiltrating granulocytes/lymphocytes, not only monocytes. What proportion of CD45+Iba1- cells are monocytes in/around the injury site? This could change the values presented in Fig. 2.

Page 11:

(8) It would be more effective to count neurons within a defined ROI and express data in Fig. 6 as neuron number/ROI. As currently formatted, the data will lead readers to believe that CCR2 deletion (and reduced monocyte influx) increases neuron number relative to wt when in fact, reducing monocyte influx is neuroprotective. Also, the effects on neuron loss would be better illustrated by showing NeuN stain alone (C"/D"). There are also small errors within the figures. For example, Fig. 6C' (WT) has the lesion outlined, but Fig 6CD' (CCR2-/-) does not. Figure 5H1 seems to be right-shifted from Fig. 5H and Figure 5i1 seems to be right-shifted from Fig. 5i, so the lesion sites don't quite match up. Fig. S1G' shows only two slices from the z stack, making it difficult to see the blood vessel staining shown in S1G. Please make S1G' a maximum intensity projection of the Z-stack to better illustrate the anatomy.

(9) The authors should refrain from using the term "wound healing". Data in this report do not provide insight to changes in "wound healing" nor is it possible to conclude that wound healing is "improved" in mice with reduced monocyte recruitment. In fact, data in Fig. 7 show reduced deposition of ECM proteins, which are essential components of "healing" wounds. Instead, it would more accurate to state that in the absence of monocytes, gliosis, glial "scarring" and associated changes in glial scar ECM molecules are reduced in the absence of infiltrating monocytes,

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implicating the latter cells in promoting scarring and ECM remodeling.

Pages 12-13:

(10) There are mismatches between the figures and the text. For example, page 13 states reduced Tn-C in CCR2 mice confirmed by immunostaining (Fig. S6), but Fig 6 shows S100β/GFAP staining. Page 13 refers to Fig. S5C, but Fig S5 contains only A and B. Page 26 refers to protein network analysis in Fig. 7B, but network analysis is in Fig. 8.

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Page 15:

(12) The rationale provided in the text for looking at AhR is confusing. Monocytes do invade stab wounds in WT mice so why look at WT mice if the goal is to show that juxtavascular astrocytes restrict monocyte invasion? Wouldn't it be more useful (and impactful) to compare monocyte numbers and spatial distribution (relative to juxtavascular astrocytes) within stab wounds of cdc42 and SMOM2 transgenic mice, i.e., mice with reduced or enhanced proliferation of juxtavascular astrocytes and enhanced/reduced monocyte infiltration, respectively?

(13) Please include all group sizes in the figure legends and provide a description in the Statistics section of how samples sizes were determined.

(14) For the most part, the text is well-written and easy to understand but there are many examples throughout where the English can be improved. I recommend that the authors have a native-English speaking person edit the document.

Referee #2:

The authors have used genetic mouse models to target proliferating astrocytes after MCAO but mostly cortical stab lesions to determine the effects on the extravasation of inflammatory cells which in turn, effects the scarring process. To my knowledge this is the first time that tight control of astroglial numbers as well as monocyte invasion and their close interactions after CNS injury have been investigated. Thus, the results are quite novel. However, there are a number of issues that the authors should address in order to strengthen the impact of this interesting research.

1) In the introduction, the statement that astrocytes recruited from ependymal stem cells are the closest equivalent to zebrafish stem cells may likely be incorrect. Indeed, the role of the ependymaI contribution to astroglial scar formation has recently and seriously been called into question by a rather convincing paper from the Sofroniew lab (Scientific Reports Jan 2017, Ren et al). This part of the introduction should be clarified.

2) While the Sofroniew lab has done notable work, their Nature paper suggesting that the astroglial component of scar is always "beneficial" since it is never a barrier and is always regeneration supportive is highly controversial. No citations from the Silver lab in the intro and elsewhere within the paper is a major omission of pertinent literature related to this controversy as well as discussion related to scar and extracellular matrix. Since the present paper does not examine axon regeneration in the vicinity of the cortical stab lesion in the various mutants, the authors should make it clear that their discussion of the "beneficial" aspects of astroglial scar formation only relates to interactions with inflammatory cells and not axons. It would have been interesting to look at serotonergic axons in the vicinity of the various lesions. Also, some of the critical findings in the present paper are quite different and novel from those of the Sorfoniew lab especially concerning the role of glial proliferation in scar formation. The observation that lack of macrophage invasion leads to more

rather than less astrocyte proliferation and, surprisingly (given the Sofroniew lab findings), less rather than more scarring is the major novel finding in the present paper. This means likely that local cell-to-cell or cell-to- matrix interactions between astrocytes and inflammatory cells are more critically important for scarring than is proliferation per se. The role of cell-cell/matrix interactions could be discussed. (See Hara et al. Nat Med 2017). Also, the conclusion that scarring in the brain is different from scarring in the spinal cord makes no sense.

3) The MCAO model involves rather profuse stem cell migration towards the stroke cavity from the SVZ which is not seen after a cortical stab lesion. Thus, the two models are quite different. Indeed, given that the scarring processes and astroglial/monocyte interactions related to the MCAO model over time are not investigated it seems that this part of the paper is superfluous and could be omitted.

4) It was not made clear what, if any, special property of astrocytes whose somata lie very near vessels might be. Why might astrocytes with slightly more distant cell bodies from the endothelium but with processes that intimately abut blood vessels also be important?

5) Scar formation has other functional consequences such as repair of BBB leakage. Have the authors examined leakiness of the blood brain barrier (BBB) after stab lesion in the various mutants? Given the known importance of a compromised BBB to scar formation and vice versa (Schachtrup et al., J Neurosci 2010) it would be interesting to know what happens to the BBB especially when astrocytes proliferate poorly. What about the opposite situation? If no data are available then some discussion of the BBB is warranted.

6) The authors have examined col 4 which is interesting but they should have also looked at col1 since this is now known to be a major instigator of astrocytic scar (see Hara et al., Nat Med).

7) Astrocyte proliferation appears to have little to do with scar formation at least in the absence of macrophage invasion. Have the authors considered that dividing astrocytes (lacking macrophage signaling) may be entering a more immature state. There is a wealth of previous information about mature versus immature astrocytes and how immature astroglia are associated with improved wound healing. Another possible point of discussion.

8) Some of the figure notations seem problematic. I see no Figure S3H., Figure S6 has nothing to do with tenascin.

Referee #3:

Using models of traumatic brain injury and MCAO ischemia, the authors show a relationship between astrocyte scar formation and monocyte infiltration. In particular, CCR2-/- mice with deficient monocyte infiltration had reduced scar formation despite increased astrocyte proliferation. Proteomic analyses guided the authors to evaluate certain ECM components and the results are discussed in the context of scar formation. The manuscript is interesting and the datasets are generally of good quality.

The following are meant to improve the manuscript:
1. Are the results of the inverse relationship reflected in blood-brain barrier disruption? For example, in CCR2-/- mice, is the integrity of the BBB (eg fibrinogen deposition) better than WT animals?
2. The distinction of microglia (CD45+Iba1+) from recently infiltrated macrophages (CD45+Iba1-) may hold in the first few hours of injury (Fig 1) but become unreliable at later (eg 3 days as used in Fig 1 or 5 days in Fig 5) time points of injury. The authors should qualify the distinctions better (eg using TMEM115 ab in the absence of available reporter mice) in their results. It is increasingly apparent that macrophages and microglia can have very different roles after injury, so the casual distinction as used here (Iba1- or +) should be better justified.
3. How are the cell types controlling or seemingly repelling one another? Recent work (Horng et al., JCI 127:3136, 2017) of astrocyte tight junctions controlling the exit of immune cells from the perivascular space into the CNS parenchyma could be cited. The role of AhR in regulating the

astrocyte results herein in Figure 9 is a correlation and a speculation at best.
4. In the context of CSPG deposition in Figure 7, what are the cellular sources? The results could be interpreted as CSPGs being produced by monocytes/macrophages to affect astrocytes, or astrocytes producing CSPGs to limit the invasion of leukocytes. The mouse anti-CSPG (Abcam) descriptor should be extended (eg targeting CS-56) and the authors should comment on mouse monoclonals being used on mouse tissues (where increased background staining is expected at sites of blood brain barrier disruption); staining controls could be better presented particularly for ECM components in areas of blood-brain barrier disruption.
5. For the proteomic analysis, the authors should be complimented for stating that they had to remove 2 outlier from each genotype (page 13). What is the basis for considering some samples as outliers and thus their deletion from the analysis?

1st Revision - authors' response 5 February 2018

Referee #1:

Frik et al. explore the relationship between astrocyte proliferation, monocyte invasion, and glial scar formation in experimental traumatic brain injury and ischemic brain injury. The authors show that increasing astrocyte proliferation by conditional genetic ablation of cdc42 or smo attenuates leukocyte invasion. A similar effect was observed when astrocyte proliferation was enhanced via transgenic overexpression of a constitutively active form of smoothened (caSMOP-YFP;GLASTCreERT2 mice) in adult astrocytes. Conversely, reducing leukocyte infiltration via genetic deletion of CCR2 also increased astrocyte proliferation.

Overall, the authors do a beautiful job of documenting the gliotic and inflammatory responses to a stab wound injury using the varied genetic mouse models described above; the anatomical presentation is top-notch and the images are compelling.
Many thanks.

Where the manuscript needs improvement is in proving that the inverse relationship between juxtsavascular astrocyte proliferation and leukocyte influx also influences microglia proliferation/activation and gliosis/scarring. Early in the paper, the data prove an inverse relationship between juxtavascular astrocyte proliferation and leukocyte infiltration but later the focus shifts to correlative studies showing the effects of reducing infiltrating monocytes on gliosis and scarring. There is no attempt to prove that juxtavascular astrocytes are affected in CCR2rfp/rfp mice or that gliosis or scarring (e.g., Figs. 5-7) are differentially affected in the cdc42, smo or SMOM2-YFP transgenic mice (as used in Figs. 2&3).
According to the reviewers suggestion we now added new data for the CCR2 KO mice showing the bias of astrocyte proliferation to juxtavascular positions (p.11). This is very interesting as these data highlight that any stimulation of astrocyte proliferation – be it intrinsic (such as by SMOM2) or extrinsic (such as by the failure of monocyte invasion) proliferation is always biased towards juxtavascular astrocytes. This fits to the concept discussed in the discussion that they are intrinsically different and biased towards proliferation and to inhibit monocyte invasion.
However, we can not examine effects on scar formation in the inducible gain- or loss-offunction mouse lines due to the recombination rate. As discussed on p.8 we recombine in about 50% of all astrocytes and only a fraction of them proliferate. Therefore effects on scar formation are expected to be rather small if detectable at all.

The immunohistochemical and proteomic analysis also are correlative and indicate that extracellular matrix (ECM) proteins synthesized by juxtavascular astrocytes may initiate scar formation and limit monocyte invasion.

We do not claim that the ECM changes observed in the CCR2KO mice are due to changes in astrocyte ECM synthesis. As we also show changes in the NG2 glia proliferation and these cells also contribute to the matrix and we also observe changes in microglia reactivity, these data highlight the cross-talk between these glial populations and the potent influence of invading monocytes on all these cell types. Indeed, ECM changes can not be attributed to a

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single glial population (Silver, 2016). The importance of these data lies in showing the increase of astrocyte proliferation (mostly juxtavascular) in the absence of monocyte invasion and nevertheless a reduced astrogliotic scar at the end. But of course we have no means of showing that the failure of monocyte invasion elicits a direct effect on astrocyte proliferation, even though they will be the first contact of invading monocytes at the glia limitans.

Additional comments are provided below and should be addressed during revision:
(1) The introduction should more clearly state the aim or hypothesis that guides studies in this report. The importance of understanding glial-monocyte interactions should be clarified, particularly since CCR2 deficiency has already been investigated in both middle cerebral artery occlusion (e.g. Dimitrijevic et al., 2006, Stroke) and traumatic brain injury (e.g. Hsieh et al., 2014, J Neurotrauma). The statement that 'virtually nothing is known about the effects of invading immune cells on macroglia reactivity' should be tempered, given progress in this field in recent years.
We have added these citations to the introduction and state our aims very clearly now. Notably, none of these publications of CCR2 deficiency examined macroglia proliferation – if at all the authors analyzed GFAP expression. We spell this out more clearly now in the introduction and also cite the recent review by Vittorio Gallo who emphasizes the need to look at glial cell heterogeneity to better understand the discrepancies between different injury models, which is exactly the point we wanted to make and applies very well also to the discrepancies found in mouse models with CCR2 deficiencies after different injury paradigms.

Page 5:
(2) Fig. S1A-D: Are there differences in proportion of GFAP vs. S100b+ astrocytes at the justavascular position (or as a function of distance from the lesion margins)? Presumably, the combined use of both markers is meant to capture a larger population of astrocytes, which are phenotypically heterogeneous cells.
In the vicinity to injury virtually all astrocytes are GFAP+, so there is not much heterogeneity in this regard. This is stated now explicitly on p. 5. We also see no differences in GFAP levels between juxtavascular or non-juxtavascular astrocytes, nor in Hes5-GFP or BLBP-RFP (data not shown). The difference in AhR is the first and so far only molecular difference we could observe between these populations.

(3) In the last sentence of the first paragraph, please add the word "all" before "astrocytes", or, specify that of proliferating astrocytes, which make up ~1/3 of all astrocytes, most (~2/3) are juxtavascular.
Done. Thanks for helping to clarify this possible misunderstanding.

Page 9
(4) Fig. S3A: There is no WT comparison shown in this figure. However, microglia morphology does appear to be different between the WT and SMOM2-YFP images provided in Figs. 3G&H. Were attempts made to quantify microglia morphology (e.g., proportional area differences) between experimental and control groups?
We did not attempt to quantify these differences, but we now show panels depicting WT in Figure EV3C and show higher magnifications to highlight the morphological differences in Figure EV3G,H. We focus here on the proliferative reaction and the increase in microglia proliferation in these mice as shown in Figure EV3E indicates their difference in reactivity.

Page 10:
(5) (first paragraph) The authors refer to NG2 glia in Fig S3 but there are no NG2 data in Fig. S3.
Sorry about this mistake. NG2 glia are now included in Figure EV3 I-K.

(6) (second paragraph) CD11b is not an ideal activation marker for microglia. Other markers should also be tested (and combined with Ki67) to more rigorously confirm this observation. Similarly, although the identification of infiltrating leukocytes as CD45+Iba1- cells is correct, some CD45+Iba1+ cells, which are exclusively considered in this report at resident microglia, may also be infiltrating Iba1+ macrophages (Jeong et al., 2013, Exp Neurobiol). The authors should address the limitations of the antibody combinations used or provide references that

unequivocally prove that CD45+Iba1+ cells are microglia that are distinct from early arriving monocyte-derived macrophages. Finally, identification of reactive microglia as Iba1+CD11b+ cells (Fig. 5) could also include monocyte-derived macrophages.
We have included now also Tmem119 immunostaining for microglia (see below). We apologize for the misunderstanding CD45+/Iba1+. We fully agree that these may also be derived from invading monocytes, but as they are transitioning already we focused our analysis on the CD45+/Iba1-negative cells, ie undoubtedly the recently invaded monocytes. This is now stated explicitely on p. 6. As there is an overlap of more than 90% between the CD45+/Iba1- cells and the RFP+ cells in the CCR2 knock-in mice, this highlights the validity of our analysis to reliably detect the invading monocytes. Moreover, we further analysed the CD45+ cells by FACS demonstrating the absence of B- and T- cells in our injury paradigm (new Appendix Figure S1).

(7) CD45+Iba1- cells could be infiltrating granulocytes/lymphocytes, not only monocytes. What proportion of CD45+Iba1- cells are monocytes in/around the injury site? This could change the values presented in Fig. 2.
We have examined this and find only invading monocytes at the stages analyzed (3 and 5 and 7dpi). Data for 3dpi are now included as new Appendix Figure S1. There are no B- and T cells detectable in our injury paradigm.
Page 11:

(8) It would be more effective to count neurons within a defined ROI and express data in Fig. 6 as neuron number/ROI. As currently formatted, the data will lead readers to believe that CCR2 deletion (and reduced monocyte influx) increases neuron number relative to wt when in fact, reducing monocyte influx is neuroprotective. Also, the effects on neuron loss would be better illustrated by showing NeuN stain alone (C"/D").
This is an excellent suggestion and we have implemented this analysis in the revised manuscript. We now show in Figure 6 only NeuN stainings for the analysis of ROIs that are now included (Figure 6H-R). This quantification of numbers of NeuN+ cells also in comparison to contralateral sides now allows us making the point much more clearly, that there is a reduction of NeuN numbers at the injury site in both genotypes, but less so in the CCR2 KOs.

There are also small errors within the figures. For example, Fig. 6C' (WT) has the lesion outlined, but Fig 6CD' (CCR2-/-) does not.
We now include the outline of the lesion also in Figure 6CD.
Figure 5H1 seems to be right-shifted from Fig. 5H and Figure 5i1 seems to be right-shifted from Fig. 5i, so the lesion sites don't quite match up. Fig. S1G' shows only two slices from the z stack, making it difficult to see the blood vessel staining shown in S1G. Please make S1G' a maximum intensity projection of the Z-stack to better illustrate the anatomy.
We have corrected this in Figure 5 and now also inserted the full maximum intensity projection comprising 10 optical sections in EV1G' of the revised manuscript.

(9) The authors should refrain from using the term "wound healing". Data in this report do not provide insight to changes in "wound healing" nor is it possible to conclude that wound healing is "improved" in mice with reduced monocyte recruitment.
We have changed the term to ‚wound ‚contraction' and in most cases actually avoid any statement about this process. What we were referring to was the small hole that is left from the injury is much smaller in the CCR2 KO mice. We state this now as such and further avoid any statements on the 'wound'.

In fact, data in Fig. 7 show reduced deposition of ECM proteins, which are essential components of "healing" wounds. Instead, it would more accurate to state that in the absence of monocytes, gliosis, glial "scarring" and associated changes in glial scar ECM molecules are reduced in the absence of infiltrating monocytes, implicating the latter cells in promoting scarring and ECM remodeling.
We have changed the text accordingly.

Pages 12-13:

(10) There are mismatches between the figures and the text. For example, page 13 states reduced Tn-C in CCR2 mice confirmed by immunostaining (Fig. S6), but Fig 6 shows S100β/GFAP staining. Page 13 refers to Fig. S5C, but Fig S5 contains only A and B. Page 26 refers to protein network analysis in Fig. 7B, but network analysis is in Fig. 8.
This has been corrected now. Many apologies for for the mistakes.

Page 14:

(11) The interpretation of the proteome analysis in the "Results" is interesting but meandering. Although the value of this analysis is evident, I don't think it is effective to bury the key findings of these assays in the supplemental data. Instead, the authors should extract key data and illustrate the protein changes and protein-protein interactions that differentiate WT vs. CCR2-/- at 5dpi (or provide insight to differences in the glial scar at later (chronic) times post-injury).
We have done this and now included the key data in Figure 8 (B) as well as an analysis of protein interactions (8C). We have also modified the text to clarify the points raised by the reviewer.

Page 15:

(12) The rationale provided in the text for looking at AhR is confusing. Monocytes do invade stab wounds in WT mice so why look at WT mice if the goal is to show that juxtavascular astrocytes restrict monocyte invasion?
Because this is a quantitative effect – in WT juxtavascular proliferating astrocytes restrict monocyte invasion as revealed when their proliferation is inhibited and even more monocytes are invading. The goal is to find a mechanism why juxtavascular astrocytes can better restrict monocyte invasion than others – a question also raised by reviewer 2. We show that juxtavascular astrocytes are AhR+ which provides a clue to what makes them special.

Wouldn't it be more useful (and impactful) to compare monocyte numbers and spatial distribution (relative to juxtavascular astrocytes) within stab wounds of cdc42 and SMOM2 transgenic mice, i.e., mice with reduced or enhanced proliferation of juxtavascular astrocytes and enhanced/reduced monocyte infiltration, respectively?
We did and still do provide numbers of monocytes in both of these transgenic mice (Figures 2 and 3). As momocytes migrate within the tissue it will be difficult to conclude much from their distribution in the tissue. However, we of course fully agree with the reviewer that it will be important to examine the interaction of the juxtavascular astrocytes and invading monocytes more closely in the future – ideally by live in vivo imaging. The reviewer will surely understand that this clearly exceeds the scope of this manuscript and is also not doable in all these transgenic mice.

(13) Please include all group sizes in the figure legends and provide a description in the Statistics section of how samples sizes were determined.
Group size had been included already in the Figure legends of the previous manuscript but we carefully controlled that this is the case for all once again. Sample size has been determined for our animal license and is used accordingly as stated in the Methods now.

(14) For the most part, the text is well-written and easy to understand but there are many examples throughout where the English can be improved. I recommend that the authors have a native-English speaking person edit the document.
Adam O'Neill is a native speaker and has corrected the manuscript for English language.

Referee #2:

The authors have used genetic mouse models to target proliferating astrocytes after MCAO but mostly cortical stab lesions to determine the effects on the extravasation of inflammatory cells which in turn, effects the scarring process. To my knowledge this is the first time that tight control of astroglial numbers as well as monocyte invasion and their close interactions after CNS injury have been investigated. Thus, the results are quite novel. However, there are

a number of issues that the authors should address in order to strengthen the impact of this interesting research.
1) In the introduction, the statement that astrocytes recruited from ependymal stem cells are the closest equivalent to zebrafish stem cells may likely be incorrect. Indeed, the role of the ependymaI contribution to astroglial scar formation has recently and seriously been called into question by a rather convincing paper from the Sofroniew lab (Scientific Reports Jan 2017, Ren et al). This part of the introduction should be clarified.
We have included this citation in the introduction but would like to point out that our work is in the forebrain while this study addresses spinal cord ependymal cell recruitment. To our knowledge the work of Benner et al 2013 has not been called into question.

2) While the Sofroniew lab has done notable work, their Nature paper suggesting that the astroglial component of scar is always "beneficial" since it is never a barrier and is always regeneration supportive is highly controversial. No citations from the Silver lab in the intro and elsewhere within the paper is a major omission of pertinent literature related to this controversy as well as discussion related to scar and extracellular matrix. Since the present paper does not examine axon regeneration in the vicinity of the cortical stab lesion in the various mutants, the authors should make it clear that their discussion of the "beneficial" aspects of astroglial scar formation only relates to interactions with inflammatory cells and not axons. It would have been interesting to look at serotonergic axons in the vicinity of the various lesions. Also, some of the critical findings in the present paper are quite different and novel from those of the Sorfoniew lab especially concerning the role of glial proliferation in scar formation. The observation that lack of macrophage invasion leads to more rather than less astrocyte proliferation and, surprisingly (given the Sofroniew lab findings), less rather than more scarring is the major novel finding in the present paper. This means likely that local cell-to-cell or cell-to- matrix interactions between astrocytes and inflammatory cells are more critically important for scarring than is proliferation per se. The role of cell-cell/matrix interactions could be discussed. (See Hara et al. Nat Med 2017).
These are very good points and we definitely did not want to give a unilateral view – we just wanted to be brief and focus on the astrocyte aspects. We have amended this now and include the Silver 2016 citation but also the new review from Vittorio Gallo that nicely discusses how heterogeneity in glial cell populations may well explain some of the discrepancies seen in the field which is exactly our main motivation to look at proliferative and juxtavascular astrocytes here.
Also, the conclusion that scarring in the brain is different from scarring in the spinal cord makes no sense.
We have removed this sentence as we agree with the reviewer that this is not our main point here.

3) The MCAO model involves rather profuse stem cell migration towards the stroke cavity from the SVZ which is not seen after a cortical stab lesion. Thus, the two models are quite different. Indeed, given that the scarring processes and astroglial/monocyte interactions related to the MCAO model over time are not investigated it seems that this part of the paper is superfluous and could be omitted.
If possible, we would like to keep these data as they show that the bias of juxtavascular astrocytes to proliferation is not restricted to TBI, but of more general relevance despite all the differences between these injury models.

4) It was not made clear what, if any, special property of astrocytes whose somata lie very near vessels might be. Why might astrocytes with slightly more distant cell bodies from the endothelium but with processes that intimately abut blood vessels also be important?
Because the former have AhR signalling and the latter not. As AhR has been shown to inhibit Ccl2 expression we suggest that juxtavascular astrocytes could form a sheet of 'nonattracting' cells. For these data please see Figure 9 and discussion in the text.

5) Scar formation has other functional consequences such as repair of BBB leakage. Have the authors examined leakiness of the blood brain barrier (BBB) after stab lesion in the various mutants? Given the known importance of a compromised BBB to scar formation and vice versa (Schachtrup et al., J Neurosci 2010) it would be interesting to know what happens to the BBB especially when astrocytes proliferate poorly. What about the opposite situation? If no

data are available then some discussion of the BBB is warranted.
This is an excellent point, and we incude now an entirely new set of experimental data examining BBB leakage at 3dpi (ie just before the resealing of BBB in our lesion model in WT) in comparison between CCR2 KO and WT using cadaverine iv injection. Indeed, we see a significant reduction in the cadaverine labelled region in CCR2 KO stab wound injured brains suggesting faster resealing of the BBB in the absence of monocyte invasion. These important new data are now included as Appendix Figure S4 and in the results on p.12. Note, however, that astrocyte (and any other cell) proliferation is not yet increased at 3dpi in the CCR2 KO mice, suggesting that it is unlikely that the proliferation of glial cells mediates the earlier resealing of the BBB. However, the earlier closure of the BBB may of course affect the scar forming properties of cells as we now discuss on p.22/23 especially for the mechanism suggested by Schachtrup et al.

6) The authors have examined col 4 which is interesting but they should have also looked at col1 since this is now known to be a major instigator of astrocytic scar (see Hara et al., Nat Med).
We now also included immunostaining for collagen 1 as suggested by the reviewer as Appendix Figure S5 and describe the results on p.14. Notably, we see some differences between WT and CCR2 KO mice in collagen I, but much less pronounced compared to collagen IV.

7) Astrocyte proliferation appears to have little to do with scar formation at least in the absence of macrophage invasion. Have the authors considered that dividing astrocytes (lacking macrophage signaling) may be entering a more immature state. There is a wealth of previous information about mature versus immature astrocytes and how immature astroglia are associated with improved wound healing. Another possible point of discussion.
This is an excellent suggestion and we discuss this interesting possibility on p. 22 of the revised manuscript.

8) Some of the figure notations seem problematic. I see no Figure S3H., Figure S6 has nothing to do with tenascin.
Sorry about these mistakes that were of course corrected now.

Referee #3:

Using models of traumatic brain injury and MCAO ischemia, the authors show a relationship between astrocyte scar formation and monocyte infiltration. In particular, CCR2-/- mice with deficient monocyte infiltration had reduced scar formation despite increased astrocyte proliferation. Proteomic analyses guided the authors to evaluate certain ECM components and the results are discussed in the context of scar formation. The manuscript is interesting and the datasets are generally of good quality.

The following are meant to improve the manuscript:
1. Are the results of the inverse relationship reflected in blood-brain barrier disruption? For example, in CCR2-/- mice, is the integrity of the BBB (eg fibrinogen deposition) better than WT animals?
Yes, indeed it is. Many thanks for this excellent suggestion. Indeed, we had attempted before already to examine such possible differences in BBB closure, but had done so with tracers that did not look very convincing to us, and hence we omitted this from the manuscript. Now we performed a series of new experiments using cadaverine injection and found indeed a significant reduction in the cadaverine-labelled region at 3dpi in CCR2 cKO mice compared to WT. These important new data are now included as Appendix Figure S4 and on p.12 and discussed on p.22/23. We also stained for fibrinogen and observed a similar trend, but less clear effect, due to some staining in the subpial area. Unfortunately, we did not have sufficient number of mutant animals within the 3 months revision time to determine the fibrinogen effects conclusively. However, the cadaverine results are clear and answer this important question.

2. The distinction of microglia (CD45+Iba1+) from recently infiltrated macrophages

(CD45+Iba1-) may hold in the first few hours of injury (Fig 1) but become unreliable at later (eg 3 days as used in Fig 1 or 5 days in Fig 5) time points of injury. The authors should qualify the distinctions better (eg using TMEM115 ab in the absence of available reporter mice) in their results. It is increasingly apparent that macrophages and microglia can have very different roles after injury, so the casual distinction as used here (Iba1- or +) should be better justified.
We assume the reviewer rather meant Tmem119 and we include a staining now in the appendix Figure S3. In our hands this staining is rather weak and not suitable for quantifying cell numbers. Nevertheless it supports the trend of higher activation of microglia in CCR2 KO mice. The justification of using CD45+ (Iba1-) cells is that this staining virtually completely overlapped with the RFP in the CCR2 heterozygous knock-in mice. As RFP persists for a few days and can hence be used as a short-term lineage marker, we conclude that CD45 immunostaining is a reliable marker for the vast majority of CCR2+ invading monocytes as mentioned on p. 10 of the revised manuscript.

3. How are the cell types controlling or seemingly repelling one another? Recent work (Horng et al., JCI 127:3136, 2017) of astrocyte tight junctions controlling the exit of immune cells from the perivascular space into the CNS parenchyma could be cited. The role of AhR in regulating the astrocyte results herein in Figure 9 is a correlation and a speculation at best.
Excellent point and we would like to thank the reviewer for reminding us of this interesting manuscript. This is included now in the discussion on p. 20 of the manuscript. Yes, the expression of AhR in juxtavascular astrocytes after injury is a correlation, but the reviewer will agree that using another conditional deletion (GlastCreERT2/flAhR mice) will go beyond the scope of this manuscript. Moreover, it is an important finding as we have been searching for years for a difference in any of the known markers, including GFAP, Hes5 and BLBP, between juxtavascular and non-juxtavascular astrocytes and failed to find anything. We are therefore very excited to provide this new data here on the difference in AhR immunostaining between juxtavascular and non-juxtavascular astrocytes that also provides an interesting model given the regulation of Ccl2 by Aryl hydrocarbon receptor signaling.

4. In the context of CSPG deposition in Figure 7, what are the cellular sources? The results could be interpreted as CSPGs being produced by monocytes/macrophages to affect astrocytes, or astrocytes producing CSPGs to limit the invasion of leukocytes. The mouse anti-CSPG (Abcam) descriptor should be extended (eg targeting CS-56) and the authors should comment on mouse monoclonals being used on mouse tissues (where increased background staining is expected at sites of blood brain barrier disruption); staining controls could be better presented particularly for ECM components in areas of blood-brain barrier disruption.
We now include the information that this antibody recognizes the CS-56 epitope in the text and Figure 7. Moreover, we now include confocal pictures of the control stainings using only the secondary antibodies in Figure EV5. The secondary antibodies used for ECM immunostainings are mostly mouse IgM and rabbit which have not much background as shown in the respective panels. The origin of CSPGs is probably multicellular and we have no way to dissect this in the present manuscript.

5. For the proteomic analysis, the authors should be complimented for stating that they had to remove 2 outlier from each genotype (page 13). What is the basis for considering some samples as outliers and thus their deletion from the analysis?
These ‘outliers’ are 2 samples that were prepared and run at a different time and hence were not as well comparable as the 4 samples run at the same time point. We now explain this on p. 15/16 of the revised manuscript. Many thanks for alerting us to this which does indeed need explanation!

2nd Editorial Decision 27 February 2018

Thank you for the submission of your revised manuscript to EMBO reports. I have now received the reports from three referees that were asked to re-evaluate your study, which can be found at the end of this email.

As you will see, all three referees now support the publication of your work in EMBO reports. However, before we can proceed with formal acceptance, I have a couple of editorial requests that need to be addressed in a final revised version:

Please upload editable TIFF or EPS-formatted individual figure files in high resolution for main figures and the EV figures.

Please provide the manuscript text as word file, and the abstract written in present tense.

Please add the PRIDE accession number to the respective paragraph in the methods section.

Please provide the appendix with page numbers, and also add page numbers to the Appendix TOC.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this revised manuscript, Frik et al. explore the relationship between astrocyte proliferation, monocyte invasion, and glial scar formation in experimental traumatic brain injury and ischemic brain injury. The authors have done a fabulous job responding to all major and minor critiques. Their responses were thorough, appropriate and scholarly. I have no further concerns related to technique, data presentation or data interpretation. This paper will be a valuable contribution to the field of neural injury/repair, neuroinflammation and glial biology.
One minor point that I noticed in the revised document was that the authors refer to data in both "expanded view" and "supplemental figures", with the latter linked to an Appendix. I did not find any supplemental or appendix material in the revised merged PDF. I believe that supplementary material should be consolidated into the "EVC" data. Thus, some revision is needed in the text to refer to the appropriate figures.

Referee #2:

The authors have adequately attended to the suggested revisions.

Referee #3:

The authors have addressed my concerns

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Magdalena Götz; Swetlana Sirko
EMBO Reports
EMBOR-2017-45294V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The group size was determined to allow statistical evaluation of differences between two groups with the power indicated in the manuscript.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The minimum sample size for experiments was n=3. In most cases sample size was larger. We always used statistical analysis as described in the methods section.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	we did not exclude any data from the analysis. In case of proteomics we performed the focused statistical analysis and GO term analysis on the samples that were prepared and runsimultaneaously at the mass spec as stated in the manuscript.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable as the mice we used had different genotypes and were allocated according to their genotypes.
For animal studies, include a statement about randomization even if no randomization was used.	In this studies we used mostly transgenic mice, no pharmacological treatments. We randomized in the sense that we always lesioned WT or transgenic/mutant animals radomly as they became available.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Most analysis was done by taking confocal pictures. Quantification of the cells on the pictures was done without checking the genotype.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No specific blinding was done, but the genotype was typically assessed after the countings were completed.
5. For every figure, are statistical tests justified as appropriate?	yes, see methods section
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	we tested for normal distribution and used the accordingly appropriate tests. See method section for more detailed description
Is there an estimate of variation within each group of data?	yes, standard error of the mean
Is the variance similar between the groups that are being statistically compared?	GraphPad Prism was used for statistical analysis. The software uses F-test to compare variances while performing t-Test or mann Whitney test. Variance can be different for different genotypes. However in our study these differences were minimal.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	A supplementary table with antibody information and catalog numbers is provided.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	not applicable

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All mice were used at yound adult age (2-3 months old) when the experimental treatment began. They were kept under standard housing conditions with access to water and food ad libidum. We used mostly the GLASTCreERT2 mouse line that we had generated ourselves and described in Mori et al., 2006. This was crossed with the fl cdc42 generated by Wu et al., 2006 as described in Bardehle et al., 2013 and Robel et al., 2011. The fl smo mouse line was used in Sirko et al., 2013 and generated by Long et al. 2001. The CCR2 RFP knock-in line was purchased from Jackson lab (Cat. 017586) like the mouse line to conditionally activate SMOM2 (Gt(ROSA)26Sortm(Smo/EYFP)Amc/J (Cat. 005130).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animals handling and experimental procedures were performed in accordance with German and European guidelines and approved by the State of upper Bavaria.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	we comply with the ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	proteomics data have been deposited at PRID with the identifier PXD008906.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	A Supplementary document with Excel Tables of raw Data will be deposited in datadryad.org after manuscript acceptance
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No