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Lineage tracing clarifies the cellular origin of tissue resident macrophages in the developing heart

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Re: JCB manuscript #202108093

Prof. Bin Zhou
State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, University of Chinese Academy of Sciences
320 Yueyang road, Life Science Research Building A-2112
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China

Dear Prof. Zhou,

Thank you for submitting your manuscript entitled "Cellular origin of tissue resident macrophages in the developing heart". Your manuscript has been assessed by expert reviewers, whose comments are appended below. We apologize for the extensive delay in providing you with a decision and thank you for your patience.

You will see that the two reviewers find that your premise that cardiac macrophages do not generate from endothelial cells of the endocardium is very interesting but note it is not adequately supported by the data. They appreciate the elegant work that you have done using several lineage tracing mouse models. Nonetheless, to unequivocally prove that the endocardial endothelium does not have hemogenic potential and thus refute what previously reported in the field, it would be required, as suggested by reviewer #1, that you perform lineage tracing studies in a mouse model of a specific marker of endothelial cells of the endocardium but not of the YS and AGM - maybe publicly available scRNAseq data could help identify such marker - or that you use an indirect mouse model, as suggested by reviewers #1 and #2, to prove the yolk sac (CXCR4-CreERT2) or the AGM (Mds1CreERT2, Hlf or Mecom) derived origin of cardiac macrophages. Like the reviewers, we understand that the work with a new mouse model may turn out too difficult or effort and time consuming, so we agree with them that, given that your data is exciting, an alternative path would be performing more descriptive analyses to reinforce your observations, as outlined by reviewers (rev #1 p1 & rev #3 paragraph 3: testing in vitro the hemogenic potential of Nfact1+ endothelial cells from different parts of the embryo + rev #1 p3: stain for macrophages on the Mef2c and Np3 reporter lines at earlier timepoints to rule out that the 'endocardial-derived' macrophages important for valve remodeling have been replaced), and in parallel significantly tone down your claims. We leave up to you which path you wish to pursue during revisions, and we also hope that you will be able to address each of the reviewers' other issues as well. Taking the first path, which we suspect you may have already initiated, would truly maximize the impact of your excellent work in general and this manuscript in particular.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers, as well as our editorial concerns. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ira Mellman, Ph.D.
Editor
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The paper from Liu et al. focuses on the cellular origin of tissue resident macrophages in the embryonic heart. The authors did an impressive amount of work with several different mouse models to identify the ontogeny of endocardial macrophages in the developing heart. The paper is clear and well written and the experiments are technically well performed.

However, the authors fail to prove the point that cardiac macrophages do not derive from endocardial hematopoiesis.

In particular:
1- The authors efficiently prove that Nfact1 is not a specific marker for the endothelial cells of the endocardium by showing its expression in the endothelium of YS and AGM. This is an interesting piece of data, but it doesn't exclude that the Nfact1+ endothelium of the endocardium might generate hematopoietic cells (and macrophages) independently from YS and AGM. There is also the possibility that the Nfact1+ endothelial cells are hemogenic in the heart but not in YS and AGM. The only thing that these data prove is that there are Nfact1+ endothelial cells in different parts of the embryo, but they do not say anything about their hemogenic potential. To have an idea about this last point, the authors could sort the Nfact1+ cells from the different tissues and check for their hemogenic potential at least in vitro (co-culture on stromal cells, methyl cellulose assay).
2- The authors show that Mef2c and Np3 are tissue specific for the endocardium because they are not present in the YS and AGM. Again, this is elegantly show, but the TdT reporting for Mef2c or Np3 is not expressed in the totality of PECAM+ cells (as shown in Figure 4E and 5E), hence there is a fraction of the endothelium that doesn't express these two markers. The authors cannot exclude that is the Mef2c-/Np3- endothelium the responsible for cardiac hematopoiesis.
3- The staining for F4/80 on the Mef2c and Np3 reporter lines are made at E15.5. By that time, the cardiac macrophages that are important for valve remodelling (Shigeta 2019) could already be replaced by YS- or AGM-derived macrophages. By using this time point, the authors cannot exclude that the endocardial-derived macrophages (if they exist) are transiently present in the heart (around E10/E11) and they get replaced after that.
4- The Cdh5-2A-CreER mouse model efficiently label the endothelial cells of the YS, but also of the heart (Fig 6D). So, the authors cannot exclude that (as already discussed in the point 1 above), the Cdh5+ endothelial cells of the heart can have

hemogenic potential independently from the YS or the AGM and that the endocardium can contribute to local hematopoiesis.

To really prove that the endothelium of the endocardium does not contribute to the generation of cardiac macrophages, the authors should explore different mouse models.
For example:
1- Find a marker that is expressed in 100% of the endothelial cells of the endocardium without being expressed by YS or AGM and prove that macrophages are not derived from that kind of endothelial cells.
2- Use an indirect way to reach the same conclusion. For example they could use the CXCR4-CreERT2 (https://pubmed.ncbi.nlm.nih.gov/32783932/
) to exclude (or not) YS-origin or the newly published Mds1CreERT2 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8428393/) to prove (or not) that cardiac macrophages derive from AGM-derived adult type HSCs.
3- Use a circulation deficient mouse model that is still alive at the time of cardiac development/valve remodelling and assess the macrophage compartment of the endocardium. If there are no macrophages in the endocardium without circulation it means they do not have a local origin.

If the authors do not wish to explore additional mouse models to prove their conclusion, then they have to tone down their conclusions. The paper still presents interesting data that can complement (but do not disprove) the current literature on the topic, but it cannot be published in its present form because the conclusions are not supported by the data shown.

Minor points:
1- When presenting flow cytometry data, the authors should show representative dotplots in addition to the mean of the different experiments. The authors are looking at extremely low number of cells, also 10 events can make a difference during development.
2- When showing FACS histograms, the authors should show also the unstained or the FMO controls otherwise the data are not informative (see Fig4 and Fig5).
3- Page 10, line 9, the Figure is the 3C and not the 2C.
4- The authors should comment on why the Cre and the Dre Nfact1 reporter models give such a difference in % of TdT+ cells. To understand which is the most accurate model they should perform a direct staining for Nfact1 to understand its expression in different embryonic tissues.

Reviewer #2 (Comments to the Authors (Required)):

Understanding the developmental origins of differentiated cell types relies currently on the generation of genetic lineage-tracing models, as well as the appropriate interpretation of the data. These studies complicated by the expression of targeted genes not only in developmental space and time, but also by the fact that targeted genes are typically expressed by multiple cell types. As such, it is useful to study multiple genetic models and also to examine cells outside of their presumed tissue specific target organs. This latter point is a significant strength of this carefully performed and carefully analyzed study.

It as well established that blood cells arise during embryogenesis from hemogenic endothelial sources primarily in the yolk sac, and subsequently in large arterial vessels. Interestingly, other vascular beds, including the endocardium, have been proposed to be sites of hematopoietic stem and progenitor cell (HSPC) emergence. It is also well established that hematopoietic progenitors arising in the yolk sac give rise to populations of tissue-resident macrophages that persist long-term in multiple organs. Here, the authors re-examine the developmental origins of tissue-resident macrophages in the heart, and also examine whether the endocardium in fact does contain hemogenic potential, as currently suspected.

In this well-organized and clearly written paper, the authors carefully examine the Nfatc1-Cre lineage-tracing mouse model, revealing that endothelial and blood cells in the E9.5 yolk sac are labeled (Fig. 2G-I). While a sizable proportion of cardiac tissue-resident macrophages were also labeled, these data clearly indicate that Nfatc1 is not a specific gene marker for targeting of the endocardium, instead also targeting endothelial cells in known sites of hemogenic endothelium. These results were confirmed using an Nfatc1-Dre mouse model, as well as an inducible Nfatc1-CreER mouse model. The authors go on to show that primary monocytes and macrophages in the developing heart express Nfatc1, raising the possibility that the Nfatc1c lineage-tracing mouse models may lead to direct labeling of cardiac macrophages (Fig. 3). While these studies do not prove that the endocardium is not hemogenic, taken together the findings significantly weaken the case that it is hemogenic for cardiac macrophages. Determining if Nfatc1-labeled endothelial cells in the yolk sac and endocardium also co-express nuclear Runx1, thus confirming their hemogenic endothelial identity (or not), could strengthen the conclusions drawn.

The authors next demonstrate that specific labeling of endocardium with either Mef2c-Cre or Npr3-CrER, failed to lineage trace cardiac macrophages. In addition, labeling of epicardium with Wt1-CreER failed to label cardiac macrophages. Taken together, these data supprt the novel paradigm that the developing heart does not generate its own macrophages. This is a highly significant finding.

In a final set of experiments a new Cdh5-CreER mouse model is used to determine if cardiac tissue-resident macrophages are

derived from the yolk sac or "AGM". TAM treatment at E7.5 labeled endothelium, microglia, and the large majority of cardiac macrophages. In contrast, treatment at E10.5 labeled a minority of cardiac macrophages and no microglia. While these results are interpreted as cardiac macrophages being derived from "primitive" hematopoiesis in the yolk sac and "definitive" hematopoiesis in the AGM, concerns are raised over this interpretation. While it is well accepted that microglia are derived from "primitive" macrophages (also referred in the Introduction as "early" EMP), it is not clear that the new Cdh5-CreER mouse model induced at E7.5 might not also begin to label the "transient" definitive wave (so-called "late" EMP) as well. In addition, it is very likely that TAM treatment at E10.5 would also label the "transient" definitive wave of hematopoietic progenitors that emerge in the yolk sac from hemogenic endothelium, as well as HSCs emerging in the aorta. If the "transient" definitive wave were being lineage traced with TAM at either E7.5 or E10.5, then definitive erythroid cells in the E12.5 fetal liver would also be labeled, though microglia would not be labeled. To more clearly label hemogenic endothelium in the aorta (AGM), mouse models such as Hlf or Mecom, as recently published, and thought to be specific for HSCs, would need to be used. As it stands, the data can be interpreted as cardiac macrophages being derived from "primitive" and from "definitive" hematopoiesis, if the latter is meant to also refer to "transient" definitive and "AGM/HSC" definitive hematopoiesis. In that case, the terminology in the Introduction ("early" vs. "late" EMP) would need to be modified to match the terminology of the Discussion.

Minor

1. Page 3, lines 25-26: HSCs do not begin to colonize the fetal liver until E11.5-E12.5.
2. Page 8, line 4: "exam" should be "examine".
3. Page 12, line 15: what does "minimally" mean here?
4. Page 14, line 28: "wildly" probably should be "widely".

Response to Reviewers' Comments

Reviewer #1 (Comments to the Authors (Required)):

The paper from Liu et al. focuses on the cellular origin of tissue resident macrophages in the embryonic heart. The authors did an impressive amount of work with several different mouse models to identify the ontogeny of endocardial macrophages in the developing heart. The paper is clear and well written and the experiments are technically well performed.

However, the authors fail to prove the point that cardiac macrophages do not derive from endocardial hematopoiesis.

We thank the reviewer for all the valuable comments and suggestions to advance our work. We have performed additional experiments to strengthen our conclusion.

In particular:

1- The authors efficiently prove that Nfact1 is not a specific marker for the endothelial cells of the endocardium by showing its expression in the endothelium of YS and AGM. This is an interesting piece of data, but it doesn't exclude that the Nfact1+ endothelium of the endocardium might generate hematopoietic cells (and macrophages) independently from YS and AGM. There is also the possibility that the Nfact1+ endothelial cells are hemogenic in the heart but not in YS and AGM. The only thing that these data prove is that there are Nfact1+ endothelial cells in different parts of the embryo, but they do not say anything about their hemogenic potential. To have an idea about this last point, the authors could sort the Nfact1+ cells from the different tissues and check for their hemogenic potential at least in vitro (co-culture on stromal cells, methyl cellulose assay).

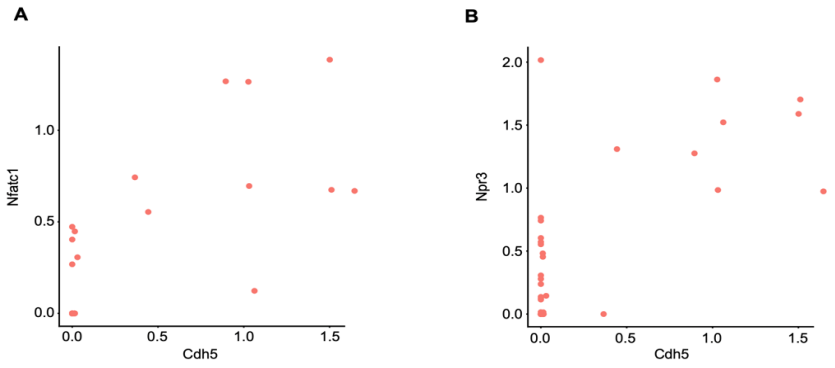
We thank the reviewer for raising this important point. We agree with the reviewer that our previous data did not fully exclude that the hematopoietic potential of Nfatc1^{+} endocardium. Runx1 is an important regulator of the hemogenic endothelium to control activation of hematopoietic gene expression (North et al., 2002, Immunity). To test whether these Nfatc1^{+} endocardial cells express the hemogenic endothelial cell marker Runx1, we performed co-immunostaining for VE-cad, Runx1, and tdTomato (tdT) on E9.5 *Nfatc1-ires-Cre;R26-tdTomato* embryonic sections (Fig. 4 A). The results showed that the tdT^{+} VE-cad^{+} Runx1^{+} endothelial cells were detected in the yolk sac region, but not in the OFT/heart region (Fig. 4, B and C). Furthermore, we detected many tdT^{+} Runx1^{+} VE-cad^{-} cells in the vascular lumen and heart chambers, and co-immunostaining data revealed that most of these cells expressed CD45, suggesting that these committed blood progenitor cells may be originated from other Runx1^{+} hemogenic endothelium through circulation (Fig. 4, D and E). The absence of endocardial expression of the hemogenic endothelial marker Runx1 *in vivo* suggested that the endocardium was less likely to have hematopoietic activity.

Because current genetic tools do not enable us to exclusively trace Nfatc1^{+} endocardial cells *in vivo*, we then performed *ex vivo* hematopoietic colony-forming assays, as suggested by the reviewer. We used E8.0 *Nfatc1-ires-Cre;R26-tdTomato* embryos for *ex vivo* experiments because the circulatory system has not yet been fully established at this stage. Briefly, the yolk sac, caudal half, head, and heart regions of E8.0 *Nfatc1-ires-Cre;R26-tdTomato* embryos were dissected and pre-cultured on OP9 stromal cells for 4 days, and were then transferred to methylcellulose medium for 10 days to test their hemogenic potential according to the protocols described by Nakano and colleagues (Fig.

5 A). The yolk sac and caudal half were included as the positive control groups, and the head was used as the negative control group. Our *ex vivo* results showed that the tdTomato$^+$ macrophage colonies were generated from both the yolk sac and caudal half groups, but neither from the head nor the heart groups (Figure 5, B and C). Immunostaining for tdTomato and F4/80 or CD45 confirmed that Nfatc1$^+$ cells contributed to macrophages in yolk sac and caudal half groups but not in head or heart groups (Figure 5, D and E). Collectively, these data supported that the Nfatc1$^+$ endocardial cells did not have hemogenic potential. We have added these data in the revised manuscript. For details, please refer to Page 10, Paragraph 2, Line 27 of the revised manuscript.

2- The authors show that Mef2c and Np3 are tissue specific for the endocardium because they are not present in the YS and AGM. Again, this is elegantly show, but the TdT reporting for Mef2c or Np3 is not expressed in the totality of PECAM+ cells (as shown in Figure 4E and 5E), hence there is a fraction of the endothelium that doesn't express these two markers. The authors cannot exclude that is the Mef2c-/Np3- endothelium the responsible for cardiac hematopoiesis.

We thank the reviewer for providing us with valuable comments. In Atsushi Nakano's 2013 paper (Nakano et al., 2013, Nature Communications), by using the *Nkx2.5-ires-Cre* line, they showed that the endocardium of outflow cushion and atria contributes to transient definitive hematopoiesis. In Atsushi Nakano's 2019 paper (Shigeta et al., 2019, Developmental Cell), they showed that the Nfatc1$^+$ endocardium of endocardial cushion region was the hemogenic site, capable of contributing to ~60% cushion macrophages that migrate to other locations during development. However, the labeled Mef2c$^+$ and Npr3$^+$ endocardial cells in our study did not support their hematopoietic potential. It is possible that Nkx2.5$^+$Nfatc1$^+$Mef2c$^-$Npr3$^-$endocardial subpopulation that has the hematopoietic potential. Nevertheless, re-analysis of the public single-cell sequencing data of E9.5 endothelium/endocardium showed that almost all Cdh5-expressing endocardial cells expressed Nfatc1 and Npr3 (see figure blow, and reference come from: DeLaughter, D.M., Bick, A.G., Wakimoto, H., McKean, D., Gorham, J.M., Kathiriya, I.S., Hinson, J.T., Homsy, J., Gray, J., Pu, W., et al. (2016). Single-Cell Resolution of Temporal Gene Expression during Heart Development. Dev Cell 39, 480-490. 10.1016/j.devcel.2016.10.001.).



While the *Mef2c-AHF-Cre* and *Npr3-CreER* lines did not label all PECAM$^+$ endocardial cells, we found that they randomly labeled the cushion and atrial endocardial cells, which were proposed to have hemogenic potential as previously reported by Nakano (Nakano et al., Nat Commun 2013; Shigeta et al., Dev Cell 2019). We showed that ~81% and ~55% endocardial cells of the outflow

cushion were randomly labeled by *Mef2c-AHF-Cre* and *Npr3-CreER* respectively, and ~95% of the endocardium of the atria were randomly labeled by *Npr3-CreER*. As the majority of these endocardial cells were labeled, we did not find any hematopoietic cell in the cardiac valves and circulation. To independently address this issue, we performed *ex vivo* hematopoietic colony-forming assays. Culture of the tissues containing all the endocardial cells, including the potential Nkx2.5^{+}Nfatc1^{+}Mef2c^{-}Npr3^{-} subpopulation, showed that the endocardium did not have hemogenic potential (Fig. 5). Moreover, we did not detect any Runx1 expression (hemogenic endothelial cell marker) that regulates activation of hematopoietic gene expression in any VE-cad^{+} endocardium (Fig. 4). Taken together, these data demonstrated that the endocardium was not hemogenic during embryogenesis. We have added this data and also discussion in the revised manuscript. For details, please refer to Page 10, Paragraph 2, Line 27.

3- The staining for F4/80 on the Mef2c and Np3 reporter lines are made at E15.5. By that time, the cardiac macrophages that are important for valve remodelling (Shigeta 2019) could already be replaced by YS- or AGM-derived macrophages. By using this time point, the authors cannot exclude that the endocardial-derived macrophages (if they exist) are transiently present in the heart (around E10/E11) and they get replaced after that.

We thank the reviewer for raising this important point. During heart development, the resident macrophages could be slowly replaced by YS- or AGM-derived macrophages. As suggested by reviewers, we collected embryos of *Mef2c-AHF-Cre;R26-tdTomato* and *Npr3-CreER;R26-tdTomato* at earlier stage (E10.5) for analysis. Immunostaining for tdTomato and F4/80 on tissues sections showed that neither F4/80^{+} macrophages in E10.5 *Mef2c-AHF-Cre;R26-tdTomato* tissues nor in E10.5 *Npr3-CreER;R26-tdTomato* tissues expressed tdTomato (Fig. S3, A-C; Fig. S4, A-C). These data indicated that the endocardium did not transiently give rise to cardiac macrophages at early developmental stage. We have added these data in the revised manuscript. For details, please refer to Page 12, Line 17 and Page 13, Line 19.

4- The Cdh5-2A-CreER mouse model efficiently label the endothelial cells of the YS, but also of the heart (Fig 6D). So, the authors cannot exclude that (as already discussed in the point 1 above), the Cdh5+ endothelial cells of the heart can have hemogenic potential independently from the YS or the AGM and that the endocardium can contribute to local hematopoiesis.

We thank the reviewer for raising this important question. The results collected from *Cdh5-2A-CreER* mouse model needs to be interpreted with the above endocardial lineage tracing results in our study. In the first part of the manuscript, we used three Nfatc1 tools to demonstrate that Nfatc1 is not an endocardial specific marker (Fig. 2 and 3; Fig. S1 and S2). Independently, the hematopoietic colony-forming assays showed that the Nfatc1^{+} endocardial cells did not have hemogenic potential *ex vivo* (Fig. 5). In the second and third parts, we used another two genetic tools (*Mef2c-AHF-Cre* and *Npr3-CreER*), which were more specific in targeting the endocardium, that further demonstrated that the endocardium did not contribute to cardiac macrophages nor circulating blood cells (Fig. 6 and 7; Fig. S3 and S4). In the fourth part, our findings had also ruled out the possibility that the epicardium contributed to cardiac macrophages (Fig. 8). Based on these results showing that the endocardium has no hemogenic activity, the endocardium labeled by *Cdh5-2A-CreER* would unlikely have hemogenic activity. Therefore, in the last part, we used *Cdh5-2A-CreER* line to determine whether the hemogenic endothelium of YS and AGM were cell sources of

cardiac macrophages in the developing heart. We found *Cdh5-2A-CreER* could trace the hematopoietic cells derived from the endothelium of YS or AGM after tamoxifen treatment. Our fate mapping results suggested that cardiac macrophages were mostly derived from the primitive/transient definitive hematopoiesis of the endothelium of YS with some being generated from definitive hematopoiesis in the endothelium of AGM region (Fig. 9; Fig. S5). Collectively, by using multiple genetic tools and also *in vitro* experiments, we found that cardiac macrophages were derived from the endothelium of YS and AGM, but not the endocardium in the developing heart.

To really prove that the endothelium of the endocardium does not contribute to the generation of cardiac macrophages, the authors should explore different mouse models.
For example:
1- Find a marker that is expressed in 100% of the endothelial cells of the endocardium without being expressed by YS or AGM and prove that macrophages are not derived from that kind of endothelial cells.
2- Use an indirect way to reach the same conclusion. For example they could use the CXCR4-CreERT2 (https://pubmed.ncbi.nlm.nih.gov/32783932/) to exclude (or not) YS-origin or the newly published Mds1CreERT2 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8428393/) to prove (or not) that cardiac macrophages derive from AGM-derived adult type HSCs.
3- Use a circulation deficient mouse model that is still alive at the time of cardiac development/valve remodelling and assess the macrophage compartment of the endocardium. If there are no macrophages in the endocardium without circulation it means they do not have a local origin.

If the authors do not wish to explore additional mouse models to prove their conclusion, then they have to tone down their conclusions. The paper still presents interesting data that can complement (but do not disprove) the current literature on the topic, but it cannot be published in its present form because the conclusions are not supported by the data shown.
We thank the reviewer for raising these important points and we fully agree with the reviewer's suggestions. Indeed, more genetic tools could be employed to validate our conclusion. For the reviewer's first suggestion, screening for a marker which targets 100% endocardial cells will be important for specifically and efficiently tracing endocardial lineage. However, we searched public databases but did not find such a gene that is suitable for driving constitutively active Cre specifically in the endocardium but not in YS or AGM. Based on our experience in many mouse constructions, we found that the cell types labeled by the gene promoter-driven Cre lines do not necessarily match exactly to the cells expressing their transcripts as one gene that is specific to a cell type at one time that could be transiently expressed by another cell type at another time. There is a high risk in generating mouse models based on the screened genes. Although our *Mef2c-AHF-Cre* and *Npr3-CreER* models did not label 100% endocardial cells, they did randomly label most of the hemogenic endocardium of outflow cushion and atrium that mentioned by Nakano (Nakano et al., 2013, Nature Communications). Our *in vitro* hematopoietic colony-forming assay data also showed that the Nfatc1^{+} endocardium did not have hemogenic potential. Collectively, these data suggested that the endocardium has no hematopoietic activity.
Regarding the reviewer's second suggestion, *CXCR4-CreERT2* and *Mds1-CreERT2* are useful models for studying the cellular origins of cardiac macrophages. Since we did not have these mice,

and transfer of these mice from overseas labs really takes time during the COVID-19 pandemic. We again appreciate the reviewer for this constructive suggestion, and believe that it is valuable to perform new lineage tracing experiments using these mice in future. We thank the reviewer for suggesting that using circulation defect model to study endocardium could rule out the effects of other regions. *Ncx1*-mutant is a circulation defect mouse model and the mutant embryos could not survive beyond E10.5 (Nakano et al., 2013, Nature Communications). It is difficult to find such mutant for studying valve remodeling that occurs between E12.5-E15.5. We are also very sorry that we did not have *Ncx1*-mutant mice to generate circulation deficient mouse model. Nevertheless, we have added *ex vivo* hematopoietic colony-forming assays in the revised manuscript, which could also rule out the effects of circulation. We found that the Nfatc1^{+} endocardium did not have hemogenic potential, compared with YS and AGM compartment. We have added the discussion in the revised manuscript. For details, please refer to Page 10, Paragraph 2, Line 27.

Minor points:

1- When presenting flow cytometry data, the authors should show representative dotplots in addition to the mean of the different experiments. The authors are looking at extremely low number of cells, also 10 events can make a difference during development.

We thank the reviewer for raising this important point. As suggested by the reviewer, we have showed the representative dotplots of our FACS data. These data were added in the revised manuscript. For details, please refer to Fig. 3, Fig. S1, Fig. S3, and Fig. S4 in the revised manuscript.

2- When showing FACS histograms, the authors should show also the unstained or the FMO controls otherwise the data are not informative (see Fig4 and Fig5).

We thank the reviewer for raising this important point. As suggested by the reviewer, we have showed the unstained controls of our FACS histograms. These data were added in the revised manuscript. For details, please refer to Fig. 6 and Fig. 7.

3- Page 10, line 9, the Figure is the 3C and not the 2C.

We thank the reviewer for pointing out this error. We have corrected it in the revised manuscript. For details, please refer to Page 10, Line 4 of the revised manuscript.

4- The authors should comment on why the Cre and the Dre Nfact1 reporter models give such a difference in % of TdT+ cells. To understand which is the most accurate model they should perform a direct staining for Nfact1 to understand its expression in different embryonic tissues.

We thank the reviewer for raising this point. Indeed, in our results, the % of tdT^{+} cells from constitutive *Nfatc1-ires-Cre* and constitutive *Nfatc1-2A-Dre* were different. Because the Cre-loxP and Dre-rox are two different systems driven by two different recombinant enzymes, we think that this difference likely came from the difference in the recombination efficiency in these two systems. In our previous research, we found that in some mice, even though Cre-loxP and Dre-rox were inserted after the same promoter, their recombination efficiencies (reporter readout) were different. Whole-mount epi-fluorescence and sectional immunostaining showed that the tdT^{+} endothelial cells of *Nfatc1-ires-Cre* were higher than that of *Nfatc1-2A-Dre* (Fig. 2, F, G, M, N), indicating that the recombination efficiency of *Nfatc1-ires-Cre* was higher. As Nfatc1 gene is expressed in

macrophages and monocytes, the proportion of these immune cells labeled by *Nfatc1-ires-Cre* should also be higher than that by *Nfatc1-2A-Dre*, which explained this difference (Fig. 3). We also used *Nfatc1-2A-CreER* to label endocardial cells *in vivo*. We think the differences between all these genetic tools are due to different recombination efficiency. The purpose of using all these Nfatc1 tools in our study is to demonstrate its broad expression in cells such as YS, AGM, immune cells, in addition to endocardial cells. This part has been included in the Discussion of the revised manuscript. For details, please refer to Page 17, Paragraph 2, Line 25.

Reviewer #2 (Comments to the Authors (Required)):

Understanding the developmental origins of differentiated cell types relies currently on the generation of genetic lineage-tracing models, as well as the appropriate interpretation of the data. These studies complicated by the expression of targeted genes not only in developmental space and time, but also by the fact that targeted genes are typically expressed by multiple cell types. As such, it is useful to study multiple genetic models and also to examine cells outside of their presumed tissue specific target organs. This latter point is a significant strength of this carefully performed and carefully analyzed study.

It as well established that blood cells arise during embryogenesis from hemogenic endothelial sources primarily in the yolk sac, and subsequently in large arterial vessels. Interestingly, other vascular beds, including the endocardium, have been proposed to be sites of hematopoietic stem and progenitor cell (HSPC) emergence. It is also well established that hematopoietic progenitors arising in the yolk sac give rise to populations of tissue-resident macrophages that persist long-term in multiple organs. Here, the authors re-examine the developmental origins of tissue-resident macrophages in the heart, and also examine whether the endocardium in fact does contain hemogenic potential, as currently suspected.

In this well-organized and clearly written paper, the authors carefully examine the Nfatc1-Cre lineage-tracing mouse model, revealing that endothelial and blood cells in the E9.5 yolk sac are labeled (Fig. 2G-I). While a sizable proportion of cardiac tissue-resident macrophages were also labeled, these data clearly indicate that Nfatc1 is not a specific gene marker for targeting of the endocardium, instead also targeting endothelial cells in known sites of hemogenic endothelium. These results were confirmed using an Nfatc1-Dre mouse model, as well as an inducible Nfatc1-CreER mouse model. The authors go on to show that primary monocytes and macrophages in the developing heart express Nfatc1, raising the possibility that the Nfatc1c lineage-tracing mouse models may lead to direct labeling of cardiac macrophages (Fig. 3). While these studies do not prove that the endocardium is not hemogenic, taken together the findings significantly weaken the case that it is hemogenic for cardiac macrophages.

We thank the reviewer for appreciating our work and raising valuable comments to strengthen our manuscript. In addition to the *in vivo* genetic lineage tracing data, we also performed *ex vivo* hematopoietic colony-forming assays to determine whether the endocardium has hematopoietic activity. Briefly, we collected the yolk sac, caudal half, head, and heart regions of E8.0 *Nfatc1-ires-*

Cre;R26-tdTomato mice and pre-cultured them on OP9 stromal cells for 4 days, and then transferred them into methylcellulose medium for 10 days to study their hemogenic potential (Fig. 5 A). We set the yolk sac and caudal half as positive control groups, and the head as negative control group. Our *ex vivo* results showed that the tdTomato$^+$ macrophage colonies could be detected in both the yolk sac and caudal half groups, but not in the head nor the heart groups (Figure 5, B and C). Furthermore, immunostaining for tdTomato and F4/80 or CD45 showed that the Nfatc1$^+$ cells contribute to macrophages in yolk sac and caudal half groups but not in head nor heart groups (Figure 5, D and E). Collectively, these *ex vivo* data demonstrated that the Nfatc1$^+$ endocardium does not have hemogenic potential. We have added these data in the revised manuscript. For details, please refer to Page 10, Paragraph 2, Line 27 of the revised manuscript.

Determining if Nfatc1-labeled endothelial cells in the yolk sac and endocardium also co-express nuclear Runx1, thus confirming their hemogenic endothelial identity (or not), could strengthen the conclusions drawn.

We thank the reviewer for raising this important point. It has been previously reported that the endocardial hematopoietic activity was relatively high at around E9.5 (Nakano et al., 2013, Nature Communications; Shigeta et al., 2019, Developmental Cell). We therefore collected E9.5 *Nfatc1-ires-Cre;R26-tdTomato* embryos for analysis (Fig. 4 A). Immunostaining for VE-cad, Runx1, and tdTomato on tissues sections showed that the tdT$^+$ VE-cad$^+$ Runx1$^+$ endothelial cells were detected in yolk sac but not in OFT/heart region. (Fig. 4, B and C). The absence of the hemogenic endothelial marker Runx1 that regulates activation of hematopoietic gene expression in endocardium suggested that it was less likely for the endocardium to have hematopoietic activity. We found tdT$^+$ Runx1$^+$ cells only in the vascular lumen and heart chambers, and immunostaining data revealed that they are circulating hematopoietic cells expressing CD45, suggesting that these committed blood progenitor cells may have recently been generated and circulated from other Runx1$^+$ hemogenic endothelium through blood circulation (Fig. 4, D and E). Collectively, these data suggested that the Nfatc1-labeled endocardium does not have hemogenic potential. We have added these data in the revised manuscript. For details, please refer to Page 10, Paragraph 2, Line 27 of the revised manuscript.

The authors next demonstrate that specific labeling of endocardium with either Mef2c-Cre or Npr3-CrER, failed to lineage trace cardiac macrophages. In addition, labeling of epicardium with Wt1-CreER failed to label cardiac macrophages. Taken together, these data supprt the novel paradigm that the developing heart does not generate its own macrophages. This is a highly significant finding.

We thank the reviewer for appreciating our work.

In a final set of experiments a new Cdh5-CreER mouse model is used to determine if cardiac tissue-resident macrophages are derived from the yolk sac or "AGM". TAM treatment at E7.5 labeled endothelium, microglia, and the large majority of cardiac macrophages. In contrast, treatment at E10.5 labeled a minority of cardiac macrophages and no microglia. While these results are interpreted as cardiac macrophages being derived from "primitive" hematopoiesis in the yolk sac and "definitive" hematopoiesis in the AGM, concerns are raised over this interpretation. While it is

well accepted that microglia are derived from "primitive" macrophages (also referred in the Introduction as "early" EMP), it is not clear that the new Cdh5-CreER mouse model induced at E7.5 might not also begin to label the "transient" definitive wave (so-called "late" EMP) as well. In addition, it is very likely that TAM treatment at E10.5 would also label the "transient" definitive wave of hematopoietic progenitors that emerge in the yolk sac from hemogenic endothelium, as well as HSCs emerging in the aorta. If the "transient" definitive wave were being lineage traced with TAM at either E7.5 or E10.5, then definitive erythroid cells in the E12.5 fetal liver would also be labeled, though microglia would not be labeled.

We thank the reviewer for raising this important point to improve our work. To examine whether 4-hydroxytamoxifen (4OHT) treatment at E7.5 or E10.5 could label the “transient” definitive wave, we collected E12.5 embryos for analysis. Immunostaining for F4/80 and tdTomato of E7.5 treated tissues showed that the macrophages of the yolk sac and the microglia of the brain were almost all labeled by tdTomato, indicating that the “primitive” hematopoiesis was efficiently tracked (Fig. S5, A and B). As suggested by the reviewer, we found the majority of Ter-119^{+} erythroid cells of the fetal liver were also labeled by tdTomato, indicated that 4OHT induction at E7.5 could label “transient” definitive wave (Fig. S5, C and D). Immunostaining of E10.5 treated tissues showed that neither macrophages of the yolk sac nor the microglia of the brain were labeled by tdTomato (Fig. S5, E and F). In fact, it has been recently reported that HSCs do not contribute significantly to embryonic erythropoiesis and most red blood cells were derived from the primitive or transient definitive wave (Soares-da-Silva et al., JEM, 2021, 218:e20201729). Indeed, we also did not detect any Ter-119^{+} tdTomato^{+} definitive erythroid cell in E12.5 fetal liver, indicating that 4OHT treatment at E10.5 could no longer track primitive or “transient” definitive hematopoiesis in our mouse model (Fig. S5, G and H). These data suggested that induction of 4OHT at E7.5 could track YS region-derived primitive hematopoiesis and transient definitive hematopoiesis, and induction at E10.5 could track AGM/HSC definitive hematopoiesis. We have added these data in the revised manuscript. For details, please refer to Page 15, Line 10 and Line 21 of the revised manuscript.

To more clearly label hemogenic endothelium in the aorta (AGM), mouse models such as Hlf or Mecom, as recently published, and thought to be specific for HSCs, would need to be used. As it stands, the data can be interpreted as cardiac macrophages being derived from "primitive" and from "definitive" hematopoiesis, if the latter is meant to also refer to "transient" definitive and "AGM/HSC" definitive hematopoiesis. In that case, the terminology in the Introduction ("early" vs. "late" EMP) would need to be modified to match the terminology of the Discussion.

We thank the reviewer for raising this suggestion and we agree with the reviewer on this point. The Hlf and Mecom are good candidate genes for targeting HSCs, but considering that it would take a lot of time and effort for generating a new mouse tool or transporting mice from overseas laboratories under the COVID-19 pandemic restrictions, these genetic mouse lines are not available to us. We used *Cdh5-2A-CreER* model for more careful study in the revision. We have shown that 4OHT treatment at E7.5 could label primitive hematopoiesis and transient definitive hematopoiesis, and treatment at E10.5 could clearly label AGM/HSC definitive hematopoiesis. We have modified the terminology of Introduction to match that of the Discussion in the revised manuscript. For details, please refer to Page 3, Line 20-24 of Introduction Section and Page 16, Line 10 of Discussion Section of revised manuscript.

Minor

1.Page 3, lines 25-26: HSCs do not begin to colonize the fetal liver until E11.5-E12.5.

We thank the reviewer for spotting this mistake. We have revised this sentence accordingly. For details, please refer to Page 3, Line 28 of the revised manuscript.

2.Page 8, line 4: "exam" should be "examine".

We thank the reviewer for carefully reading our manuscript. We have revised this accordingly. For details, please refer to Page 8, Line 8 of the revised manuscript.

3.Page 12, line 15: what does "minimally" mean here?

We thank the reviewer for spotting this error. We have revised this sentence accordingly. For details, please refer to Page 12, Line 24 of the revised manuscript.

4.Page 14, line 28: "wildly" probably should be "widely".

We thank the reviewer for spotting this mistake. We have revised this accordingly. For details, please refer to Page 16, Line 12 of the revised manuscript.

April 7, 2022

RE: JCB Manuscript #202108093R

Prof. Bin Zhou
State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, University of Chinese Academy of Sciences
320 Yueyang road, Life Science Research Building A-2112
Shanghai 200031
China

Dear Prof. Zhou:

Thank you for submitting your revised manuscript entitled "Cellular origin of tissue resident macrophages in the developing heart". The reviewers have now assessed your revised manuscript and are satisfied with revisions, thus we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully. Please go through all the formatting points paying special attention to those marked with asterisks.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures. Please note that main text figures should be provided as individual, editable files.

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

*** Scale bars must be present on all microscopy images, including inset magnifications. Please include scale bars in main Figs 1E (inset magnification), 2B (inset magnification), 2K (inset magnification), 2V (inset magnification), 6L-M (inset magnifications), 9G (inset magnification), 9K (inset magnification) and supplemental Figs 2B-D (inset magnification), 3B (inset magnification), 4B (inset magnification).

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please ensure that the particular red and green hues used in micrographs in main Figs 1E, 1G, 2G, 2I, 2N, 2O, 2S-T, 2X, 3H, 4B-E, 6C, 6F, 6I, 7C-D, 7F-G, 7J-K, 8C, 9C, 9E, 9I and supplemental Figs 5B, 5F are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

4) Statistical analysis:
Error bars on graphic representations of numerical data must be clearly described in the figure legend.

The number of independent data points (n) represented in a graph must be indicated in the legend.

Statistical methods should be explained in full in the materials and methods.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

*** Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or

two-sided, etc.). Please indicate in figure legends the statistical tests used in your experiments where appropriate.

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title:
The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

*** The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership. While your current title is concise and accessible to a general readership, we feel that sounds a bit like front matter, so we would suggest something along the lines of "Lineage tracing clarifies the cellular origin of tissue resident macrophages in the developing heart".

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods.

*** You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please include species for all of your antibodies.

8) Microscope image acquisition:
The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
*** f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials:
There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

*** Please note that supplemental figures and tables should be provided as individual, editable files.

A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

11) eTOC summary:
*** A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement:
JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial

interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts.

*** All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature is encouraged (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

15) Materials and data sharing: As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay.

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

16) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

If your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. Please let us know if any complication preventing you from meeting this deadline arises and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Ira Mellman, Ph.D.
Editor
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This reviewer finds the revised version of the manuscript much improved. I am happy with the additional experiments performed by the authors to address my previous comments and I understand that some of the experiments suggested could not be done in a limited time frame. Nevertheless, the manuscript is much stronger now and it nicely complements/challenges the current literature on macrophages in the developing heart.

Reviewer #2 (Comments to the Authors (Required)):

The authors have added significant new data in response to the critiques of the reviewers. Importantly, new lineage labeling experiments have been added, as well as studies looking for Runx1+ cells in various endothelial beds. The added data supports the notion, not only that cardiac endothelium isn't hemogenic, but that cardiac macrophages are derived both from yolk sac progenitors as well as HSCs.

Response Letter to Reviewers

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We thank the reviewer for all the valuable comments and suggestions to advance our work.

Reviewer #2 (Comments to the Authors (Required)):

The authors have added significant new data in response to the critiques of the reviewers. Importantly, new lineage labeling experiments have been added, as well as studies looking for Runx1+ cells in various endothelial beds. The added data supports the notion, not only that cardiac endothelium isn't hemogenic, but that cardiac macrophages are derived both from yolk sac progenitors as well as HSCs.
We thank the reviewer for all valuable comments and constructive suggestions.