MyD88-dependent TLR signaling oppositely regulates hematopoietic progenitor and stem cell formation in the embryo

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Original submission

First decision letter

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MS TITLE: MyD88-dependent TLR signaling oppositely regulates hematopoietic progenitor and stem cell formation in the embryo

AUTHORS: Nancy A Speck, Laura F Bennett, Melanie D Mumau, and Yan Li

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This study by Bennett and collaborators explores the role of MyD88 during embryonic blood progenitors emergence in the AGM. Overall, this is a well written manuscript reporting interesting and novel findings on the role of MyD88 - Toll like signalling in the composition of progenitors within the intra-aortic clusters generated in the aorta upon endothelial to hematopoietic transition.

Comments for the author

While this is a well conducted study, several aspects of the data presented need to be further explained and investigated.

Specific comments:
RUNX1 positivity is taken as a measure of HE numbers at E9.5 and E10.5. However, not all RUNX1+ endothelial cells are HE and will undergo EHT. The number of HE should be determined functionally by assessing the frequency of RUNX1+ endothelial cell undergoing EHT via single cell depositing or limiting dilution analysis.
The authors claim that “Inefficient EHT is unlikely as it would have resulted in an accumulation of HE cells”. This is very speculative as inefficient EHT could result in alternative fate such as HE becoming endothelial cells or undergoing apoptosis.

The timeline of EHT might be altered in Tlr4-/- embryos, what is the number of IAHC cells at later stages of development?
The authors claim that “MyD88-dependent TLR signaling increases the generation of LMPs at the expense of long-term multilineage-repopulating HSCs.” and that “MyD88 dependent TLR signaling regulates the balance between stem cells and progenitors with different potentials”. There are no evidences that LMPs are generated at the expense of HSCs or that there is a balance between progenitors and HSCs generation. The two observations could be totally independent. LMPs and HSCs might be generated from different HE subsets and independently regulated by TLR signalling.

What does % CD62E+ means (Fig 3H)? Is this the frequency of cells or expression level? Flow data should be shown.
Is TLR4 expressed by endothelial cells as measured by qRT-PCR or immuno-fluorescent staining? Is the lack of detection by flow cytometry a technical issue?
Figure 2B: it really looks like loss of Ticam1 increase the frequency of IAHC cells /mm. It is surprising that this doesn’t reach statistical significance.

Reviewer 2

Advance summary and potential significance to field

It has been known that IAHCs have significant heterogeneity in hematopoietic potential. However, it has not been reported whether there are any genes that can play different or even opposite functions for the ontogeny of these populations in embryonic aorta. Interestingly, this study gives such an example, which reveals the opposite role of MyD88-dependent TLR signaling in the development of intra-embryonic HSC and LMP through careful and reliable phenotype analysis.

Comments for the author

A few issues are expected to be addressed in the revised version.
1. Whether Tlr4 deficiency affect the number of E10.5 AGM-pre-HSC, which may be analyzed by more surface markers, such as CD41 and EPCR, as well as that of E12.5 FL-HSC?
2. Fig2 E: In E10.5 WT AGM, the average number of LMPs per ee reached 1500, far exceeding the number of IAHCs. How to explain the data?
3. P10: Why purify endothelial cells with the phenotype of CD31hiVEC+ESAM+CD44-CD45-Ter119-Mac1-CD41-? CD44 has recently been shown as the marker of arterial ECs (Oatley, Nat Commun. 2020; Hou, Cell Res. 2020).

4. In the discussion of P11: the description that RUNX1 levels are normal in mutant embryos at E9.5, is inconsistent with the data shown in the Fig1 G.

First revision

Author response to reviewers' comments

Reviewer 1
Specific comments:

1. RUNX1 positivity is taken as a measure of HE numbers at E9.5 and E10.5. However, not all RUNX1+ endothelial cells are HE and will undergo EHT. The number of HE should be determined functionally by assessing the frequency of RUNX1+ endothelial cell undergoing EHT via single cell depositing or limiting dilution analysis.

We agree with Reviewer 1 that quantifying HE cells by confocal microscopy cannot substitute for a functional assay. We performed limiting dilution HE assays using CD44+ endothelial cells purified from E9.5 embryos. We found no difference in the frequencies of functional HE cells, confirming the phenotypic analysis. These data are presented in Figure 1F.

2. The authors claim that “Inefficient EHT is unlikely as it would have resulted in an accumulation of HE cells”. This is very speculative as inefficient EHT could result in alternative fate such as HE becoming endothelial cells or undergoing apoptosis.

We thank the reviewer for this comment and have changed the wording of our statement to the following:

“The decreased number of IAHC cells at E10.5 could be caused by inefficient EHT, decreased proliferation, or increased apoptosis. We observed no decrease in specified HE cells at E9.5 nor accumulation of HE cells at E10.5, suggesting that the decrease in IAHC cells at E10.5 is not due to inefficient EHT, although we cannot rule this out since inefficient EHT might cause HE cells to lose RUNX1 expression, assume an alternative cell fate, or undergo apoptosis.”

3. The timeline of EHT might be altered in Tlr4-/- embryos, what is the number of IAHC cells at later stages of development?

We enumerated the number of IAHCs in wild type and TLR4 mutant embryos at E9.5 and E11.5. These new data are in Figure 1I.

4. The authors claim that “MyD88-dependent TLR signaling increases the generation of LMPs at the expense of long-term multilineage-repopulating HSCs.” and that “MyD88 dependent TLR signaling regulates the balance between stem cells and progenitors with different potentials”. There are no evidences that LMPs are generated at the expense of HSCs or that there is a balance between progenitors and HSCs generation. The two observations could be totally independent. LMPs and HSCs might be generated from different HE subsets and independently regulated by TLR signalling.

The reviewer raised a valid point. We changed the language throughout the text by removing the word “balance” and simply noted that we observe fewer LMPs and increased HSCs without tying those fates together. We also added information from the recent publication of Dignum et al. showing committed progenitors and HSCs arise from distinct subsets of HE. We added text to discuss the possibility of TLR signaling differentially impacting distinct HSPC subsets.

5. What does % CD62E+ means (Fig 3H)? Is this the frequency of cells or expression level? Flow data should be shown.

We added flow data showing the sorting strategy and a representative histogram of the percentage of CD62E+ endothelial cells following stimulation with LPS to Supplementary Figure 3. The text and

axis label in Fig. 3H (now Fig. 3K) have been updated to reflect that % of CD62E+ represents the percentage of cells that are CD62E+ and not the amount of CD62E on each cell.

6. Is TLR4 expressed by endothelial cells as measured by qRT-PCR or immuno-fluorescent staining? Is the lack of detection by flow cytometry a technical issue?

TLR4 expression is very low on embryonic endothelial cells as previously determined by RNA-seq (Li et al. Genes Dev. 28, 2597, 2014; Gao et al. Genes Dev. 34, 950, 2020) and cell surface TLR4 was difficult to detect by flow (see the formatted PDF for figures). TLR4 on macrophages, on the other hand, was easy to detect in the same experiments. A search of the literature shows that many types of endothelial cells express TLR4. Our results demonstrating the dependence of TLR signaling on the MyD88 and not TRIF signaling adaptors also support a role for cell surface TLR4 signaling, despite the observation that the levels of the receptor are low on the cell surface.

7. Figure 2B: it really looks like loss of Ticam1 increase the frequency of IAHC cells /mm. It is surprising that this doesn't reach statistical significance.

We thank the reviewer for pointing this out. This was an oversight on our part. It is indeed significant, and the text and figure have been updated accordingly.

Reviewer 2

Specific Comments:

1. Whether Tlr4 deficiency affect the number of E10.5 AGM-pre-HSC, which may be analyzed by more surface markers, such as CD41 and EPCR, as well as that of E12.5 FL-HSC?

We analyzed both AGM pre-HSCs and E12.5 and E13.5 FL HSCs as suggested by the reviewer, and those data have been included in the manuscript in Fig. 2E, F. We analyzed type I and type II pre-HSCs at E11.0 (39-44 somite pairs), when pre-HSCs are more abundant using CD31, Kit, CD41, and CD45 using a previously described strategy (Zhou et al. Nature, 2016). Very few cells in the type I or type II populations could be classified as EPCRhi (< 5 cells), and we therefore restricted our classification to strictly type I and type II using CD41 and CD45 to allow for more robust and consistent analysis. Loss of TLR4 did not significantly impact the phenotypic populations of either pre-HSCs in the AGM region or long-term repopulating HSCs in the fetal liver.

2. Fig2 E: In E10.5 WT AGM, the average number of LMPs per ee reached 1500, far exceeding the number of IAHCs. How to explain the data?

The number of IAHC cells shown in Fig. 1E (now Fig. 1G) at E10.5 represents the number of IAHC cells counted specifically within a region spanning 5-6 somites of the dorsal aorta centered around its connection with the vitelline artery, and represented as IAHC cells per mm. The LMP assay, on the other hand was performed using the entire AGM region plus the umbilical and vitelline arteries and the data displayed as LMPs per embryo. We realized this was not clear in the text and figure legends and changed the text accordingly.

3. P10: Why purify endothelial cells with the phenotype of CD31hiVEC+ESAM+CD44-CD45-Ter119-Mac1-CD41-? CD44 has recently been shown as the marker of arterial ECs (Oatley, Nat Commun. 2020; Hou, Cell Res. 2020).

We agree that purification and subsequent stimulation of CD44+ arterial endothelial cells would have been ideal. However, we encountered several challenges with that approach which led us to use only the CD44- fraction of endothelial cells. Initially, we sorted CD44- and CD44+ fractions of primary endothelial cells. We were able to isolate very few CD44+ ECs (fewer than 4K, generally speaking from multiple pooled litters) and this made splitting the population for stimulation and subsequent flow cytometry for CD62E unfeasible. Another hurdle was that we were simultaneously examining basal levels of CD62E in wild type embryos and observed an increased frequency of CD62E+ cells in the CD44+ fraction compared with the CD44- fraction (almost 80% of CD44+ cells are CD62E+ compared with 50% of CD44-) which made us question whether stimulation with LPS would

have any effect on this population that, for the most part, already expressed the marker. Both factors led us to examine the CD44- fraction for our stimulation of endothelial cells with LPS. Given that CD44+ cells comprise less than 10% of the total endothelial cell population (CD31+VEC+ESAM+CD41lo/-Ter119-CD45-Mac1-), we concluded that including these cells in subsequent experiments would not change the results.

4. In the discussion of P11: the description that RUNX1 levels are normal in mutant embryos at E9.5, is inconsistent with the data shown in the Fig1 G.

We thank the reviewer for bringing this to our attention and have changed the text on page 11 to reflect accurately what is shown in Fig 1G (now Fig. 1E).

“The numbers of phenotypic RUNX1+ HE cells were equivalent in E9.5 Tlr4+/+ and Tlr4-/- embryos (Fig. 1D), and the levels of RUNX1 in Tlr4-/- HE cells were slightly increased (Fig. 1E).”

We again thank the reviewers for their very thoughtful comments.

Second decision letter

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AUTHORS: Nancy A Speck, Laura F Bennett, Melanie D Mumau, and Yan Li
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

NA

Comments for the author

The authors have addressed all my comments satisfactorily.

Reviewer 2

Advance summary and potential significance to field

This study provided solid phenotypical evidence showing opposite regulation on emergence of multi-potent HPCs and HSCs from hemogenic endothelial precursors by the same signaling pathway.

Comments for the author

All the concerns have been fully addressed.