Derivation of extra-embryonic and intra-embryonic macrophage lineages from human pluripotent stem cells

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MS TITLE: Derivation of Extraembryonic and Intraembryonic Macrophage Lineages from Human Pluripotent Stem Cells

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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. At this point, I would suggest that the experiments requesting co-culture with neurons to evaluate differentiation to microglia, although interesting, could be considered being beyond the scope of this manuscript, and not required.

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

The paper by Bredemeyer et al., describes derivation of macrophages from human PSCs in the presence of WNT inhibitor IWP2, or WNT agonist CHIR99021 + TGFb inhibitor SB431452. These two waves of hematopoiesis were defined as WNT-independent (WNTi) and WNT-dependent (WNTd). Using scRNAseq, the authors revealed that these two culture conditions produced macrophages which specify through distinct developmental pathways and consequently acquire a distinct transcriptional signature. In WNTd cultures, macrophages develop through intermediates with a CD14-CD123+CD45R+ CMP phenotype, while attempts to detect progenitors with a similar phenotype in WNTi macrophage cultures failed.
However, scRNA analysis of WNTi cultures revealed two macrophage progenitors with a CD14-CXCR4+CLEC1B- or CD14-CXCR4+CLEC1B+ phenotype. Overall, these studies are well executed and make a significant novel contribution to our understanding of macrophage development from human PSCs.

Comments for the author

Major Comments:
1. As shown by scRNA analysis (Fig. 3C), more than 70% of cells in WNTi macrophage differentiation culture are red blood cells and megakaryocytes. In contrast, WNTd cultures are composed of mostly myeloid progenitors. Thus, a correct comparison of CD45RA expression in CD14-CD64- population in WNTi and WNTd cultures should exclude erythroid and megakaryocytic cells and/or include gated CD34+ population when analyzing WNTi cultures.
2. Based on scRNA analysis, authors speculate that progenitor 1 and 2 may possess granulocytic and megakaryocytic potentials. It would be important to assess granulocytic and megakaryocytic potential of these progenitors to prove this suggestion.
3. Are there any differences in the yield of macrophages from WNTi and WNTd cultures? It seems that WNTi macrophage cultures contains very few proliferating cells.
4. Yolk sac primitive macrophages develops in a c-Myb-independent manner and shows higher expression of Maf and Mafb. Are the any differences in c-Myb, Maf and Mafb expression in WNTd and WNTi macrophages?
5. Fig.1B shows distinct CCR2 expression in WNTi and WNTd macrophage populations. What is the distribution of CCR2 in UMAP plots? Is there any overlap between CCR2- macrophage populations from WNTi and WNTd cultures?
6. Fig. 1D and Fig.4I shows a single experiment. These findings should be confirmed using independent biological replicates.
7. Authors should be careful equating WNTd hematopoiesis with intraembryonic hematopoiesis without showing expression of other critical markers of intraembryonic hematopoiesis such as HLF, SPINK2, MECOM and MLLT3. In addition intraembryonic hematopoiesis originates mostly from subset of arterial endothelial cells. However, it is unclear whether specification of WNTd hematopoiesis is associated with arterialization of endothelium in the described differentiation system.
8. Since in addition to regulation of Wnt signaling, GSK3b is involved in NOTCH phosphorylation and modulation of NOTCH signaling, attributing CHIR effect solely to WNT signaling modulation is questionable.

Minor:
1. Prior studies have demonstrated that primitive macrophages display decreased mitochondrial respiratory and glycolytic activity compared to fetal liver monocytes DOI: 10.15252/embj.2019103205. Those findings are in contrast with this manuscript, which reports a higher respiratory capacity in WNTi macrophages. Any explanation for these contradictory findings?

2. Reference regarding selective expression of CCR2 in intraembryonic macrophages but not primitive macrophages should be added on page 4.

Reviewer 2

Advance summary and potential significance to field

Bredemeyer et al. characterize two distinct developmental pathways from iPSCs to macrophages via manipulation of WNT signalling. Using scRNA-seq approaches, they describe the developmental trajectories of these developing and mature iPSC cultures.

The study is timely and very interesting for the developmental immunology community since the debate of macrophage ontogeny and its potential for the cell's function is an ongoing debate. The presented work may serve as a basis to study the distinct human hematopoietic waves and the impact on macrophage functions in the future.

Comments for the author

The study would greatly profit from and improve by comparisons with already published data sets describing human ex vivo immune/progenitor cells. Several studies, including fetal liver and yolk sac progenitors (e.g. PMID: 32499656 PMID: 31597962), provide a basis to extend the here presented single-cell data set, and a detailed bioinformatic analysis would allow to draw more concrete conclusions on which hematopoietic waves are present within the WNTi and WNTd cell culture conditions.

Further points that should be addressed:
1. Studies in mice show that most tissue-resident macrophages arise from erythro-myeloid progenitors (EMP) in the yolk sac (PMID: 25470051). While the early wave of macrophages differentiates mainly from so-called pre-macrophages (pMacs, PMID: 27492475), EMP can also give rise to monocytes that in turn could differentiate to macrophages (PMID: 25902481). Thus, monocyte-derived and yolk-sac derived macrophages could – in theory – be the same thing since EMP move from the yolk sac to the fetal liver, where they constantly give rise to immune cells. Additionally, HSCs in the fetal liver will give rise to monocytes; however, these are unlikely the source for fetal macrophages. The literature about macrophage ontogeny is somewhat controversial. Nevertheless, the authors should extend their introduction, describe the three hematopoietic waves in more detail and refer to original papers and not reviews. Finally, the authors should place their work in the context of this debate since it is of high interest to the community to understand whether macrophage differentiation in mice and human are comparable.
2. Heterogeneity of macrophages should be confirmed on protein level to some extent (e.g. TREM2/LYVE1 should work well in flow cytometry).
3. From mouse work, one would expect that WNTi macrophage progenitors would be Cxcr4- or that the authors stain rather a multipotent mesoderm-like population in their culture since Cxcr4 distinguishes the early yolk sac hematopoietic wave from the definitive wave that gives rise to HSCs (PMID: 32042176). Could the authors superimpose EMP, pMac, fetal (pre-)HSCs to characterize the two progenitors better? Along these lines, both progenitors give rise to macrophages.
But are these macrophages different in morphology or function? What are the other CD14- cells that differentiate from these progenitors? This could give a hint on which kind of progenitors these are. Of note, Cxcr4 is quickly internalized leading to an inefficient surface staining when performed on ice. Staining at 37°C may increase the efficient cell sorting of these progenitors.
4. Consider moving some information from the supplemental files to the main figures and the other way around (e.g. putting the histograms/dot plots in figure 1A/B to supplement and instead represent MFI to the main figure). The scheme describing the approach to produce the cultures could be in the main figures as well. Additionally, a ratio of cell input/macrophage output would be helpful for the reader to understand the in vitro system immediately without the need to go through already published work.
5. WNTi and WNTd cultures do not generate two distinct types of macrophages.
Which cells were taken for the Seahorse/ROS/LPS assays? Since the resulting cell types are quite heterogeneous, the differences in mitochondrial/immune response activity could result from different cell types present in these mixed cultures.

Reviewer 3

Advance summary and potential significance to field

Bredemeyer et al. established a cell culture system based on hPSCs that enables the generation of macrophages derived from extra-embryonic-like (WNT-independent) and intra-embryonic-like (WNT-dependent) hematopoietic progenitors. These distinct lineages share common characteristics with tissue-resident macrophages or monocyte-derived macrophages and the distinct populations were characterized in terms of morphology, transcriptional profile and functional properties. The authors provide a comprehensive scRNA sequencing analysis demonstrating the differences between the two generated macrophage populations and their developmental trajectories. This very well characterized cell culture system is of great interest and very valuable for future studies on human macrophages.
That said, this referee very much hopes that the comments below will be helpful to improve the clarity of the manuscript:

Comments for the author

The authors demonstrate the macrophage character of the generated cells by using typical markers like MerTK and CD14 in FACS, by electron microscopy showing the macrophage lysosomes and phagosomes as well as by the phagocytosis assay using pHrodo E.coli BioParticles. The scRNA analysis should also reflect the macrophage phenotype. This reviewer additionally would like to see feature plots (not z-score) depicting expression of common macrophage genes like CD68, CD163 or CD206 and also the genes already shown as z-score feature plots e.g. CD36, TREM2, LYZ.
The scRNA sequencing (Figure 2) showed that the two macrophages derived from extra- or intra-embryonic-like progenitors are transcriptionally different. A very prominent cluster of proliferating cells consists of cells of both of the populations. Would the cells redistribute into the other clusters after regression of cell cycle related genes before clustering or will they form again a separate cluster representing remaining progenitor cells?
For analysis of the progenitor cells, the cell cycle regression was performed. This referee would wish to see feature plots of cell cycle genes e.g. MKI67 and Top2A of Figure 4 A and D.
The authors used different stimuli like LPS, SOZ and PMA to investigate the functional role of the distinct macrophage populations. Phagocytosis was demonstrated using pHrodo-E.coli BioParticles. This referee would wish to see if the phagocytic capacity differs between the two populations. This can be analysed by FACS showing how many Particles were taken up (mfi). In line with that, it would be very interesting to see if there is a specialization regarding the type of particle that has to be phagocytosed (phagocytosis of labelled necrotic cell, phagocytosis of labelled apoptotic cells) - as CD36 (expressed mainly on WNTi cells) is involved in the uptake of apoptotic cells.
Takata et al 2017 demonstrated that iPSC derived macrophages acquire a microglia-like phenotype after coculture with neurons. It would be very interesting to see if WNTi and WNTd macrophages are equally able to acquire a microglia phenotype in coculture with Neurons.
In Figure 2D it is written: “Genes used to calculate the z-score are indicated below the feature plots.” Gene names are on the right.
The authors state:” Unlike what we observed after 14 days of culture, while the two cultures had clearly distinct clusters, there was also a significant observable overlap between the two differentiation cultures (Fig. 3E).” - This referee can not agree on that statement. Indeed, the clusters are not very much separated from each other. But they are also not really overlapping with the exception of eosinophils and maybe megakaryocytes.

First revision

Author response to reviewers' comments

Major changes to the manuscript have been highlighted in blue.

Reviewer 1 Advance Summary and Potential Significance to Field:
The paper by Bredemeyer et al., describes derivation of macrophages from human PSCs in the presence of WNT inhibitor IWP2, or WNT agonist CHIR99021 + TGFb inhibitor SB431452. These two waves of hematopoiesis were defined as WNT-independent (WNTi) and WNT-dependent (WNTd). Using scRNAseq, the authors revealed that these two culture conditions produced macrophages which specify through distinct developmental pathways and consequently acquire a distinct transcriptional signature. In WNTd cultures, macrophages develop through intermediates with a CD14-CD123+CD45R+ CMP phenotype, while attempts to detect progenitors with a similar phenotype in WNTi macrophage cultures failed. However, scRNA analysis of WNTi cultures revealed two macrophage progenitors with a CD14 CXCR4+CLEC1B- or CD14-CXCR4+CLEC1B+ phenotype. Overall, these studies are well executed and make a significant novel contribution to our understanding of macrophage development from human PSCs.

Thank you for your positive comments. They are greatly appreciated.

Reviewer 1 Comments for the Author:

1. As shown by scRNA analysis (Fig. 3C), more than 70% of cells in WNTi macrophage differentiation culture are red blood cells and megakaryocytes. In contrast, WNTd cultures are composed of mostly myeloid progenitors. Thus, a correct comparison of CD45RA expression in CD14-CD64- population in WNTi and WNTd cultures should exclude erythroid and megakaryocytic cells and/or include gated CD34+ population when analyzing WNTi cultures.

We have repeated this analysis including antibodies specific for erythroid (CD235A) and megakaryocyte (CLEC1B) populations, and the results are now shown in Fig. S5A. Excluding the CD235A+ and CLEC1B+ cell populations from the IL3RA and CD45RA surface expression analysis did not substantially alter the percentages of cells positive for CD45RA.

2. Based on scRNA analysis, authors speculate that progenitor 1 and 2 may possess granulocytic and megakaryocytic potentials. It would be important to assess granulocytic and megakaryocytic potential of these progenitors to prove this suggestion.

We have performed this experiment and found that granulocyte potential is indeed restricted to progenitor 2. Both progenitor 1 and 2 have megakaryocyte potential, although progenitor 1 appears to have higher efficiency of generating megakaryocytic cells. These results are now shown in Fig. S8 E-H.

3. Are there any differences in the yield of macrophages from WNTi and WNTd cultures? It seems that WNTi macrophage cultures contains very few proliferating cells.

The number of macrophages generated from the WNTd and WNTi pathways have been added to the text (page 4, paragraph 2). Overall, both cultures yield around 1-2x10^6 macrophages per differentiation (starting with 5x10^6 hPSCs).

4. Yolk sac primitive macrophages develop in a c-Myb-independent manner and shows higher expression of Maf and Mafb. Are there any differences in c-Myb, Maf and Mafb expression in WNTd and WNTi macrophages?

Violin plots for MAF and MAFB expression in the mature macrophages have been added to Fig. S2E. MAF expression is slightly higher in the WNTi macrophages than the WNTd macrophages (log_2FC = 0.3), but below our cutoff of log_2FC = 0.5 for differentially expressed genes. We did not see a significant difference in the expression of MAFB. A feature plot of MYB expression in the WNTi progenitor culture has been added to Figure 4. Myb is not expressed in macrophages. Myb is

expressed in progenitor 1 and progenitor 2 from the WNTi cultures. This expression level is similar to the level in WNTd progenitors (shown below).

5. Fig.1B shows distinct CCR2 expression in WNTi and WNTd macrophage populations. What is the distribution of CCR2 in UMAP plots? Is there any overlap between CCR2- macrophage populations from WNTi and WNTd cultures?

A density plot of CCR2 expression has been added to Fig. S2. CCR2 is found only in the WNTd culture, with highest expression in the monocyte cluster. The WNTi and WNTd CCR2- macrophages do not have substantial overlap, except in the proliferating cell cluster.

6. Fig. 1D and Fig.4I shows a single experiment. These findings should be confirmed using independent biological replicates.

These experiments are representative of at least 3 biological replicates and the figure legends have been edited to reflect this. The authors apologize for the oversight.

7. Authors should be careful equating WNTd hematopoiesis with intraembryonic hematopoiesis without showing expression of other critical markers of intraembryonic hematopoiesis such as HLF, SPINK2, MECOM and MLLT3. In addition, intraembryonic hematopoiesis originates mostly from subset of arterial endothelial cells. However, it is unclear whether specification of WNTd hematopoiesis is associated with arterialization of endothelium in the described differentiation system.

We have added feature plots of HLF, SPINK2, MECOM and MLLT3 expression in the WNTd and WNTi progenitors to Fig S7. The WNTd CD34+ progenitors express each of these genes. MECOM and MLLT3 are expressed in RBCs within the WNTi culture, while SPINK2 and HLF are absent from the WNTi cells. The arterial gene signature of hPSC-derived CD34+ cells with this WNT/ACTIVIN-patterned differentiation system has been previously described by others (Ng et al., Nature Biotechnology 2016). WNTd CD34+ progenitors recapitulate a HOXA+ arterial hemogenic endothelial population.

8. Since in addition to regulation of Wnt signaling, GSK3b is involved in NOTCH phosphorylation and modulation of NOTCH signaling, attributing CHIR effect solely to WNT signaling modulation is questionable.

Thank you for raising this point. We have revised the results section accordingly (page 4, paragraph 2).

Minor:

1. Prior studies have demonstrated that primitive macrophages display decreased mitochondrial respiratory and glycolytic activity compared to fetal liver monocytes DOI:10.15252/embj.2019103205. Those findings are in contrast with this manuscript, which reports a higher respiratory capacity in WNTi macrophages. Any explanation for these contradictory findings?

The WNTi macrophages in our study most resemble the yolk-sac-derived macrophages that come from the EMP, which gives rise to some fetal monocytes, rather than the primitive macrophages of the first wave of yolk sac hematopoiesis. Thus, our results concur with the high respiratory capacity found in the murine EMP and fetal monocytes. We have modified the discussion to clarify the relationship of the WNTi culture to the primitive and EMP waves of yolk sac hematopoiesis.

2. Reference regarding selective expression of CCR2 in intraembryonic macrophages but not primitive macrophages should be added on page 4.

We have added references for both human (DOI: 10.1038/s41591-018-0059-x) and mouse (DOI: 10.1016/j.immuni.2013.11.019).

Reviewer 2 Advance Summary and Potential Significance to Field:
Bredemeyer et al. characterize two distinct developmental pathways from iPSCs to macrophages via manipulation of WNT signalling. Using scRNA-seq approaches, they describe the developmental trajectories of these developing and mature iPSC cultures.

The study is timely and very interesting for the developmental immunology community since the debate of macrophage ontogeny and its potential for the cell's function is an ongoing debate. The presented work may serve as a basis to study the distinct human hematopoietic waves and the impact on macrophage functions in the future.

Thank you for your positive comments. They are greatly appreciated.

Reviewer 2 Comments for the Author:

The study would greatly profit from and improve by comparisons with already published data sets describing human ex vivo immune/progenitor cells. Several studies, including fetal liver and yolk sac progenitors (e.g. PMID: 32499656, PMID: 31597962), provide a basis to extend the here presented single-cell data set, and a detailed bioinformatic analysis would allow to draw more concrete conclusions on which hematopoietic waves are present within the WNTi and WNTd cell culture conditions.

We informatically compared our data to the fetal liver and yolk sac datasets found in PMID: 31597962. By reference mapping, we compared the MPPs from the yolk sac and the HSC/MPPs from the fetal liver to our WNTd and WNTi progenitor cultures. We found high correlation between the WNTd CD34+ progenitors and the fetal liver HSCs/MPPs. In contrast, the human yolk sac MPP mapped poorly to the WNTd CD34+ cells. Mapping the same populations onto our WNTi dataset showed no correlation between fetal liver HSCs/MPPs and any WNTi cluster. The yolk sac MPPs mapped to the endothelial clusters in both WNTd and WNTi, and weakly to WNTd GMPs. These comparisons are shown in Fig. S7E. We also show mapping of the WNTd and WNTi datasets to the integrated fetal liver and yolk sac dataset to confirm that our cell type annotations correlate well with the previously published data (Fig. S7A).

As no megakaryocyte or granulocyte clusters were identified in the human yolk sac dataset, and the fetal liver samples were all taken at time points after HSC formation, identification of populations analogous to progenitor 1 and progenitor 2 was challenging. To attempt to identify the wave of hematopoiesis represented by the WNTi culture, we mapped the murine EMP, pre-macrophage (pMac) and macrophage gene signatures from Mass, et al., 2016 (PMID: 27492475) onto the WNTi progenitors (Fig 4J). The gene lists used for this comparison are in Table S3. We also added feature plots for characteristic EMP genes MYB and KIT, showing that both progenitor 1 and progenitor 2 express MYB, while only progenitor 1 expresses KIT. This analysis indicates that progenitor 1 has an EMP/pMac gene expression profile. We speculate that progenitor 2 may be analogous to the EMP-derived progenitors that generate early fetal monocytes.

Further points that should be addressed:

1. Studies in mice show that most tissue-resident macrophages arise from erythro-myeloid progenitors (EMP) in the yolk sac (PMID: 25470051). While the early wave of macrophages differentiates mainly from so-called pre-macrophages (pMacs, PMID: 27492475), EMP can also give rise to monocytes that in turn could differentiate to macrophages (PMID: 25902481). Thus, monocyte-derived and yolk-sac derived macrophages could – in theory – be the same thing since EMP move from the yolk sac to the fetal liver, where they constantly give rise to immune cells. Additionally, HSCs in the fetal liver will give rise to monocytes; however, these are unlikely the source for fetal macrophages. The literature about macrophage ontogeny is somewhat controversial. Nevertheless, the authors should extend their introduction, describe the three hematopoietic waves in more detail and refer to original papers and not reviews. Finally, the authors should place their work in the context of this debate since it is of high interest to the community to understand whether macrophage differentiation in mice and human are comparable.

We thank the reviewer for the suggestion to extend and clarify our discussion of the waves of hematopoiesis and where our hPSC cultures fit in this context. We have added additional

information about the 3 waves of hematopoiesis (primitive, EMP, HSC) to the introduction (page 3, paragraph 3), and we have clarified the conclusion that the WNTi culture appears to be analogous to the EMP wave of yolk sac hematopoiesis in the discussion (page 10, paragraph 1). To support this conclusion, we have added a comparison of WNTi progenitors with murine EMP gene signatures to Fig. 4 (see above). While it is possible that WNTi progenitor 1 and progenitor 2 represent the early and late EMPs (described in PMID: 25902481), respectively, we do not have sufficient evidence to support this conclusion.

2.Heterogeneity of macrophages should be confirmed on protein level to some extent (e.g. TREM2/LYVE1 should work well in flow cytometry).

We have compared LYVE1 and TREM2 protein expression in WNTd and WNTi macrophages by confocal microscopy. These results confirmed our scRNAseq data and are included in Fig. S4.

3.From mouse work, one would expect that WNTi macrophage progenitors would be Cxcr4- or that the authors stain rather a multipotent mesoderm-like population in their culture since Cxcr4 distinguishes the early yolk sac hematopoietic wave from the definitive wave that gives rise to HSCs (PMID: 32042176). Could the authors superimpose EMP, pMac, fetal (pre-)HSCs to characterize the two progenitors better?

Along these lines, both progenitors give rise to macrophages. But are these macrophages different in morphology or function? What are the other CD14- cells that differentiate from these progenitors? This could give a hint on which kind of progenitors these are. Of note, Cxcr4 is quickly internalized, leading to an inefficient surface staining when performed on ice. Staining at 37°C may increase the efficient cell sorting of these progenitors.

It is possible that the mouse and human progenitors differ in expression of CXCR4, as both human progenitor analyses cited above show expression of CXCR4 in yolk-sac-derived myeloid progenitors. Alternatively, CXCR4 expression may be substantially higher in HSC-derived progenitors than in EMPs and other yolk-sac progenitors. We have superimposed the EMP, pMac and Mac gene signatures from Mass, et al. (2016) onto the WNTi progenitors in Fig 4J, and compared human fetal liver MPPs/HSCs to WNTd and WNTi progenitors (Fig. S7), as discussed above.

We have compared the morphology of the macrophages derived from the two WNTi progenitors and found that they are similar in size and appearance (Fig S8D). Unfortunately, performing functional assays on these sorted populations was not possible due to limited cell numbers. The CD14- cell types that can be generated by progenitor 1 and 2 are shown in Fig. S8. We find that progenitor 1 can give rise to mast cells and megakaryocytes, while progenitor 2 can additionally give rise granulocytes (CD32+CD15+CD16+ neutrophils).

4.Consider moving some information from the supplemental files to the main figures and the other way around (e.g. putting the histograms/dot plots in figure 1A/B to supplement and instead represent MFI to the main figure). The scheme describing the approach to produce the cultures could be in the main figures as well. Additionally, a ratio of cell input/macrophage output would be helpful for the reader to understand the in vitro system immediately without the need to go through already published work.

We have made the requested changes and added the cell input/output of macrophages to the results section text.

5.WNTi and WNTd cultures do not generate two distinct types of macrophages. Which cells were taken for the Seahorse/ROS/LPS assays? Since the resulting cell types are quite heterogeneous, the differences in mitochondrial/immune response activity could result from different cell types present in these mixed cultures.

The Seahorse, ROS, and LPS assays compare mature macrophages obtained from WNTi and WNTd cultures. For reference, these are the mature macrophage cultures shown in Figures 1 (flow cytometry) and Figure 2 (single cell RNA seq). >90% of cells in each culture were macrophages. The single cell RNA seq analysis indicates that WNTi and WNTd macrophages are transcriptionally

distinct. Our contention is that the WNTd and WNTi macrophages are functionally distinct and represent different macrophage subtypes or states. This conclusion is supported by the presented cell morphology, gene expression, flow cytometry, and functional data.

Reviewer 3 Advance Summary and Potential Significance to Field:
Bredemeyer et al. established a cell culture system based on hPSCs that enables the generation of macrophages derived from extra-embryonic-like (WNT-independent) and intra-embryonic-like (WNT-dependent) hematopoietic progenitors. These distinct lineages share common characteristics with tissue-resident macrophages or monocyte-derived macrophages and the distinct populations were characterized in terms of morphology, transcriptional profile and functional properties. The authors provide a comprehensive scRNA sequencing analysis demonstrating the differences between the two generated macrophage populations and their developmental trajectories. This very well characterized cell culture system is of great interest and very valuable for future studies on human macrophages. That said, this referee very much hopes that the comments below will be helpful to improve the clarity of the manuscript.

Thank you for your positive comments. They are greatly appreciated.

Reviewer 3 Comments for the Author:

The authors demonstrate the macrophage character of the generated cells by using typical markers like MerTK and CD14 in FACS, by electron microscopy showing the macrophage lysosomes and phagosomes as well as by the phagocytosis assay using pHrodo E.coli BioParticles. The scRNA analysis should also reflect the macrophage phenotype. This reviewer additionally would like to see feature plots (not z-score) depicting expression of common macrophage genes like CD68, CD163 or CD206 and also the genes already shown as z-score feature plots e.g. CD36, TREM2, LYZ.

We have added feature plots for CD68, CD163 and MRC1 to Fig. S2, demonstrating broad expression across the macrophage clusters. Violin plots for the genes comprising the WNTd and WNTi signatures (CD36, TREM2, LYVE1, etc.) are provided in Figure S3, which we feel allows for easier comparison of expression level than the individual feature plots. The fold change values of the DEGs for each macrophage state cluster are in Supplemental Table 1.

The scRNA sequencing (Figure 2) showed that the two macrophages derived from extra- or intra-embryonic-like progenitors are transcriptionally different. A very prominent cluster of proliferating cells consists of cells of both of the populations. Would the cells redistribute into the other clusters after regression of cell cycle related genes before clustering or will they form again a separate cluster representing remaining progenitor cells? For analysis of the progenitor cells, the cell cycle regression was performed. This referee would wish to see feature plots of cell cycle genes e.g. MKI67 and Top2A of Figure 4 A and D.

The proliferating cells in Figure 2 are macrophages, as evidenced by their expression of C1Q genes, CD68, CD163 and MRC1 (Fig. 2 and Fig. S2). They also express the characteristic genes for the sample (WNTi or WNTd) that they belong to (Fig. 2D). The cell cycle regression for this dataset does not fully redistribute the proliferating macrophages back to their respective WNTi and WNTd clusters. This appears to be due to incomplete removal of the cell cycle signature that is strongly expressed in a small number of cells (Fig. S2B). For this reason, we have chosen to present this dataset without cell cycle regression.

The progenitor datasets in Fig 3 are made up of transcriptionally diverse cell types and large numbers of proliferating cells are present. In this scenario, the cell cycle regression was effective at redistributing proliferating cells into their appropriate clusters. MKI67 and TOP2A feature plots for the progenitor datasets are shown in Fig. S7B.

The authors used different stimuli like LPS, SOZ and PMA to investigate the functional role of the distinct macrophage populations. Phagocytosis was demonstrated using pHrodo-E.coli BioParticles. This referee would wish to see if the phagocytic capacity differs between the two populations. This can be analysed by FACS showing how many Particles were taken up (mfi). In line with that, it would be very interesting to see if there is a specialization regarding the type of particle that has

to be phagocytosed (phagocytosis of labelled necrotic cell, phagocytosis of labelled apoptotic cells) - as CD36 (expressed mainly on WNTi cells) is involved in the uptake of apoptotic cells.

We thank the reviewer for the helpful suggestion to analyze different particles for phagocytosis. Flow cytometry of WNTd and WNTi macrophages that have phagocytosed pHrodo Red E. coli particles did not show a difference in phagocytic capacity – this data is presented in Fig. S1D. Incubating the macrophages with apoptotic mouse thymocytes, however, showed a clear difference between WNTd and WNTi macrophages, with the WNTi macrophages having a higher phagocytic capacity for these cells. This data is shown in Figure 5B and C.

Takata et al 2017 demonstrated that iPSC derived macrophages acquire a microglia-like phenotype after coculture with neurons. It would be very interesting to see if WNTi and WNTd macrophages are equally able to acquire a microglia phenotype in coculture with Neurons.

While this is a question we are very interested in investigating, we agree with the editor that it is outside the scope of this paper. Understanding how the tissue microenvironment influences the differentiation of WNTi and WNTd macrophages is the subject of our next study. Similarly, we hope this manuscript will enable the broader community to assess WNTi and WNTd macrophages across a wide range of co-culture microenvironments.

In Figure 2D it is written: “Genes used to calculate the z-score are indicated below the feature plots.” Gene names are on the right.

We have fixed this error.

The authors state:” Unlike what we observed after 14 days of culture, while the two cultures had clearly distinct clusters, there was also a significant observable overlap between the two differentiation cultures (Fig. 3E).” - This referee can not agree on that statement. Indeed, the clusters are not very much separated from each other. But they are also not really overlapping with the exception of eosinophils and maybe megakaryocytes.

The authors agree that this statement did not accurately reflect the data and it has been removed.

Second decision letter

MS ID#: DEVELOP/2021/200016

MS TITLE: Derivation of Extraembryonic and Intraembryonic Macrophage Lineages from Human Pluripotent Stem Cells

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ARTICLE TYPE: Techniques and Resources Article

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

This paper identified two different pathways of macrophage development from human pluripotent stem cells and described for the first time differences in transcriptional profile and function of macrophages originating from two distinct waves of embryonic hematopoiesis.

Comments for the author

The authors have responded appropriately to my comments. I recommend to accept this paper for publication in Development.

Reviewer 2

Advance summary and potential significance to field

The new analyses improve the paper even further.

Comments for the author

All points raised have been addressed. the paper should be published without delay.

Reviewer 3

Advance summary and potential significance to field

Bredemeyer et al. significantly improved the clarity of the manuscript. All my comments and questions were addressed in a satisfactory way.

Comments for the author

This referee thanks the authors for taking his suggestions into account and is looking forward to see exciting data about how the tissue microenvironment influences the differentiation of WNTi and WNTd macrophages in the next study.