Immune landscape of human placental villi using single-cell analysis

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First decision letter

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MS TITLE: Human placental villi immune cells help maintain homeostasis in utero

AUTHORS: Liza Konnikova, Jessica M. Toothaker, Oluwabunmi Olaloye, Blake T. McCourt, Collin C. McCourt, Rebecca M. Case, Peng Liu, Dean Yimlamai, and George Tseng

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

Using suspension mass cytometry and imaging techniques, the authors report a complex immune system within the mid18 gestation (17-23 weeks) human placental villi (PV). The authors have carefully separated maternal decidua and fetal chorionic/amniotic membranes covering the chorionic plate from the placental villi with a forceps under a dissecting microscope. By analysing cytokine production, they have stated that PV T cells are responsive to TCR stimulation.

Comments for the author

The message of this manuscript is unclear and confusing. The only immune cell population found in abundance in healthy human placental villi are macrophages. In the current format, the authors, imply that the placental villi are enriched with other cell types such as T cells, ILC and NKs. This is clearly not the case, as indicated through many publications of histological evidence. Any T cells that are found in normal healthy placental villi are rare, and sparsely populate the villi. Given this, I find that the conclusions the authors reach in this manuscript will only add confusion to the field.
Cytof data: Analysis of placental villi digests is difficult due to the possibility of maternal decidual and blood and fetal blood cell contaminants. Unfortunately, the authors have not made sufficient efforts to exclude these cells from their cytof data sets. For all of the cytof immune cell data, it is unclear as to whether they are describing placental villi cells or fetal blood or maternal cells.
The evidence that their placental villi digests are contaminated with non-placental villi cells is strongly indicated in Figure 2. It is well described in the literature that macrophages are the most highly abundant immune cell population in the placental villi. Any other immune, including T cells are rare to not present. Yet, in Figure 2 J, the authors provide data indicating that placental villi macrophages make up only 20% of CD45+ cells and CD8+ T cells make up 5% of CD45+ cells. This is not reflective of what is actually seen in the tissue by histology.
Also, it seems that the authors have not gated on some populations correctly. For example, in Figure S7, it seems more likely that the CD8T cells are sparser CD3bright, CD8 brighter population, and not all the cells that have been gated.
Microscopy data: Histological localisation data throughout the paper is not convincing, e.g. the (p)ZAP70 microscopy data (Figure 7A). Also, to say a cell is in the villous stroma and not a capillary is very difficult, for example, due to sectioning artefacts.
The placental villi microscopy data does not align with the cytof data. For example, from the microscopy data T cells are obviously rare in the placental villi. However, the cytof data implies these cells can be found in abundance.

Reviewer 2

Advance summary and potential significance to field

This paper uses RNA-seq and CYTOF to analyze fetal immune cell content in fetal villi. The authors were careful to verify that they carefully separated immune cells in the villi from maternal immune cells in the decidua. This catalog of immune cell subtypes and gene expression will be valuable for future studies of immune function of fetal cells in the decidua.

Comments for the author

My main problem with the manuscript is over-interpretation of immune cell function from phenotype. The regulation of immune reactivity in the placenta is very tight and I do not think it is really possible to infer function from phenotype. For example, decidual NK cells have very high levels of granzymes and perforin, but are very poorly cytotoxic. Immune regulation goes well beyond PD-1 and PD-L1 expression. In any event the differences in these markers in the different layers is not very impressive.
In particular the title is not a fair representation of the content of the manuscript - there are no experiments that show that PV immune cells maintain homeostasis.
I am not convinced that the activation experiments make sense and the gating may or may not be appropriate (especially in Fig. 6 and 7). For example in 6C there doesn't seem to be a big difference

of CD69 with stimulation. It is not always possible to compare cell types of different size and complexity with the same gate and the gates do not clearly separate a negative and a positive population (such as for IFNg and TNFa). I couldn't make out Figure 7 but there again I wasn't convinced the gating was right in the contour plots and the differences with gestation and stimulation were unimpressive.
I do not understand what the basis is for the conclusion of an inflammatory response in the Pv in the abstract or final paragraph from the data in the paper. My strong suggestion is to either remove Figs 6 and 7 and reserve for another paper with better functional data or at a minimum to tone down the functional conclusions

Minor points:
scale bars are missing in some of the images.
I couldn't figure out what "DEs" are in Figure 5
"down-regulation" in lines 332 and 333 should really be "low expression"

First revision

Author response to reviewers' comments

Dear Florent and Reviewers,

We appreciate your time reviewing our submitted manuscript “Human placental villi immune cells help maintain homeostasis in utero,” constructive feedback on the manuscript, and allowing us the chance to revise according to the reviewers’ comments.

In response to the reviewers comments we have composed a summary of how we have addressed their concerns in the current submission. All changes from the previous version appear in blue ink in the revised manuscript. Please note that we have added an author (Tatiana Silva) to the manuscript who has been instrumental with the revisions.

Reviewer 1.

1. The message of this manuscript is unclear and confusing. The only immune cell population found in abundance in healthy human placental villi are macrophages. In the current format, the authors, imply that the placental villi are enriched with other cell types such as T cells, ILC and NKs. This is clearly not the case, as indicated through many publications of histological evidence.

While it is true that macrophages (Hofbauer cells) are the dominant immune population in the healthy human placenta, we respectfully disagree that they are the only immune cell population. Several studies cited in our manuscript have identified other immune cell subsets, both in single cell suspension (Pique-Regi eLife, 2020) and histologically (Bonney Gynecol Obstet Invest 2000). We agree that many histological publications identify macrophages as the predominant immune cell population in the placenta, however, to our knowledge there is not a large body of literature specifically looking for T cells by histology and failing to detect any in the placental villi. In fact, the aforementioned study by Bonney et al. detected T cells by histology in the healthy first trimester placental villi.

Moreover, we specifically show CD45+ (immune) CD163- (non-Hofbauer) populations by histology in this manuscript in Figure 1D.

2. Any T cells that are found in normal healthy placental villi are rare, and sparsely populate the villi. Given this, I find that the conclusions the authors reach in this manuscript will only add confusion to the field.

We do not argue that T cells are not rare in the placental villi, however rare in abundance is not equivalent to non-important or biologically irrelevant. For example, innate lymphoid cells are rare by abundance in the gastrointestinal tract, yet their importance has been demonstrated in both

healthy and disease contexts. This manuscript adds to a growing body of literature that there are other cell types in the PV beyond HBC that might be biologically relevant. Moreover, we use numerous independent techniques including CyTOF, flow and histology to show the presence of T cells in the PV.

3. Cytof data: Analysis of placental villi digests is difficult due to the possibility of maternal decidual and blood and fetal blood cell contaminants. Unfortunately, the authors have not made sufficient efforts to exclude these cells from their cytof data sets. For all of the cytof immune cell data, it is unclear as to whether they are describing placental villi cells or fetal blood or maternal cells.

We respectfully disagree with this assessment. We showed via qPCR, bulk RNA-seq, flow and in situ hybridization that the immune cells analyzed in the placental villi are likely of fetal origin. This point was validated by reviewer 2's comment that the placental layers were well separated. We do agree that it is possible immune cells in the villi may represent circulating fetal cells. However, we have shown via histology that T cells are present both inside and outside of the fetal vasculature in Figure 5A. Additionally, we observed that many T cells express CD69 at baseline and lack the expression of CCR7 and CD45RA. The combination of these surface markers is associated with tissue-resident phenotypes. Specifically, CD69 expression was not found on T cells in the blood (Buggert et al Cell 2020).

4. The evidence that their placental villi digests are contaminated with non-placental villi cells is strongly indicated in Figure 2. It is well described in the literature that macrophages are the most highly abundant immune cell population in the placental villi. Any other immune, including T cells are rare to not present. Yet, in Figure 2 J, the authors provide data indicating that placental villi macrophages make up only 20% of CD45+ cells and CD8+ T cells make up 5% of CD45+ cells. This is not reflective of what is actually seen in the tissue by histology.

The first two sentences in this comment have been addressed in the sections above. In regard to the concerns with T cell/macrophage immune abundance, I think the reviewer mis-understood the figure as we are only showing 1 of the numerous macrophage and T cell populations that are present in the CyTOF data of the PV. The point of this figure was to show the populations that are specifically enriched in the PV. In Figure 2H, we show all the macrophage populations combined (bright green) and that represents 50-60% of all the CD45% cells. Figure S1B shows IF for CD45 cells (red) and HBC (CD163, white) with the tabulation of the data to the right suggesting that HBS represent ~60% of all immune cells present. The two datasets are remarkably similar.

To make the comparison to histology clearer, we have added Figure S2C that shows the abundance of each immune subset as a proportion of all cells in addition to as a proportion of CD45+ cells. This allows for direct comparison to what is seen by histology when immune cells would be assessed as a proportion of all cells in the placental villi.

5. Also, it seems that the authors have not gated on some populations correctly. For example, in Figure S7, it seems more likely that the CD8T cells are sparser CD3bright, CD8 brighter population, and not all the cells that have been gated.

We have removed Figures 6 and 7 and their accompanying supplemental figures from the manuscript per reviewer's 2 suggestion. For the flow data remaining in the manuscript, , we have displayed all flow gating next to appropriate single color controls to confirm gating accuracy. Though it is of note that reviewer 1 acknowledges the presence of CD3bright cells, which is reflective of T cells being present in the placental villi.

6. Microscopy data: Histological localisation data throughout the paper is not convincing, e.g. the (p)ZAP70 microscopy data (Figure 7A). Also, to say a cell is in the villous stroma and not a capillary is very difficult, for example, due to sectioning artefacts.

Per request of reviewer 2, we have removed Figure 7A from the manuscript.

7. The placental villi microscopy data does not align with the cytof data. For example, from the microscopy data T cells are obviously rare in the placental villi. However, the cytof data implies these cells can be found in abundance.

We respectfully disagree with this point. The two actually align very well. Please see reply to comment 4 above. Our CyTOF data does not suggest that T cells make up the majority of the immune cells, but rather shows that it is a minority population. As stated up above, we have added Figure S2C that shows the abundance of each immune subset as a proportion of all cells in addition to as a proportion of CD45+ cells. This allows for direct comparison to what is seen by histology when immune cells would be assessed as a proportion of all cells in the placental villi.

Review 2:

1. My main problem with the manuscript is over-interpretation of immune cell function from phenotype. The regulation of immune reactivity in the placenta is very tight and I do not think it is really possible to infer function from phenotype. For example, decidual NK cells have very high levels of granzymes and perforin, but are very poorly cytotoxic. Immune regulation goes well beyond PD-1 and PD-L1 expression. In any event the differences in these markers in the different layers is not very impressive.

We appreciate this point and agree that phenotype is not equivalent to immune cell function and that immune regulation goes beyond PD-1 and PD-L1 expression. We have softened the language throughout the manuscript in which function is inferred from phenotype and focus on the phenotype findings.

2. In particular the title is not a fair representation of the content of the manuscript - there are no experiments that show that PV immune cells maintain homeostasis.

We have update the title to reflect this is a phenotype characterization focused manuscript. Proposed new title “Immune landscape of human placental villi using single cell analysis.”

3. I am not convinced that the activation experiments make sense and the gating may or may not be appropriate (especially in Fig. 6 and 7). For example in 6C there doesn't seem to be a big difference of CD69 with stimulation. It is not always possible to compare cell types of different size and complexity with the same gate and the gates do not clearly separate a negative and a positive population (such as for IFNg and TNFa). I couldn't make out Figure 7 but there again I wasn't convinced the gating was right in the contour plots and the differences with gestation and stimulation were unimpressive.

We appreciate reviewer’s feedback and per additional suggestion in point 4, we have removed all cytokine/CD69 flow from Figure 6 and completely removed Figure 7 from the manuscript. For the proliferation flow experiment that remains in Figure 6 we have added appropriate single-color controls to the supplement.

4. I do not understand what the basis is for the conclusion of an inflammatory response in the Pv in the abstract or final paragraph from the data in the paper. My strong suggestion is to either remove Figs 6 and 7 and reserve for another paper with better functional data or at a minimum to tone down the functional conclusions

Thank you for the suggestion and feedback. We have removed all cytokine/CD69 experiments from Figure 6 and fully removed Figure 7 from the manuscript.

Second decision letter

MS ID#: DEVELOP/2021/200013

MS TITLE: Immune Landscape of Human Placental Villi Using Single-cell Analysis

AUTHORS: Jessica M Toothaker, Oluwabunmi Olaloye, Blake T McCourt, Collin C McCourt, Tatiana Silva, Rebecca M Case, Peng Liu, Dean Yimlamai, George Tseng, and Liza Konnikova

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

The authors have made a number of improvements to the manuscript. However, a number change still need to made.

The authors have acknowledged that T cells are rare in the PV. Yet their abstract and introduction does not reflect this. They need make significant changes to their abstract, so it more accurately reflects their findings.

In the discussion the authors acknowledge that PV contains blood contaminants. This needs to be made clearer in the introduction and results section, i.e. the PV data is not representative of the placental villi stroma, but of fetal blood and the placental villi stroma.

Comments for the author

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In the discussion the authors acknowledge that PV contains blood contaminants. This needs to be made clearer in the introduction and results section, i.e. the PV data is not representative of the placental villi stroma, but of fetal blood and the placental villi stroma.

Response to authors comments:

1. The authors have not correctly interpreted the work of Bonney et al. 2000. In Table 2 of this study the results show that T cells are not found in the first trimester and full term PV. While the study did identify T cells in the second trimester placenta, the representative images provided, are not convincing at all. I suggest they remove this reference. The work of Pique-Regi et al. 2019 does not provide any supporting T cell localisation data, so it is impossible to determine if the cells identified are found in PV. The human placenta is embedded into the maternal decidua and bathed in maternal blood. In addition, the PV are highly vascularised, and placental digests contain fetal blood cells.
The authors state that "we specifically show CD45+ (immune) CD163- (non-Hofbauer) populations by histology in this manuscript in Figure 1D". Yet in Line 167 – 168, the authors state that there are CD163- macrophages in the PV. So the CD45+CD163- cells could be CD163- Hofbauer cells
2. If the authors agree that T cells and immune cells other than macrophages, are rare in the placental villi, then they need to state this more clearly in the abstract and introduction. In the current form, the authors have implied that placental villi are enriched with a broad immune cell repertoire, when this is clearly not the case.
3. Only a small proportion of the T cells identified in PV express CD69 (Figure 5E), indicating the majority are blood T cells. The authors need to state more clearly in the text that the PV Cytof contains fetal blood cells.
4. The numbers provided by the authors do not make sense. To state that Hofbauer cells only make up of 60% of immune cells in the placental villi, goes against accepted literature on these cells. In addition, the authors state in Line 167 – 168, that there are CD163- macrophages in the PV, indicating that by using CD45 and CD163 alone, they are not accurately capturing all the macrophages in the PV. I suggest the authors repeat this analysis with CD45 and CD14. CD14 is in their IMC panel.

Other comments:
Lines 59-66: The authors need to state that these previous studies lack histology data, making it difficult to determine where the cells are found that had been identified, and whether they are fetal blood or PV cells.
Figure 1e and S1c – this staining is poor. What is the bright green smudge? Couldn't the authors find a better field to image.
Figure B-D – the authors don't explain PanKer and why they used it.
Figure 2D – The authors have outlined where they think trophoblasts of the placental villi are found. However, within the trophoblast layer, there seems to be HLA-DR+, CD14+ and CD31+ cells. I don't understand how this is possible. Unless this section is actually decidual tissue and not placental. Some of the CD14+ cells are outside the "trophoblast cells", and so must be maternal contaminants?
Figure 2G- I cannot see any positive staining for GATA3 in the cell labelled as IL-C2.
Line 101 – the authors have incorrectly cited Bonney et al 2000, they actually found that T cells are not present in first trimester and full term PV. So they need to remove this citation.
Line 163 - 164 – the authors refer to Figure 1H and Figure 2B, however plot 1H is not provided and data referred to in the text is not provided in Figure 2B. The text seems to be referring to incorrect figures.
Line 167 – 168 – the authors suggest that there are CD163neg macrophages in the PV. However, in Line 99 – 103, the authors imply that CD163 is classic Hofbauer cell marker, implying that the CD45posCD163lo cells identified in Figure 1D, must be immune cells that are not macrophages. The text in Line 99-102 is misleading and does not align with the text provided in Lines 167 – 168.
Line 166 – 168 – the heterogeneity observed in the macrophage populations, could be due fetal blood monocytes.
Line 170 – the authors state that their CD163 cytof data aligns with CD163 RNAseq Decidual data shown in Figure 1D. However, in Figure 1D, the CD163 gene is not highlighted. The authors need to add that gene to the list indicated in Figure 1D, so the reader can interpret the data.

Text referring to Figure 3 – I think it is misleading to refer to cells identified in the PV, as PV, as most likely many of these immune cells are fetal blood contaminants. The text needs to more clearly indicate that fetal blood cells are likely to be contaminating this data set.
Line 187 – it seems that authors have referred to the incorrect table. It should be Table S7 not S8.
Figure S4E – what do the authors mean by the label fetal on the X-axis. It is not explained in the Figure legend or consistent with Figure 4.
Figure 5B – why is the image so pixelated? It is impossible to interpret this data.
Figure S5B – can the authors explain why the trophoblast cells are also positive for the T cell marker, CD3.
Line 259 – 262: If CD69 expression is a marker for tissue T cells, then this indicates that in Figure 5E, the majority of the T cells analysed are blood contaminates and their cytof analysis is highly contaminated with blood cells. They authors need to be more clearly state this in the text.

Second revision

Author response to reviewers' comments

Dear Editors and Reviewers,

We appreciate your time re-reviewing our submitted manuscript “Immune Landscape of Human Placental Villi Using Single-cell Analysis,” your constructive feedback on the manuscript, and allowing us the chance to revise according to the reviewers’ comments.

In response to the reviewers comments we have composed a summary of how we have addressed their concerns in the current submission. All changes from the previous version appear in blue ink in the revised manuscript.

Essential revision:
The authors have acknowledged that T cells are rare in the PV. Yet their abstract and introduction does not reflect this. They need make significant changes to their abstract, so it more accurately reflects their findings.

We have updated the abstract and introduction to include that the T cell populations detected were rare.

In the discussion the authors acknowledge that PV contains blood contaminants. This needs to be made clearer in the introduction and results section, i.e. the PV data is not representative of the placental villi stroma, but of fetal blood and the placental villi stroma.

We have updated the introduction and results section to improve clarity that this data set contains cells in both the blood and PV stroma.

Response to authors comments:
1. The authors have not correctly interpreted the work of Bonney et al. 2000. In Table 2 of this study the results show that T cells are not found in the first trimester and full term PV. While the study did identify T cells in the second trimester placenta, the representative images provided, are not convincing at all. I suggest they remove this reference. The work of Pique-Regi et al. 2019 does not provide any supporting T cell localisation data, so it is impossible to determine if the cells identified are found in PV. The human placenta is embedded into the maternal decidua and bathed in maternal blood. In addition, the PV are highly vascularised, and placental digests contain fetal blood cells.

We apologize for the error and have corrected the Bonney reference to refer only to detection of T cells in the second trimester. The work of Pique-Regi refers to their dataset with cells isolated from the “PV”, and as such still believe that it is a fair reference. We believe that our reference in the text is true to what has been published in the manuscript, which indeed refers to these cells as being isolated from the PV.

2. The authors state that “we specifically show CD45+ (immune) CD163- (non-Hofbauer) populations by histology in this manuscript in Figure 1D”. Yet in Line 167 – 168, the authors state that there are CD163- macrophages in the PV. So the CD45+CD163- cells could be CD163- Hofbauer cells

CD163 is a classical marker for HBC. We have updated the text throughout to refer to the CD45+CD163- cells as potential non-classical Hofbauer cells.

3. If the authors agree that T cells and immune cells other than macrophages, are rare in the placental villi, then they need to state this more clearly in the abstract and introduction. In the current form, the authors have implied that placental villi are enriched with a broad immune cell repertoire, when this is clearly not the case.
We respectfully disagree, as no terminology with enrichment or high abundance was used. However, we have added text to explicitly acknowledge the T cell population is rare.

4. Only a small proportion of the T cells identified in PV express CD69 (Figure 5E), indicating the majority are blood T cells. The authors need to state more clearly in the text that the PV Cytof contains fetal blood cells.

We have added back in Flow plots of unstimulated T cells from PV showing ~40% constitutively express CD69. Similarly, using our CyTOF data, we calculated that ~40% of PV T cells express CD69 without stimulations. Implying that ~40% are resident to the PV and the rest could be either resident to PV or from fetal blood. This has been added to the text starting on line 286 “We calculated the abundance of CD69+ T cells from both CyTOF and flow cytometry analysis and ~40% of T cells had constituent CD69 expression by both measures (Fig S5F), and could be concluded to be PV resident T cells, while the rest were likely a mixture of PV and fetal blood cells.”

5. The numbers provided by the authors do not make sense. To state that Hofbauer cells only make up of 60% of immune cells in the placental villi, goes against accepted literature on these cells. In addition, the authors state in Line 167 – 168, that there are CD163- macrophages in the PV, indicating that by using CD45 and CD163 alone, they are not accurately capturing all the macrophages in the PV. I suggest the authors repeat this analysis with CD45 and CD14. CD14 is in their IMC panel.

As stated above, we have updated the text through the manuscript to identify that the CD163- CD45+ populations may also include Non-classical Hofbauer cells.

Other comments:
Lines 59-66: The authors need to state that these previous studies lack histology data, making it difficult to determine where the cells are found that had been identified, and whether they are fetal blood or PV cells.

We have added text to include that prior studies did not include histological data. As can be seen starting on line 66. “Of note, these single-cell surveys lacked histological data and as such there is a gap in knowledge as to the localization (fetal blood or PV stroma) of the immune sell types.”

Figure 1e and S1c – this staining is poor. What is the bright green smudge? Couldn’t the authors find a better field to image.

The staining that we refer to in the image is in focus and has a different speckled pattern staining the Y chromosome. The “green smudge” observed are red blood cells which autofluorescence in the green channel. For clarity, we have added a label to the image identifying this. Please see updated figure.

Figure B-D – the authors don’t explain PanKer and why they used it.

We have added a sentence to the figure legend explaining that PanKer is a marker for trophoblasts. “PanKer = pankeratin, a trophoblasts marker.”

Figure 2D – The authors have outlined where they think trophoblasts of the placental villi are found. However, within the trophoblast layer, there seems to be HLA-DR+, CD14+ and CD31+ cells. I

don’t understand how this is possible. Unless this section is actually decidual tissue and not placental. Some of the CD14+ cells are outside the “trophoblast cells”, and so must be maternal contaminants?

The trophoblast outline is simply a hand drawn outline drawn for clarity and orientation for the image. The noted CD14+ cells “outside” the trophoblast cells simply represent a minor corner of trophoblast that was missed. There are no independent nuclei, or CD14+ nuclei shown in this image outside the trophoblast layer. Furthermore, paraffin sections of the PV are one plain of a 3D object and likely represent multiple intravillous trees that make be positioned above and below the field of sectioning thus accounting for the multiple markers staining the trophoblasts. For this reason, we had used PanKer to mark the trophoblast layers which all express PanKer while the interior cells clearly lack PanKer further confirming this outlined layer are trophoblasts. Additionally, we confirmed in the previous round of revisions that HLA-DR expression specifically is detected on trophoblasts.

Figure 2G- I cannot see any positive staining for GATA3 in the cell labelled as IL-C2.

We appreciate your concern and have enhanced the green staining.

Line 101 – the authors have incorrectly cited Bonney et al 2000, they actually found that T cells are not present in first trimester and full term PV. So they need to remove this citation.

This point has been addressed above.

Line 163 - 164 – the authors refer to Figure 1H and Figure 2B, however plot 1H is not provided and data referred to in the text is not provided in Figure 2B. The text seems to be referring to incorrect figures.

We apologize for the error and have updated the figure reference to be 2A and 2H.

Line 167 – 168 – the authors suggest that there are CD163neg macrophages in the PV. However, in Line 99 – 103, the authors imply that CD163 is classic Hofbauer cell marker, implying that the CD45posCD163lo cells identified in Figure 1D, must be immune cells that are not macrophages. The text in Line 99-102 is misleading and does not align with the text provided in Lines 167 – 168.

Please see above where the same point was addressed.

Line 166 – 168 – the heterogeneity observed in the macrophage populations, could be due fetal blood monocytes.

We have added this possibility to the text.

Line 170 – the authors state that their CD163 cytof data aligns with CD163 RNAseq Decidual data shown in Figure 1D. However, in Figure 1D, the CD163 gene is not highlighted. The authors need to add that gene to the list indicated in Figure 1D, so the reader can interpret the data.

We have highlighted this gene on the figure.

Text referring to Figure 3 – I think it is misleading to refer to cells identified in the PV, as PV, as most likely many of these immune cells are fetal blood contaminants. The text needs to more clearly indicate that fetal blood cells are likely to be contaminating this data set.

Please see this point addressed up above. The text has been updated starting on line 169. “The increased granularity of focusing on HLA-DRneg innate cells, revealed that the PV contains CD163hi Mϕs likely Hofbauer cells, and contained a significant proportion CD163lo Mϕs, presumably other non-classical Hofbauer cell or other Mϕs; Hofbauer cells that had down regulated CD163 expression; or fetal blood monocytes/Mϕs (Fig 3B)”.

Line 187 – it seems that authors have referred to the incorrect table. It should be Table S7 not S8.

We apologize for the error and have corrected the table number.

Figure S4E – what do the authors mean by the label fetal on the X-axis. It is not explained in the Figure legend or consistent with Figure 4.

We apologies for the oversight and have updated the label to “mid-gestation”

Figure 5B – why is the image so pixelated? It is impossible to interpret this data.
Figure S5B – can the authors explain why the trophoblast cells are also positive for the T cell marker, CD3.

This data is from IMC, which is generated by a laser ablation of small regions of the slide from scanning across a tissue section, detections of the metals in the ablated segment and then reconstructing the positive signal (metals detected) pixel by pixel until a larger image is created. As such IMC images are naturally pixelated examined close up.

Line 259 – 262: If CD69 expression is a marker for tissue T cells, then this indicates that in Figure 5E, the majority of the T cells analysed are blood contaminates and their cytof analysis is highly contaminated with blood cells. They authors need to be more clearly state this in the text.

This was addressed in point 4 above. Please see our comments there.

Third decision letter

MS ID#: DEVELOP/2021/200013

MS TITLE: Immune Landscape of Human Placental Villi Using Single-cell Analysis

AUTHORS: Jessica M Toothaker, Oluwabunmi Olaloye, Blake T McCourt, Collin C McCourt, Tatiana Silva, Rebecca M Case, Peng Liu, Dean Yimlamai, George Tseng, and Liza Konnikova

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

This study provides new insight into human placental biology. In particular, using high dimensional techniques they provide new insight into placental T biology.

Comments for the author

The authors have addressed many of my suggestions.
However,the authors have not added the gene CD163 to figure 1D, as I previously suggested, although below they state they have.
“Line 170 – the authors state that their CD163 cytof data aligns with CD163 RNAseq Decidual data shown in Figure 1D. However, in Figure 1D, the CD163 gene is not highlighted. The authors need to add that gene to the list indicated in Figure 1D, so the reader can interpret the data.
We have highlighted this gene on the figure.”

Third revision

Author response to reviewers' comments

Thank you for your feedback. However, the comment that CD163 is not on the heatmap in the revised document in Fig 1 is incorrect. It had been added to the list on the resubmission. It appears after CD14 and before CD68. However, the sub-figure is mislabeled in the text and should be figure 1F not 1D. This has been corrected.

Fourth decision letter

MS ID#: DEVELOP/2021/200013

MS TITLE: Immune Landscape of Human Placental Villi Using Single-cell Analysis

AUTHORS: Jessica M Toothaker, Oluwabunmi Olaloye, Blake T McCourt, Collin C McCourt, Tatiana Silva, Rebecca M Case, Peng Liu, Dean Yimlamai, George Tseng, and Liza Konnikova
ARTICLE TYPE: Research Article

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