Response to editorial and reviewer comments (Lopez-Yrigoyen et al)

Thank you for giving us the opportunity to revise our manuscript. We have altered the manuscript in accordance all the Editorial comments including the addition of all authors' email addresses, a short summary and highlighted parts of the protocol that would be suitable for filming. These changes have been tracked as track changes in the revised manuscript.

We are very grateful to the reviewers for their productive comments and respond to these as follows.

Reviewer 1

Reviewer 1 states that 'It has to be mentioned however, that the present study is the first study "produced" in a video format'. We have now included this in our introduction (Page 3, lines 114-5)

Major Concerns:

1. We acknowledge that the naming of the steps used in the differentiation protocol could be misleading and the reviewer is correct in stating that we have not characterised the cells generated at each step of the process in detail. We have therefore renamed the first two steps to "Generation of embryoid bodies" and 'Emergence of haematopoietic cells in suspension" as this more accurately reflects the process and will avoid misleading readers (Page 2, lines 68-70). We have reworded the description of EB formation to reflect the protocol that we describe.
2. We acknowledge that the role of IL3 during embryonic development is not clear. Thus, we have reworded our text to emphasise that our statement about IL3 refers only to our in vitro culture conditions. We speculate that *in these conditions*, IL3 *likely supports* haematopoietic progenitor formation and proliferation (Page 2, line 83-4).
3. As stated above, we have also re-named the steps in the differentiation protocol in sections 3.2 and 3.3 to more accurately reflect the process. We have removed the word "expansion" from this step because we do not provide data to support this statement (Page 6).
4. The reviewer is correct in stating that we do not provide data to show that these cells are myeloid precursors and we agree that some of these might be more mature myeloid cells. To avoid misleading readers, we have renamed the cells generated at this point in the protocol to "haematopoietic cells in suspension" (Page 6, line 251-2).

Reviewer 2

1. We now make a clear statement that the protocol we describe is based on that described by van Wilgenburg and we now cite this reference more clearly and upfront (Page 3, line 116). The minor modifications that we made to this published protocol are clearly stated (Page 3 lines 116-121). We make the statement that this is the first time the protocol has been described in video format (Page 3 lines 114-5).
2. We have altered the title to "Production and characterization of human macrophages from Pluripotent Stem Cells". As this is the first time the protocol is described in video format, we justify retaining the word "Production" in the title.
3. The reviewer is incorrect in stating that 25F9 it is an antibody clone that detects CD163. The marker, 25F9 was first described by Zwandlo et al in 1985[1] and has since been further characterised and used by many labs as a distinct macrophage marker[2,]

[3]. 25F9 has been define as a protein of 86 k-Da found on mature macrophages on the cell surface and in intracellular vesicular structures, while CD163 is a 130-kDa membrane protein with a short cytoplasmic tail. Expression of CD163 and 25F9 has been independently measured in many publications and the percentage and/or level of CD163 and 25F9 expression is not equal in the cells tested [3–6].

4. We have replaced x-axis values with day of differentiation. As reviewer two suggests, it is more informative
5. We have replaced green with pHrodo green
6. We have received permission from the publisher to re-use figures. We attach a copy of the email communication with the publisher on this point.
7. ND stands for not detected. We have now added this information to the legend of Figure 5A.
8. We have included the following sentences in the discussion to reflect that fact that iPSCs used for differentiation should be regularly subjected to genome quality control. *'Human iPSC cultures can become overrun with karyotypically abnormal subpopulations over several passages and so robust curation of iPSC stocks and large batch master stock subjected to genome quality control is recommended. In our hands, the maintenance protocols described here can maintain karyotypically normal iPSCs for up to 2 months in continuous culture, but this may vary for different cell lines and in different laboratories. If problems are encountered, it is advisable to use a fresh vial of undifferentiated iPSCs for each differentiation experiment.'* (Page 11/12, lines 490-496).

References

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2. Fraser, A.R. *et al.* Development, functional characterization and validation of methodology for GMP-compliant manufacture of phagocytic macrophages: A novel cellular therapeutic for liver cirrhosis. *Cytotherapy*. **19** (9), 1113–1124, doi: 10.1016/j.jcyt.2017.05.009 (2017).
3. Pilling, D., Fan, T., Huang, D., Kaul, B., Gomer, R.H. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. *PloS one*. **4** (10), e7475, doi: 10.1371/journal.pone.0007475 (2009).
4. Moore, J.K. *et al.* Phenotypic and functional characterization of macrophages with therapeutic potential generated from human cirrhotic monocytes in a cohort study. *Cytotherapy*. **17** (11), 1604–1616, doi: 10.1016/j.jcyt.2015.07.016 (2015).
5. Lopez-Yrigoyen, M. *et al.* A human iPSC line capable of differentiating into functional macrophages expressing ZsGreen: A tool for the study and in vivo tracking of therapeutic cells. *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi: 10.1098/rstb.2017.0219 (2018).
6. Lopez-Yrigoyen, M. *et al.* Genetic programming of macrophages generates an in vitro model for the human erythroid island niche. *Nature Communications*. 1–11, doi: 10.1038/s41467-019-08705-0 (2019).