Rebuttal letter

Isolation of uterine innate lymphoid cells for analysis by flow cytometry

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Point-by-point response

We thank the editors and the reviewers for their comments and suggestions. Please find below (in blue), our point-by-point response.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Spelling and grammar reviewed.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Addressed in the whole document.

3. Please define all abbreviations before use (gd). Addressed in the whole documents.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Falcon, Heraeus Megafuge 40R, Percoll, BD Perm/Wash, BD Pharm Lyse, Liberase, etc. Addressed in the whole document except for Percoll, Liberase DH and Liberase TM. "Percoll", "Liberase DH" and "Liberase TM" cannot be replaced with generic terms without significant alteration of the protocol to the point that readers will not be able to reproduce it. For instance, replacing "Percoll" with "separation medium" does not make any sense, as all physical properties of this "separation medium" have to be listed, which is not possible to do as it is not disclosed by the company. Replacing Liberase TM / Liberase DH also does not make sense: replacing them with the phrase "digestion medium containing Collagenase I, II, Thermolysin / Dispase" is not good enough as we do not know the proportion of enzymes in these mixes (it is not disclosed by the company). Therefore, the

concentration of “digestion medium containing Collagenase I, II, Thermolysin / Dispase” cannot be specified.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. Done

6. For SI units, use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2. Addressed in the whole document.

7. Line 134/141: Please replace “bijoux” by “microcentrifuge” tubes or any other generic term. Done.

8. Line 136: Please provide more details to the euthanasia step. Please mention how animals are anesthetized and how proper anesthetization is confirmed prior to cervical dislocation. Done, lines 149

9. Line 144: When times is used as a unit (i.e., for concentrations and magnifications), use lowercase x throughout after the numerical value. Examples: 10x magnification, 2x concentration. Done

10. Line 177-182/ 236-242/276-278: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings. Consider adding these lines under “NOTES” Done.

11. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Done.

12. Please move the Figure and Table legends to the end of Representative Results. Done.

13. Please do not use the &-sign or the word “and” when listing authors in the references. Please title case and italicize journal titles and book titles. Do not use any abbreviations. References reviewed and edited accordingly.

14. Please obtain explicit copyright permission to reuse any figures from a previous

publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We have A permission to reproduce figures from the Nature group is included.

15. Figure 2: Please consider revising the x axis legend to "3 weeks, 5 weeks, etc. Provide the define the term "gd" in the figure legend description. Changing "w" for "weeks" on the x axis impairs clarity of the figure, thus "gd " and "w" were clearly defined in the figure legend.

16. Figure 3: Please remove the commercial terms (Falcon, Percoll, BD Pharm Lyse) from the figure and replace them with generic terms. Addressed except for Percoll; please see the explanation above (point 4).

17. Figure 6: Please replace the commercial term Percoll by generic term. Not replaced, please see the explanation above (point 4).

18. Figure 7/10: Please define the numbers represented in the quadrants in the Figure/Figure description as percentage. Done.

19. Figure 9: Please remove the commercial term "Liberase" from the figure and replace it with generic term. Not replaced, please see the explanation above (point 4).

20. Table 1: Please remove trademark (™) and registered (®) symbols from the Table. Done.

21. Please sort the Table of Materials in alphabetical order. Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript described the method to isolate and phenotype mouse group 1 uterine innate lymphoid cells (g1 uILCs) by flow cytometry. The description is clear and the Protocols are useful.

Major Concerns:

Most researchers know the protocols to isolate uterine innate lymphoid cells, however, they may be more interested in how to isolate Myo/ MLAp and decidua lymphoid cells respectively. The authors isolate Myo/ MLAp and decidua lymphoid cells respectively in their previously published article (PMID: 26371244), but the description is not very clear.

So it is better to add the protocols of isolating Myo/MLAp and decidua lymphoid cells respectively in this manuscript.
The protocol is applicable for the isolation of both decidual and Myo/MLAp lymphoid cells. The method to dissect the decidua from the Myo/MLAp has been described before in reference 3 and, in more details, in Croy et al, 2010, PMID: 20033660. We include this paper in the list of references.

Minor Concerns:

line 284: cNK (CD49a-s) should be cNK (CD49a-Eomes+) edited, see line 408.

Table 1 should not be separated in two pages. Table 1 was not designed to be separated in two pages. The table is provided as an excel file as requested by JoVE.

Reviewer #2:
Manuscript Summary:
This manuscript clearly outlines in text and figures a protocol to obtain lymphoid cells from pregnant and non-pregnant mouse uterine tissue. Whilst mainly focussed on assessment of cells by flow cytometry, and centred on g1 uILCs, the protocol is clearly indicated to be readily adaptable to obtain other cell types, and for other assessment purposes such as omics or functional assays.

Major Concerns:
None

Minor Concerns:
Is the protocol 'designed' for isolation of cells from individual uterus, or pooled uteri?
The protocol is designed for isolation of cells from individual uterus. When the yield is low, we pool cells from multiple uteri, however each uterus is treated individually. See lines 106-107 and 582-583.

Reasons why step 12 and 13 are included in the enzymatic digestion protocol section should be included. Done, see lines 190, 192, 205.

A reader may wonder why cells move from 37 degrees for digestion, to 4 degrees, then back to 37 degrees for resuspension, then additional 37 degree incubation in buffer and 10s vortex, then pushed through a filter with plunger. We appreciate the concern of the reviewer. The digestion is done at 37C to allow for the enzymatic reaction. The passage at 4C is intended to stop the enzymatic reaction. We agree with the reviewer that the subsequent step at 37C may not be necessary, however, because we have not tested other temperatures, we advise to use this step at 37C. Readers may want to test them.

In Figure 3, minor protocol steps are suggested to be added throughout, eg '...carefully collect the ring of leukocytes' could be changed to '...carefully collect the ring of leukocytes and spin' to aid clarity. This then aids interpretation of the next group of

steps. Another eg, 'Incubate the bijoux tubes for 30 min at 37 °C with agitation' is suggested to be changed to 'Add digestion mix and incubate for 30 min at 37 °C with agitation'. It is recommended to revisit all text in this figure to add additional words and phrases and remove those that are duplicated. Details were added to legend and the text has been clarified.

In part 6, it should be clarified why no azide, Tris, or protein is recommended in the PBS. See line 324-327.

The figure legend for figure 2 indicates that Liberase TM was used - is this a mistake? If not, then it should be indicated somewhere why this is still appropriate.
Not a mistake, both can be used. Liberase TM was used for that experiment as it leads to higher yield than Liberase DH and did not damage any crucial epitopes used in this specific experiment. We have also added a comment in the protocol stating that both can be used but the choice must be guided by their effects on important epitopes involved in antibody binding (see line 165-167).

It is suggested that the software created X-axis title of the overlay histograms in Figure 8 be replaced for clarity. Done.

Table 1 includes paraformaldehyde, however this is not referred to in any section. Added, see line 350.

In the discussion, it is suggested that the authors may like to include reference to cytological evaluation of vaginal smears as a way of increasing plugging rates. Done, lines 535-536.

Page 8, the authors should elaborate on why beads are not recommended. This would be of benefit to the reader, especially those attempting to assess low numbers of cells in this way for the first time. Done, lines 553-555.

Page 8, perhaps the authors could expand on why flow cytometric assessment within 24h is optimal, ie what happens after this, and if intracellular staining is not to be performed, indicate whether still recommend some sort of fixation, and what this might be. Done, lines 561-562.

Sincerely,
Francesco Colucci (for the Authors)