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Vidhya Iyer Ph.D.
Review Editor
JoVE

August 4, 2021

Dear Dr. Iyer,

We are pleased to submit our revised manuscript titled “Flow cytometric analysis for identification of the innate and adaptive immune cells of murine lung” (MS# 62985).

In this revision, all the comments of the editors and the reviewers have been taken into consideration and the manuscript has been amended accordingly. All the changes and additions have been highlighted in the text and have been outlined in our point-by-point response to the reviewers’ comments.

Sincerely,

A handwritten signature in blue ink that reads "VA Boussiotis".

Vicki A. Boussiotis, M.D., Ph.D.

Response to Reviews

We would like to thank the editor and the reviewers for their thoughtful suggestions for the improvement of our manuscript. All the comments of the reviewer and the editor have been taken into consideration and the manuscript has been revised accordingly. Below please find a point-by-point response to the reviewer's comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please revise your title to better describe the protocol in the paper and the video. Maybe something like "Flow cytometric analysis for the identification of the innate and adaptive immune cells of murine lung."
3. Please provide an email address for each author.
4. Please keep the word count of the summary between 10 and 50 words.
5. Please keep the word count of the abstract between 150 and 300 words.
6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.
7. Use numbered steps in your protocol 1., 1.1., 1.1.1., 1.1.1.1.; no letters, bullets, dashes.
8. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
9. 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.
10. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
11. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
12. Please remove the embedded Tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the

Representative Results in the manuscript text.

13. Please consider providing solution composition as Tables in separate .xls files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

14. Please include a title and a description of each figure and/or table in its legend. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

16. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

17. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

19. Please sort the Materials Table alphabetically by the name of the material.

20. Files uploaded to the "Supplemental Files (as requested by JoVE)" section of your Editorial Manager account are only for JoVE's internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the "Supplemental Code Files" section of your Editorial Manager account.

A: In response to the editorial comments we have:

- 1) Modified the title*
- 2) Provided email addressed for all authors*
- 3) Shortened the summary length*
- 4) Reformatted the reference presentation in the text into a superscript number.*
- 5) Renumbered the protocol steps to keep only numbers and not letters as indicators of sections and subsections.*
- 6) Revised the text to remove any personal pronouns*
- 7) Revised the protocol description so that is all in imperative tense.*
- 8) Removed embedded tables and provided them as separate excel files.*

9) *Added acknowledgments and conflict of interest section.*

Reviewer #1:

Manuscript Summary:

The authors provide a detailed method for obtaining a single cell suspension from lung tissue and identifying immune cells in this organ during homeostasis. For the detection of these different immune cells they propose a small flow cytometry staining panel that makes optimal use of the selective surface and intracellular proteins expressed on the different immune cell populations. Overall, this manuscript has been very well written and I am convinced that this will help researchers in there panel design in order to investigate lung immune cells. The main weakness is the fact that the proposed gating strategies and flow cytometry panel are very useful in steady state, however no remarks are made on how these populations behave in infection/disease. As most scientist will not only look at steady state lungs, but rather manipulate this environment somehow, it would be interesting/add significantly to the manuscript if this was mentioned/discussed in the manuscript.

Major Concerns:

-

Minor Concerns:

line 54: CD206 expression is also observed on alternatively activated alveolar macrophages. This should be mentioned in the introduction, to avoid confusion and clarity.

A: We made this clarification in the introduction lined 79-80 and added a relevant citation (ref#8).

line 61: Conventional CD103 DCs and conventional CD11b DCs are also known as cDC1 and cDC2, respectively. It would be beneficial to include this nomenclature for clarity.

A: We added this information (lines 84-85).

line 78: inflammatory eosinophils are also found in the lung interstitium, please amend.

A: We made this clarification (line 101).

line 80: lung-resident eosinophils have been suggested to have a regulatory role in allergy, in my opinion this has not been fully proven. Could the authors revise this sentence to be less strong.

A: We revised this point as requested (line 103).

line 131: It would be helpful to include a note to emphasize the need to remove all the adjacent fat from the lungs before further preparing the single cell suspension, as this could bias the read outs.

A: We emphasized this point (lines 156-157).

line 175: Please add a note in which the scientists are recommended to titrate the individual antibodies for optimal staining and to reduce costs.

A: We added this point (lines 233-234).

line 197: To me it is not clear why the FoxP3 staining kit (that is necessary for intranuclear

staining) has to be used, whereas the CD68 is located in the cytoplasm. If so it would be of note to add a sentence providing additional possibilities to stain intracellularly (e.g. low concentration PFA, or cytofix/cytoperm kit from eBioscience (or other vendors)).

A: We added this clarification (lines 235-238)

Figure 3: Within this gating strategy could the authors provide clarity on where the NKT cells would fall? It seems as if these cell would be the little blob in CD3+ cells within the NK cell gate. Although very little, the possible contamination of the NK cell (or other gate) with NKT cells should be addressed in the manuscript.

A: We added this clarification in the Figure legend of Figure 3.

Reviewer #2:

Manuscript Summary:

Thank you for the opportunity to review this manuscript. It is an excellent piece of work and a valid contribution to the field. The methods are clearly described and easy to follow. The manuscript is concise and well written.

Major Concerns:

Nil

Minor Concerns:

For the gating figures (Figures 1,2,3,4) it is hard to read the labels on plots (i.e the label of each cell population). These should be made clearer.

A: We increased the font size in all figures so that the labels in the plots are clearer to read.