

September 16, 2018

Phillip Steindel, Ph.D.  
Review Editor

Journal of Visualized Experiments

Re: Rebuttal letter for manuscript no. JoVE 58713 : Profiling the folate receptor beta macrophage and the vascular immune microenvironment in Giant Cell Arteritis: an immunohistopathologic study

Dear Dr Steindel

I am submitting the final manuscript after reviewing all editorial and peer commentaries. Please note that the manuscript, when viewed with tracked changes, reflected a comprehensive overhaul given the extent of the modification needed from citation formatting, removal of commercial language, phrases written in the imperative tense and additions to the protocol and discussion as adviced by the reviewers.

The rebuttal to the comments and questions as outlined below were duly addressed in the manuscript and typed in italicized text as below. Thank you so much.

Sincerely yours,

Shirley Albano-Aluquin, MD

Corresponding author

**Editorial comments:**  
Changes to be made by the Author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. *done.*  
2. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. *done*3. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient. *done*  
4. Keywords: Please provide at least 6 keywords or phrases. *done*  
5. Please shorten the Abstract to 150-300 words. Please focus on the method being presented rather than the results of a specific experiment. *done*6. Please change citations are superscripted numbers. *done*7. 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 and Reagents. For example: TechMate, Ventana Biotek, M071801 Dako, A0452 Agilent Dako, Envision™, Olympus, etc. *done*  
8. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc. *done*  
9. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion. *done*10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. *done*11. Step 1: What is used to cut? How to mount on plus slides? How many sections are mounted on each slide? *A microtome was used for cutting. Procedure for mounting was described. Three sections were mounted on microscope slides.*

12. Step 2: What type of water is used? How will you rehydrate? What containers are used? *Double distilled water was used. Rehydration was amended and further described in Protocol steps 2.5 to 2.7.*13. Step 3: What is the incubation temperature in the steamer? Are the slides taken out from the steamer after the 15-min incubation? *Procedure amended in step 2.7*   
14. Step 4: What is the incubation temperature*? Room temperature as noted in Step 2.9*15. Step 5: Please describe how to apply the antibodies. *Noted in Steps 2.9 and 2.10*16. Step 6: What volume of PBS is used? *200 µl of TBS used and indicated in Step 2.8*  
17. Step 7: Please specify the amount of antibody applied. *200 µl of antibody used and indicated in Step 2.9*18. Step 8, 10-15: Please describe how these are actually done. As currently written, they do not have enough detail to replicate as currently written. *done*19. Discussion: Please also discuss critical steps within the protocol. *done*  
20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. *done*  
21. References: Please do not abbreviate journal titles*. I referred to other JOVE articles and used Endnote program which both presented citations in abbreviated format.*   
  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
This manuscript describes a protocol for the immunohistochemical and histopathological examination of folate receptor beta (FRβ)-positive macrophages among other immune cell infiltrates (i.e., CD68+ macrophages and CD3+ lymphocytes) in formalin-fixed paraffin-embedded temporal artery biopsies (TAB) from 5 patients with giant cell arteritis (GCA) and 2 negative controls. The results of this study demonstrate that adventitia and media infiltration of FRβ-positive macrophages, representing approximately one third of total macrophage population, may be utilized as a novel biomarker for disease activity in GCA.  
This study is of potential clinical interest and the visual demonstration of the immunohistochemical staining procedure for FRβ-positive macrophages in TABs of GCA can also serve application and examination of various other tissues in autoimmune related diseases featuring macrophage infiltration as a hallmark of disease progression and activity. In fact, the reported results for GCA share similarities with synovial macrophage infiltration in arthritis patients with active disease.  
  
Minor Concerns:  
I want to suggest a series of (minor) technical and textual amendments to further improve the manuscript:  
# lines 115-144: Anticipating that specific details will be presented in a video presentation, I would still recommend extending on some details of the protocol in the following lines:  
# line 120: "…….rehydration with water"; please indicate volume and time. *A more elaborate procedure was described in Steps 2.2 to 2.7*# line 121: "incubating …citrate buffer pH 6.0 …"; please indicate volume. *Volume used was 200 ml and described in Step 2.7.*# line 123: indicate volume of incubation with 3% hydrogen peroxide. *Volume used was 200 ml and indicated in Step 2.8.*# line 126: indicate how many µl of diluted FRβ antibody was used. *Volume was 200 µl as noted in Step 2.9.*Also provide the original references for the antibody (JF Ross et al, Cancer 1994; and JF Ross et al, Cancer 1999). *done*It should be stated, either in the text or in the video presentation, that this polyclonal FRβ antibody is not commercially available and thus may hamper broad application of this protocol. Conceivably, this protocol may also work for any FRβ antibody when they may become commercially available. A recent report described the use of a commercially available FRβ antibody (EM-35, EXBIO Praha) for flow cytometry (C Machacek et al, J Immunol 2016;197:2229-2238) and claim it could also work for immunohistochemistry, but that has not been proven. Also caution needs to be exercised as the the immunogen for the EM-35 antibody was T cell lymphoma cell line, which, other than myeloid cells, should not express appreciable levels of FRβ. Just in case the authors have tested other FRβ antibodies, this would be worthwhile to mention. *Noted*# line 127: indicate how many µl of antibody dilution was used. *200 µl and noted in Step 2.9*# line 128: indicate how many µl of antibody dilution was used. *200 µl and noted in Step 2.9*# line 129: indicate the volume of PBS washes. *done*# line 130: indicate how many µl of secondary antibody (and dilution) was used*. 200 µl at 1:800 and noted in Step 2.11*  
# line 135: was this done according to manufacturer's instructions? Please indicate. *Procedure amended and described in Step 2.13.*

# line 136: rinsing with wash buffer; one time, multiple times? *3 times as described in Step 2.8*# line 150: It may be helpful and instructive to point out the representative examples of the indicated cell types and areas in Figure 1 with specific arrows. *Done*    
# lines 163-170: it may be helpful to point out the indicated areas in Figures 1-3. *done*    
# line 199: with respect to diagnostics, the results of this study would also open opportunities for non-invasive imaging approaches with FRβ targeted folic acid conjugated SPECT or PET imaging agents. *done*  
Finally, although beyond the context of this study, it may be worthwhile to examine FRβ expression in GCA in the context of M1-M2 macrophage polarization. *addended*  
  
**Reviewer #2:**  
Manuscript Summary:  
In this manuscript, the authors described the protocol to evaluate the immune cell infiltrations including folate receptor beta positive macrophages in the temporal artery biopsy specimens from patients with giant cell arteritis. The protocol is somewhat described well. However, I have a number of concerns as I wrote in the specific comments.  
  
Major Concerns:  
1. The protocol described in this manuscript is just of conventional immunohistochemistry. Are there any special techniques to stain temporal artery specimen or any caveat of the procedures? If there are, please describe in detail and discuss them.  
  
Minor Concerns:  
1. The procedures how to fix the obtained temporal artery specimens should also be described in the protocol. *Done and described in Steps 1.1 to 1.4*2. The paragraph of specimen selection should be amended. I think this paragraph is for their previous paper in Am J Clin Exp Immunol 2017. *done*  
3. The setting of steamer for antigen retrieval should be described. *Amended method stated in Step 2.7*  
4. The procedure #8 should be described in more detail. *Done and described in Step 2.13*5. The procedure of hematoxylin staining should be described in more detail. *Noted in Step 1.4*6. I think any tissues should be fine for positive control of hematoxylin staining. Are there any reasons to recommend placental tissue? *FRB was found expressed in myeloid leukemia cells and normal placental tissue*.