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Dear Dr. Wu and the reviewers,

We are submitting a revised version of our manuscript (JoVE59616) entitled "**A Mouse Model of Vascularized Heterotopic Spleen Transplantation for Studying Spleen Cell Biology and Transplant Immunity**" by Jiao-jing Wang, *et al*. for reconsideration for publication in the *Journal of Visualized Experiments.*

We thank the editors for the enthusiasm towards our work. We deeply appreciate the reviewers for their insightful and constructive comments. The following are our point-by-point responses (*in blue*) to reviewers' comments:

Editorial comments:

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

We have proofread the manuscript to ensure there are no spelling or grammatical issues.

2. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.

We have revised the protocol description as suggested (lines 156-190 and lines 198-201).

3. Please use 12 pt font and single-spaced text throughout the manuscript.

We have adjusted the font size and the spacing of the text as required.

4. 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]."

All figures represented in this manuscript are original.

5. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

We have added the ethics statement as suggested (please see lines 111-117).

6. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The utilization of vet ointment on eyes has been specified (please see lines 116-117).

7. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

The protocols of post-surgery analgesia treatments and recovery conditions were added in lines 203-214.

8. Discuss maintenance of sterile conditions during survival surgery.

The discussion was added (lines 120, 129, 132-133 and 156-157).

9. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

This step was specified in the protocol (line 208).

10. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

This step was specified in the protocol (line 210).

11. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text in Protocol and no “we’, “you”, or “our” was used.

12. Please add a one-line space between each of your protocol steps.

We have adjusted the spacing as required.

13. Step 4.1: Please write this step in the imperative tense.

We have rewritten this step in the imperative tense (please see lines 156-159).

14. Please do not abbreviate journal titles for all references.

The references format has been revised as required.

Reviewers' comments:

Reviewer #1:

1. The authors state in lines 1, 31 and 43 that this procedure is heterotopic model of spleen transplantation. As stated in line 148 the recipient's own spleen is being removed. Please explain why this is heterotopic and not orthotopic technique.

We considered this as a heterotopic model because the venous drainage of the transplanted spleen is different from the native spleen. While the blood of the native spleen drains to the portal vein, the blood of the transplanted spleen drains from donor portal vein to the recipient’s IVC.

2. Does the donor animal (BALB/c CD45.2) and the recipient animal (BALB/c CD45.1) are considered syngeneic or allogeneic? Although it might be obvious to you, take into account that this is not clear for other readers.

We thank the reviewer for this important point. CD45.1 and CD45.2 congenic mice have

been widely used for in vivo studies tracking the development or the origin of immune cells as they differ only at the CD45 locus (5 amino acids). Transplantation between BALB/c CD45.2 and BALB/c CD45.1 are generally considered as syngeneic models. We have specified this in the manuscript (lines 49, 103, and 280-286).

3. I have no doubt that the authors perform this procedure in a highly standards of sterility. However, it is better to add a paragraph that explain the surgical field preparation, including sterile conditions, draping and scrubbing of the animals and the surgical team.

We thank the reviewer's suggestions and have added the description to specify the sterile conditions of the surgical steps (please see lines 120, 129, 132-133 and 156-157).

4. Line 116 - is there any induction anesthesia taking place before injecting IP?

For this step, no induction anesthesia is used. We gently grab the mice and perform the i.p injection as specified in line 120.

5. Line 119 - please indicate whether the surgical platform is heated, and if yes, on what temperature?

We thank the reviewer for this suggestion. Yes, we place a heating pad and adjust the temperature to 37°C during the surgery. We have specified this step in the revised manuscript (please see line 156-157).

6. Line 124 - please indicate in which way the skin incision is made (scalpel/scissors/monopolar/etc), and what is the length of the incision?

We used the scissors to make a 3-4 cm skin incision. We have specified this step in the revised manuscript (please see lines 129-130, and 158).

7. Line 126 - how does the intestines and abdominal wall is being retracted? Do you use specific retractor?

We used the self-made retractor (using paperclips) to expose the abdominal wall and used sterile cotton swabs to gently retract the intestines. We have specified this step in the revised manuscript (please see lines 132-133).

8. Line 127 - how does the short gastric vein is being cauterized? monopolar?/unipolar?

We cauterized the short gastric vein using a sterile low temperature cautery (please see lines 134-135).

9. Line 129 - the authors described they dissect the portal vein behind the pancreatic tissue. Is there any physiologic or biologic implication of dissecting through the pancreatic tissue? For example, is there hyperamylasemia or other implications that the authors found out?

To clarify this step, we have modified the previous line 129 (now line 138 in the revised): please refer to line 138 for the changes. To avoid damaging the pancreatic tissue, extra precautions are taken while separating the portal vein from the pancreas. No hyperamylasemia or complications were observed.

10. Line 130 - could you please name the branches? Also, is "away" means "distal"? I think it is

better to use the word "distal" here.

We thank the reviewer for this suggestion. We have specified the name of branches (please see line 139). We agree with the reviewer and have replaced the word "away" with "distal" (please see line 140).

11. Line 134 - please name the two other branches.

We have specified the name of the other branches (please see lines 143-144).

12. Line 136 - how does the Heparin injection into IVC is being performed? Since it is a challenging procedure I wonder if it is possible to inject Heparin sub-cutaneous before the surgery starts and get the same effect?

We use the insulin syringe (301/2G) for the heparin injection into IVC. This step is actually less challenging when performed under the microscope. Normally we do not recommend injecting the heparin before the surgery starts because it may increase the risk of bleeding during the procedures of dissecting the portal vein and aortic-celiac-splenic artery.

13. Line 138 - "cold saline" - what is the temperature?

We used the cold saline (4^{o}C). We have specified the temperature in the manuscript (please see lines 148, 153, and 179).

14. Line 140 - where the harvested spleen is stored and in what conditions till it's being transplanted? For how long it is stored before starting anastomosis?

The spleen was stored in the cold saline (4^{o}C) before being transplanted into the recipients. The average storage time was approximately 10 mins. We have specified this information in the revised manuscript (please sees lines 153 and 270).

15. What is the ischemic time of the spleen transplant?

The average ischemic time of the spleen transplant was 30-45 mins in this study. We recommend to keep the total ischemic time <50 mins. We have specified this information in the revised manuscript (please see line 270).

16. It is not clear when and how the donor is euthanized. Please add more details to the manuscript.

We thank the reviewer for this suggestion. We have added the step to specify the method of euthanizing the donor mice (please see line 153).

17. Line 230 - the authors explain that positioning of the graft is important to prevent twisting of vessels. Please explain how do you inset the graft in place and prevent wandering of the spleen.

We have added a description of the way we inset the graft (please see lines 177-179)

18. How do you manage vasospasm during anastomosis? Do you use irrigating solution containing lidocaine or papaverine?

We did not use any irrigating solutions in this study. The anti-vasospasm drug may help improve the transplant success; however, we have not tested it yet.

19. Do you house the post-op animals separately or together?

In this study, we housed the post-operative animals separately.

20. Do you use lacrimal ointment to prevent recipient's corneal abrasion? If yes, please include in the manuscript.

Yes, we have provided this information in the manuscript (please see lines 116-117).

21. What is the diameter of the artery and vein that are anastomosed? I think this it would be good to get estimated diameters, so the readers will be able to understand how much this procedure is challenging.

The diameters of the artery and vein are approximately 0.4 mm and 0.6 mm respectively. We have specified the diameter of the artery and vein in the revised manuscript (please see line 303).

22. How does this model different / similar to Swirski's model (ref, 17)?

Overall, this model is similar to the one reported by Swirski FK et al. However, there were few technical details included in Swirski's report; it is not specifically clear how the surgery is performed. The current study provides a comprehensive step-by-step protocol of mouse spleen transplantation for interested researchers to follow and to master this technique. In addition, this protocol eliminated some unnecessary surgical steps (e.g. the bile duct ligation) described in the report by Swirski FK et al. and introduces the 11-0 suture for anastomosis, which would help shorten the surgical time and prevent the bleeding. This information was discussed in the revised manuscript (please see lines 313-318).

23. Line 192 - the authors state that success rate is defined by survival of both recipient mouse and transplanted spleen. How do you monitor the transplanted spleen while the animal is alive? Do you use doppler probe? Ultrasound? Other tests?

We thank the reviewer for raising these questions. We defined the success of the procedures based on the survival of both recipient mouse and the spleen graft to post-operative day (POD) 1 or POD7 (our study endpoint). The survival of the spleen graft was confirmed by the macroscopic appearance and flow cytometry analysis of the splenocytes. Based on our experience, the flow cytometry analysis (LIVE/DEAD Cell Viability Assays) is very sensitive to determine whether a spleen graft is survived, as the majority of the spleen cells would be dead if the spleen grafts were necrotic. We have not used doppler probe or Ultrasound, but believe these methodologies could be very helpful to in vivo longitudinal monitoring and will explore its utility if needed. This information was discussed in the revised manuscript (please see lines 220-224).

24. Table 1 - What is "step 1.5"? I could not find this on the text.

This was written in error. It has been corrected to reflect “steps 3.3, 3.4”.

25. Table 1 - What is "step 2.3"? I could not find this on the text.

This was written in error. It has been corrected to reflect “step 4.3”.

26. Table 1 - please explain what is "UW solution"?

“UW solution” refers to “University of Wisconsin cold storage solution”. We have specified

this information in our revised manuscript.

27. When harvesting the post-transplanted spleen, do you see any adhesions of fibrosis related to surgical wound healing?

We did observe some fibrotic donor pancreas tissue attached to the spleen graft, but we did not observe any adhesions related to the wound healing.

Reviewer #2:

Major Concerns:

1. The author claimed 90% of successful rate could be reached based on survival rate of animals and grafts. However in the manuscript only data from POD7 was shown and no description for long-term phenotype. How "survival " was defined? How long the animals and grafts have been monitored and analyzed after surgery?

We thank the reviewer for raising these questions. We defined the success of the procedures based on the survival of both recipient mouse and the spleen graft to post-operative day (POD) 1 or POD7 (our study endpoint). The survival of the spleen graft was determined by the macroscopic appearance and flow cytometry analysis of the splenocytes. Based on our experience, the flow cytometry analysis (LIVE/DEAD Cell Viability Assays) is very sensitive to determine whether a spleen graft is survived, as the majority of the spleen cells would be dead if the spleen grafts were necrotic. Two transplants were monitored for >POD60 with surviving spleen grafts as confirmed by histology and flow cytometry. The data are not included in this manuscript as we plan to perform additional transplants for long term monitoring.

2. In table 1 only "Anesthetic overdose" was given as the potential cause of death. Have the authors encountered other causes? Like bleeding, infection etc.

We thank the reviewer for this question. Yes, we encountered other causes such as bleeding, thrombosis, infection, etc. We have added these causes to Table 1.

Minor Concerns:

1. Protocol 3.6 states perfusing the whole body with 10ml saline through abdominal aorta. The authors should give a rate for perfusion that would damage the blood vessels in the graft.

We thank the reviewer for this suggestion. The perfusion rate is 10 ml/20 s. We have specified this step in the revised manuscript (please line 148).

2. Line 248 says " These findings suggest that the turnover of splenic lymphocytes is much slower than that of the myeloid populations, which underscores the importance of lymphocytes that originated from spleens in adaptive immunity". Please explain how this conclusion was made? Also murine spleen also contains marginal zone B cells which are part of innate immune system.

Thank you for this notion. Our results showed that a relatively high percentage of splenic lymphocytes remained of donor origin. More interestingly, these lymphocytes migrated to (repopulated) in other lymphoid compartments, e.g. Lymph node, bone marrow, and circulation. These findings prompt us to speculate that lymphocytes that originated from spleens are very important in the adaptive immunity. However, more investigations are required to delineate the distinct roles of splenic lymphocytes in adaptive versus innate immunity.

Reviewer #3:

Manuscript Summary:

Major Concerns:

1. This protocol did require a certain level of experience. They claimed the success rate for this protocol is >90% for experience one. What about that rate for a newly trained person then?

Based on our learning experience in overall mouse solid organ transplant models (e.g. mouse heart, lung, or kidney transplant), it may take 6-10 months for a newly trained person (without any experimental microsurgical technique) to skillfully master this technique.

2. For the potential applications of this protocol, the author only indicated it can be used to study the immune cell and spleen transplantation. However, the author has cited references several times to imply the spleen may also be related to cardiovascular diseases. How this protocol can be used to study cardiovascular diseases should be addressed.

We thank the reviewer for this suggestion. We have addressed this question in the revised manuscript (please see lines 246-249: For example, by using the mouse models of spleen transplantation, Swirski et al. found that in response to ischemic myocardial injury, spleen-derived monocytes increase their motility, migrate out of the spleen, adhere to injured tissue, and contribute to the wound healing.)

3.On line 109, they stated to use mice with proper age, gender and weight. Please specify what range is considered to be proper. They discussed a little about the weight in the discussion part. But they didn't talk about the age or gender.

We thank the reviewer for this question. We recommend using 8 to 14-week old mice for this model (as specified in line 256). Concerning the gender, we used male mice in this study. However, whether there is a gender difference regarding the transplant outcome requires further study. Therefore, which gender to use may depend on the study design.

4. For Figure 4, they should include a panel of control mice for comparison.

We thank the reviewer for this suggestion. With consideration of the limited timing for the revision, we are not be able to add a parallel control analysis for this particular study. Based on studies from our group and others, we found that the cellular compositions of syngeneic spleen grafts are similar to that of the spleen from a naive BALB/c mice.

5. On line 209, they mentioned monocytes play an important role. Their representative results should include a flow cytometry for monocyte to show that monocyte is also recovered.

We thank the reviewer for this suggestion. The flow cytometry data of monocyte were added to the supplementary figure 2.

Minor Concerns:

1. On line 103, they said this is a simplified and enhanced protocol compared with Swiski FK et al's protocol. They may need to clarify the difference.

We thank the reviewer for this suggestion. This information was discussed in the revised manuscript (please see lines 307-313). Overall, this model is similar to the one reported by Swirski FK et al. However, there were few technical details included in Swirski's report; it is not specifically clear how the surgery is performed. The current study provides a comprehensive step-by-step

protocol of mouse spleen transplantation for interested researchers to follow and to master this technique. In addition, this protocol eliminated some unnecessary surgical steps (e.g. the bile duct ligation) described in the report by Swirski FK et al. and introduces the 11-0 suture for anastomosis, which would help shorten the surgical time and prevent the bleeding.

2. For the abbreviation used, they need to define that first, such as i.p., POD etc.

We thank the reviewer's suggestion. We have specified these abbreviations (please see lines 121, 220).

3. On line 211. "under-recognition of the role the spleen" should be "under-reconition role of the spleen".

We thank the reviewer's suggestion. We have revised this sentence (please see lines 243-244).

Reviewer #4:

Major Concerns:

The authors only investigate host versus recipient leukocyte turnover in the spleen, 1 and 7 days after surgery. I would recommend to add a longer timepoint, such as 4 weeks, to evaluate the % of donor cells remaining in the spleen and the risk of graft rejection. For leukocyte turnover in the spleen, please refer to Liu K et al, Nat Immunol 2007 (PMID 17450143) who used parabionts and compare the rates of leukocyte turnover at 4 weeks. I would fear that grafted spleen might be massively invaded by recipient cells, which would decreases the impact of this technique for immunological questions.

Is there any fibrosis developing around the grafted spleen?

We thank the reviewer's suggestion. Two transplants were monitored for >POD60 day with surviving spleen grafts as confirmed by histology and flow cytometry. The data are not included in this manuscript as we plan to perform additional transplants for long-term monitoring to address this interesting question.

Thanks again for your consideration!

Sincerely

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