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To
The Editors/Reviewers of JOVE

December the 1st, 2019

Letter of Response regarding manuscript
"Heterotopic heart and cardiac muscle cell transplantation in rats – a simplified and a novel immunological model" (JoVE60956)

Dear Editor, dear Reviewers,

First of all, thank you for the fast review and the advice regarding our manuscript. We appreciate the possibility of re-submitting a revised and improved manuscript.
In the following sections we provide point-by-point replies to all issues raised in the review.
You will receive two revised versions of the manuscript (with and without track-change-function) with this letter as well as the edited figures and table.
We feel that the manuscript has profited considerably from the questions raised by the editor and the reviewers and the implemented changes and hope to meet your approval on the updated version.

If new information given in our replies – not yet integrated into the revised manuscript – should be added to the final version of the manuscript, we would be happy to comply.

If you need any further information, please let us know.

Best regards from Germany,

Oliver Beetz

Point-by-point replies (*Page references refer to the clean version of the revised manuscript)

Editor:

1. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Response:

All figures were transformed to a .psd format in a resolution of 300 DPI. The table containing the material list was converted to a .xlsx file.

2. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response:

The complete manuscript was reread by the authors to ensure no spelling or grammar issues.

3. Please include at least 6 keywords of phrases.

Response:

We provided six updated keyword phrases containing two or more words.

4. 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.

Response:

The formatting of the manuscript was implemented as requested.

5. 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: Axiovision Release 4.8.2.0.

Response:

Commercial language was removed or exchanged by generic terms.

6. 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."

7. Please ensure you answer the "how" question, i.e., how is the step performed?

Response to 6+7:

We reassessed the manuscript and addressed the above mentioned points.

8. 1.1., 2.1: Age sex strain of the animal used.

Response:

Information regarding the age and sex of the donors (Note above 1.1) and the recipients (Note above 2.1) have been added. The respective rat strain used depended on the chosen strain combinations and rejection model. We provided further information on the matter in the section "REPRESENTATIVE RESULTS".

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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.
Response:

We have ensured that we do not exceed the 2.75 page limit for filmable content. We apologize for the inconvenience.

10. 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]."

Response:

Since PLOS ONE works under an Open Access license, the copyright permission for previous publications is part of the editorial policy if the content is cited appropriately (see also https://journals.plos.org/plosone/s/licenses-and-copyright). We have provided the link to the PLOS ONE Licenses and Copyright section in an additional .docx file which we will upload separately. If you would like us to obtain an additional letter from the editor, we are of course happy to comply.

11. Please do not abbreviate the journal titles in the references section.

Response:

We have removed abbreviations of journal titles by applying the "JOVE Reference Output Style" to our bibliography in the "REFERENCES" section via Mendeley Software.

12. Please sort the materials table in alphabetical order.

Response*:*

The materials table has been alphabetically ordered.

Reviewer #1:

-

Reviewer #2:

1. The author thinks their models represent a practicable and well-studied approach and can be performed without further surgical training or background. However, we all know anastomose is very difficult for a freshman. So, we need more data to prove the models is easy to learn.

Response:

This is indeed a valid point; we would therefore like to provide some general remarks concerning the learnability of the model (which were also partially included into the "DISCUSSION" section): During the last decade our model has been performed by medical or PhD students in our department with no surgical experience to begin with. Since it is a general policy of our work group, we operate in pairs of surgeons, whereas the more experienced "surgeon"

is in charge of guaranteeing the success of the transplantation and at the same of gradually improving the skill set of the "freshman".
After a short training period of approximately ten graft explantations and implantations in dead animals, the "freshman" is in charge of harvesting the graft and assisting the graft implantation in approximately ten live animals. Subsequently, the "freshman" actively performs the vascular anastomoses, so that after approximately ten further transplantations, the "freshman" is usually capable of performing all critical steps of the model.
Applying this training concept, past publications from our department using heterotopic heart transplantation in rats showed no differences regarding morbidity, mortality or graft function despite several different teams of surgeons caused by a high turnover of work group members [1–7].
We agree with Reviewer #2, that performing vascular anastomoses is indeed the critical step of solid organ transplantation models in general, however, the presented modifications of our heterotopic heart transplantation model simplify the surgical steps that have to be undertaken before performing the vascular anastomoses and allow less room for critical errors.
Unfortunately, we cannot present statistical data on learning curves to measure the feasibility of our model, since our training concept for mastering the heterotopic heart transplantation represents a gradual and guided process, as stated above, impeding the collection of reliable and comparable statistical data.
Regardless of this fact, statistical data reflecting the degree of difficulty of surgical models in general, especially for transplantat models, are rarely presented in the current literature, making it difficult to compare the degree of feasibility of different models.
If it is necessary to mitigate the statements regarding the feasibility of our model due to missing statistical data, we would of course comply. Nonetheless, we hope that our newly included general statements on the matter ("Mastering the model of heterotopic heart transplantation", page 11, lines 435-452) provide relevant information when assessing the learnability and feasibility of our model.

2. The paper concluded histological analysis showed an increased infiltration of (e.g. TCR +, CD4 + or NKR-P1A/B +) cells in the allogenic grafts, whereas syngenic grafts were largely free of cell infiltration (Figure 6.A-B). We need more data to prove the results.

Response:

We agree that the presented figure (6.A-B) displaying two exemplary histological sections of a syngenic and an allogenic heart graft are not sufficient to statistically prove an increase of cell infiltration in allogenic grafts. We therefore provided further data on the matter and modified Figure 6 by adding a further figure (Figure 6.C) displaying the increase of cell infiltration in allogenic grafts by $CD4^+$ (T helper cells, monocytes), TCR^+ (T cells), NKR-$P1A/B^+$ (Monocytes/macrophages, NK cells, NKT cells) and $CD68^+$ (Monocytes/macrophages) cells by blinded evaluation of sections from five grafts of each group (syngenic reference group and allogenic group, respectively). The classification system used to quantify cell infiltration is described in the edited legend of Figure 6 (page 8-9, lines 344-352).

References:

1. Klempnauer, J. *et al.* Genetic control of rat heart allograft rejection: effect of different MHC and non-MHC incompatibilities. *Immunogenetics*. **30**, 81–88, at <https://link.springer.com/content/pdf/10.1007%2FBF02421535.pdf> (1989).
2. Bektas, H. *et al.* Differential effect of donor-specific blood transfusions after kidney, heart, pancreas, and skin transplantation in major histocompatibility complex-incompatible rats. *Transfusion*. **37** (2), 226–230, doi: 10.1046/j.1537-2995.1997.37297203529.x (1997).
3. Saiho, K.O. *et al.* Long-term allograft acceptance induced by single dose anti-leukocyte common antigen (RT7) antibody in the rat. *Transplantation*. **71** (8), 1124–1131, doi: 10.1097/00007890-200104270-00020 (2001).
4. Bektas, H. *et al.* Blood transfers infectious immunologic tolerance in MHC-incompatible heart transplantation in rats. *Journal of Heart and Lung Transplantation*. **24** (5), 614–617, doi: 10.1016/j.healun.2004.01.016 (2005).
5. Jäger, M.D. *et al.* Sirolimus promotes tolerance for donor and recipient antigens after MHC class II disparate bone marrow transplantation in rats. *Experimental Hematology*. **Volume 35** (Issue 1), Pages 164-170 (2007).
6. Timrott, K. *et al.* Application of allogeneic bone marrow cells in view of residual alloreactivity: Sirolimus but not cyclosporine evolves tolerogenic properties. *PLoS ONE*. **10** (4), 1–16, doi: 10.1371/journal.pone.0119950 (2015).
7. Beetz, O. *et al.* Recipient natural killer cells alter the course of rejection of allogeneic heart grafts in rats. *Plos One*. **14** (8), e0220546, doi: 10.1371/journal.pone.0220546 (2019).