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Production of Humanized Mouse via Thymic Renal Capsule Grafting, CD34+ Cells Injection and Cytokine Delivery

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TITLE:

Production of Humanized Mouse via Thymic Renal Capsule Grafting, $CD34^+$ Cells Injection, and Cytokine Delivery

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SUMMARY:

Humanized mouse models provide a more accurate representation of the human immune microenvironment. This manuscript describes the process in which these models are created through a renal graft of human thymus, injection of human $CD34^+$ cells, and the targeted delivery of human cytokine transgenes to promote $CD34^+$ cell proliferation and differentiation.

ABSTRACT:

Animal models provide a vital translation between *in vitro* and *in vivo* biomedical research. Humanized mouse models provide a bridge in the representation of human systems, thereby allowing for a more accurate study of pathogenesis, biomarkers, and many other scientific queries. In this method described, immune-deficient NOD-scid $IL2R\gamma^{null}$ (NSG) mice are implanted with autologous thymus, injected with liver-derived $CD34^+$ cells followed by a series of injected cytokine deliveries. In contrast to other models of a similar nature, the model described here promotes an improved reconstitution of immune cells by delivering cytokines and growth factors via transgenes encoded in AAV8 or pMV101 DNA-based vectors. Moreover, it offers long-term stability with reconstituted mice having an average lifespan of 30 weeks after $CD34^+$ injections. Through this model, we hope to provide a stable and impactful method of studying immunotherapy and human disease in a murine model, thus demonstrating the need for predictive preclinical models.

INTRODUCTION:

While animal models have created a deeper understanding of cellular and molecular systems, the challenge remains in elucidating the intricacies of species-specific systems, such as immunity,

physiology, and other areas of pathology. Non-human primates (NHP), such as chimpanzees, have historically been used to compensate for the wanting gaps in model research; however, the NHP model can be quite costly and inaccessible, particularly as their use has been banned in Europe[1].

Following a successful grafting procedure, the murine system replicates the human immune system, as demonstrated through the repopulation of the lymphoid organs. The development of mice with functional human immune systems provides the opportunity to conduct translational research on human immunity in a variety of contexts. Immunodeficient mice engrafted with human cells and tissues that can successfully replicate an operative human immune system facilitate the study of hematopoiesis, immunity, gene therapy[2], infectious diseases[3], cancer[4], and regenerative medicine[5]. Our group and some of our collaborators have published results using this model that demonstrates preclinical models of cutaneous melanoma[6]. This model is versatile enough to be applied to innumerable fields beyond the context of melanoma and immunotherapy research.

Peripheral blood mononuclear cells (PBMCs) are commonly used for humanization as they result in robust reconstitution of T cells, which have established roles in immune tolerance; however, because of their low rate of self-renewal and their high rate of mature lineage-committed cells, PBMCs are often replaced with human-stem-cell (HSC)-based products, which can be derived from fetal liver[7]. In combination with these derived HSC products, the addition of implanting the human thymus under the kidney capsules of NSG mice creates a system capable of supporting human T cell development. This model, known as the bone marrow-liver-thymus (BLT), is highly advantageous because it allows for multilineage hematopoiesis, T-cell education in the autologous thymus, and HLA restriction[8].

The model proposed in this manuscript is a modified BLT model with additional cytokine delivery. Proinflammatory cytokines have been shown to bolster the abilities of effector immune cells, specifically through IL-15 based immunotherapies[9]. $CD45^+$ lymphocytes are observed in the peripheral blood of humanized mice (Hu-mice) approximately 8–12 weeks after human $CD34^+$ cell injection, displaying an evident increase in reconstitution compared to circulating blood of regular NSG mice. Using the Adeno-Associated vector to deliver human IL-3, IL-7, and GM-CSF, the levels of human $CD45^+$ cells increased in circulation compared to those mice that do not receive the cytokines. The addition of the DNA Combo II cytokines (SCF, FLT3, CKIT, and THPO) improves T cells and myeloid cell differentiation[10]. The addition of cytokine delivery distinguishes this method amongst the other Hu-mice models, as is supported by published data[10].

The development of this model with an innate and adaptive human immune response has allowed to publish data regarding therapy resistance and the tumor microenvironment[10]. Provided laboratories can access tissue samples to utilize this method; this Hu-mice model has great potential for other labs to study similar fields as well as expand into other areas of immunotherapy and preclinical studies.

PROTOCOL:

All protocols involving the use of animals are closely monitored by the Wistar Institute's Institutional Animal Care and User Committee (IACUC). The laboratory adheres to the guidelines set by this committee and the attending veterinarian to ensure the health, safety, and wellbeing of the animals involved. Prior to following this protocol, veterinarian and IACUC approval are required, and individuals may have variations in the specific surgical techniques and animal handling compared to the protocol per the advice of those aforementioned parties involved in animal welfare.

NOTE: Tissue samples can be frozen until ready for use. Additionally, $CD34^+$ isolation can be performed the same day or the following day as the renal graft surgery. This information will inform when Busulfan will be injected: if intending to conduct surgery on the same day as isolation, Busulfan should be injected preemptively in preparation of receiving tissue. Treat thirty female 5–7 week-old NSG mice with 30 mg/kg (100 µL, PBS 1x) of a freshly made myelo-depleting drug; Busulfan injected IP 24 h before surgery.

1. $CD34^+$ cells isolation

1.1. Prepare the digestion solution by dissolving 100 mg of Collagenase/Dispase in 50 mL of RPMI1640. Use a 0.22 µm vacuum filter to sterilize the digestion solution.

1.2. Fetal liver (>20 weeks of age) is provided in 50 mL of RPMI1640 medium. Aspirate and discard the media, leaving 10 mL inside the tube containing the liver.

1.3. Wash the tissue with 45 mL of RPMI1640 + 100 µg/mL of the antibiotic Primocin twice in a 50 mL conical tube, vacuuming off the media between each wash. Be sure to let the tissue settle to the bottom of the tube before vacuuming off the media.

1.4. Place a sterile tissue sieve in a 200 mL sterile beaker.

1.5. Pour the fetal liver through the sieve and use the end of a 10 mL sterile plastic syringe to grind the tissue.

1.6. Rinse the liver cells remaining in the sieve using 50 mL of RPMI1640 + 100 µg/mL of the antibiotic Primocin followed by 50 mL of RPMI1640 Collagenase/Dispase media (2 mg/mL). The 200 mL beaker now contains the RPMI1640 with Primocin, RPMI1640 collagenase/dispase, and homogenized liver.

1.7. Aliquot the mixture into four 50 mL conical tubes and incubate them in a water bath (37 °C) for 1 h. Shake the conical tubes every 15 min.

1.8. Add 15 mL of Ficoll in four 50 mL conical tubes.

1.9. Carefully layer the homogenized liver solution on top, distributing approximately 35 mL between each tube. Do not disturb the interface.

1.10. Place the tubes in the centrifuge and spin at 800 x *g* for 1 h with minimal acceleration/brake.

1.11. Remove the tubes from the centrifuge and collect the cells from the interface (10–15 mL) into two 50 mL conical tubes.

1.12. Add PBS 1x up to 50 mL to wash the pellet, and centrifuge at 300 x *g* for 5 min. Discard the supernatant.

1.13. Resuspend the pellet in 10 mL of ACK (Ammonium-Chloride-Potassium) lysis buffer and incubate for 5–10 min. Fill the tube with 1x PBS.

1.14. Manually count the cells under the microscope using trypan blue and a hemocytometer. Centrifuge the cells at 300 x *g* for 5 min.

NOTE: The liver mononuclear cells can be injected directly from here if desired, for example, if the cell count is lower than 50 million cells.

1.15. Vacuum off the PBS and resuspend the cell pellet in 300 µL of the separation buffer (2 mM EDTA, 5 µg/mL of BSA in 1x PBS) for 100 x 10^6 cells or less. For more than 100 x 10^6 cells, use a ratio based on the above quantity of cells to determine the amount of buffer (i.e., double the cells, double the buffer).

1.16. Add 100 µL of FcR blocking reagent and incubate 2–5 min.

1.17. Add 100 µL of CD34 microbead antibodies, mix well and incubate on ice for 30 min; mechanically shake by hand after 15 min.

1.18. Add 7–10 mL of the separation buffer and filter through 100 µm, 70 µm, and 40 µm cell strainers in that order.

1.19. Place a column-based cell-separator in a magnetic separator. Place a 15 mL conical tube underneath the separator to collect the flow-through and buffer.

1.20. Fill the column with 500 µL of the separation buffer.

1.21. Pour the cell suspension through the column.

1.22. Fill the column with the flow-through and buffer that has been collected in the conical tube.

1.23. Wash the column 3 times with 500 µL of the separation buffer.

1.24. Discard the flow-through and replace the tube with a 15 mL polystyrene tube.

1.25. Fill the column with 1 mL of the separation buffer, remove the column from the magnet and flush the plunger down in one push.

1.26. Count the cells and resuspend in 1x PBS for injection. Place on ice until use.

2. Thymic processing

2.1. Wash the autologous thymus (>20 weeks of age) by adding 5–8 mL of RPMI1640, allow the tissue to settle. Carefully remove the medium by vacuum while avoiding contact with the thymus. Repeat this once more.

2.2. Suspend in 10 mL of room temperature RPMI1640 with Primocin (100 µg/mL).

2.3. Transfer thymus to a tissue-treated Petri dish. Ensure the thymus is fully coated in Primocin-media.

2.4. Incubate for 30–45 min at room temperature.

2.5. Vacuum off the Primocin-media. Wash the thymus with 5–8 mL of RPMI1640, remove the media by vacuum, and repeat this once more.

2.6. Suspend the thymus in 5–8 mL of RPMI1640.

2.7. Prepare the incubated thymus for grafting by slicing 1 mm x 2 mm segments using two scalpels.

NOTE: This will be grafted into the mice; therefore, ensure that the thymus slices can be vacuumed both in and out of the syringe and the attached 22 G Hamilton needle with relative ease.

3. Subrenal capsule grafting and CD34 cells injection

3.1. Examine the mice for their general health before the surgery. Shave the mice to expose an area (approximately 3” x 2”) on the left lateral body.

3.2. Anesthetize the mice via isoflurane (induction 5%, maintenance 2%–3%) in the chamber and then transfer individual mice from the chamber to a surgical stage with a nose cone.

3.3. Sanitize the shaved area with Chlorohexidine and alcohol prep swabs. Ensure individual anesthetization via pinch test.

3.4. Make a large incision (5–7 mm) posterior, lateral to the spine of the mouse, and fold back the skin.

3.5. Ensure that at this point, the spleen is visible beneath the fascia. Make a smaller ~2 mm incision in the fascia. Ensure that the incision is as small as possible while allowing the kidney to be fully exposed.

3.6. Determine the location of this incision, visually identifying the spleen and using it as a marker for the kidney, which is not visible.

NOTE: The murine kidney is located behind a grouping of fat in the northeast quadrant to the spleen.

3.7. From here, position the hand (surgeon) under the mouse's side opposite the incision and push to expose the kidney. Reposition any fat to fully expose the kidney.

3.8. Utilize the fingers on the same hand to stabilize the exposed kidney. If necessary, use sterile 1x PBS to lubricate the exposed kidney.

NOTE: It is important to note that the surgeon must use different fingers than those that handled the mouse's side to hold the kidney to maintain sterility. Consult with the institute's veterinarian regarding proper maintenance of sterility.

3.9. Simultaneously, let the assistant prepare a blunt syringe with the fetal thymus tissue. Gently suck up 2–3 medium-sized thymus chunks (1 mm x 2 mm) with minimal media to the tip of the needle and then hand it over to the surgeon to perform the procedure.

NOTE: The number of chunks of thymus depends on the size of the tissue, usually 2 chunks are enough to get humanized mice.

3.10. Let the surgeon insert the needle through the caudal end of the kidney capsule until the distal end is reached (~2 mm), to the point where the needle is visible, but it does not break through the cranial side of the kidney capsule. Let the assistant plunge the syringe.

3.11. Place the kidney, along with any exposed fat, back in the peritoneum. Place one absorbable suture (size 4) and close the superficial incision with a vet bond glue.

3.12. Postoperatively, administer a dose of the analgesic drug, 25 µL of sustained-release buprenorphine (1 mg/mL) via subcutaneous injection (1 mg/kg).

3.13. Check that mice begin to recover within 5 min post-operation.

3.14. Between cages, change the gloves and sterilize the instruments in a glass bead sterilizer.

3.15. From 1–3 h after surgery, inject 100 µL of human $CD34^+$ cells (approximately 100,000 cells) into the mouse by intravenous (IV) injection.

3.16. Conduct a general health check on the mice after concluding the surgery (usually 2 h after the first cage), then 24 h and 48 h afterward.

3.17. If there are any abnormalities in disposition or vital signs, monitor the mice further. Once they have cleared those checkpoints, monitor the mice less strictly (2x–3x per week).

3.18. One week after surgery, inject (IV) the mice with 100 µL of AAV8 human cytokine (IL-3, IL-7, and GM-CSF; 2 x 10^9 genome copies/mL).

3.19. Two weeks after surgery, perform IM injections of the plasmid DNA Combo II.

3.19.1. Use an electroporation machine to perform IM injections of plasmid DNA Combo II: SCF, FLT3, CKIT, and THPO. For each mouse, combine 25 µL of SCF (2 mg/mL), 25 µL of THPO (2 mg/mL), 25 µL of FLT3 (2 mg/mL), and 25 µL of CKIT (10 mg/mL).

3.19.2. Perform one injection per hind leg, i.e., two total, with 50 µL per leg, followed by electroporation on each injection site.

REPRESENTATIVE RESULTS:

Following successful surgery and appropriate postoperative injections, $CD34^+$ differentiation can be confirmed via flow cytometry. Approximately 8 weeks after surgery, mice are bled in preparation for FACS, recurring every 2 weeks until a specific threshold of human immune cells is met as is described previously[10]. Briefly, 100 mL of blood was collected in blood collection tubes coated with lithium and heparin. After the lysis of red blood cells using ACK lysis buffer, the cells were washed with FACS buffer (Phosphate buffered saline, 2% FBS, and 0.1% sodium azide) and centrifuged. The cell pellet was then resuspended in 100 mL of FACS buffer and incubated with a panel of antibodies (anti-mouse CD45, anti-human CD45, anti-CD3, anti-CD4, anti-CD8, and anti-CD20). To identify live and dead cells, cells were stained with DAPI, and the lymphocytes were gated based on the FSC-A and SSC-A parameters. A gate was generated to select the live cells and from those the single cells. From those single, live lymphocytes population, we first distinguished the human from the mouse $CD45^+$ cells. $CD20^+$ and $CD3^+$ cells were identified from the human $CD45^+$ cells, and finally, $CD4^+$ and $CD8^+$ cells were identified from the $CD3^+$ cells (T cells). To elaborate, Hu-mice are ready to be used for immunotherapy experiments when they hit the threshold of >25% $HuCD45^+$ in the total lymphocytes and >8%–10% $CD8^+$ in the human $CD3^+$ cells (**Figure 1**). In the early stages of reconstitution, $CD20^+$ levels are high, and $CD3^+$ levels are low. As cells proliferate and reconstitute with time, the $CD20^+$ levels drop as $CD3^+$ levels rise. This correlates with when the mouse thymus, mouse spleen, and the renal capsule-grafted Hu-thymus get repopulated with human lymphoid precursor cells that undergo differentiation.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow cytometry analysis of a reconstituted mouse. The analysis is gated to isolate the different populations of cells, beginning with the total population of lymphocytes, then distinguishing between live cells, single cells, and various stem cell populations with specific attention paid to the $CD45^+$ and $CD8^+$ to ascertain proximity to the thresholds, as is previously reported[10].

DISCUSSION:

This manuscript has herein described generating humanized mice via human fetal thymus grafted under the renal capsule and subsequent $CD34^+$ injection to recreate a human immune system.

While the protocol functions to create the best model possible, certain steps are essential to viability. For example, during the $CD34^+$ isolation, it is essential that one looking through the microscope can identify $CD34^+$ cells. Though it may seem redundant, automatic counting machines do not always identify these cells due to their morphology, and the machine itself may misidentify the debris as positive cells. Therefore, it is crucial to identify these cells manually. Moreover, it is essential to confirm that the thymus is truly entering the kidney capsule during renal graft surgery. This can be visualized or palpated: the surgeon should be able to feel the thymus pieces entering the kidney.

Importantly, the process has a few caveats that can determine the success of the model. The first being, the quality of the fetal tissue must be individually assessed to determine whether the isolated cells could potentially be toxic to the mouse. For example, tissue aged above 20 weeks appears firmer and cuts more precisely during preparation. There is generally less degradation. These qualities are general indicators of better caliber tissue. A second caveat of the technique regards the quantity and quality of $CD34^+$ cells; the number of cells produced is variable and sometimes may not provide enough for injections. In these instances, it is possible to circumvent the problem by injecting the liver mononuclear cells isolated earlier in the protocol. Third, after the implementation of a successful model, the mice may begin to develop graft-versus-host disease (GVH) around 25 weeks of age. While the model can last up to 30 weeks, it is around 25 weeks that the investigators should pay careful attention to the mice-looking changes in body score, loss of hair, and facial distress. Around this point, one should always consult with the attending veterinarian, determine the quality of life, and attend to the mice as dictated or euthanize.

The main difference between this protocol and other humanized models is the use of cytokines to improve the proliferation and stabilization of hematopoietic cells first and then enhance the differentiation of immune cells. The effects of the use of the cytokines is addressed by the increased levels of human $CD45^+$ cells in mouse peripheral blood circulation and aided in T-cell and myeloid cell differentiation compared to those mice with no cytokines, as previously reported[10].

In summary, the described model provides a humanized mouse with a stable lifespan and functional recapitulation of the human immune cells. The model can be recreated by others in an effort to study a myriad of questions relating to immunotherapy, viral research, regenerative

medicine, and numerous fields beyond these contexts.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no competing interests with this manuscript.

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Figure tiff

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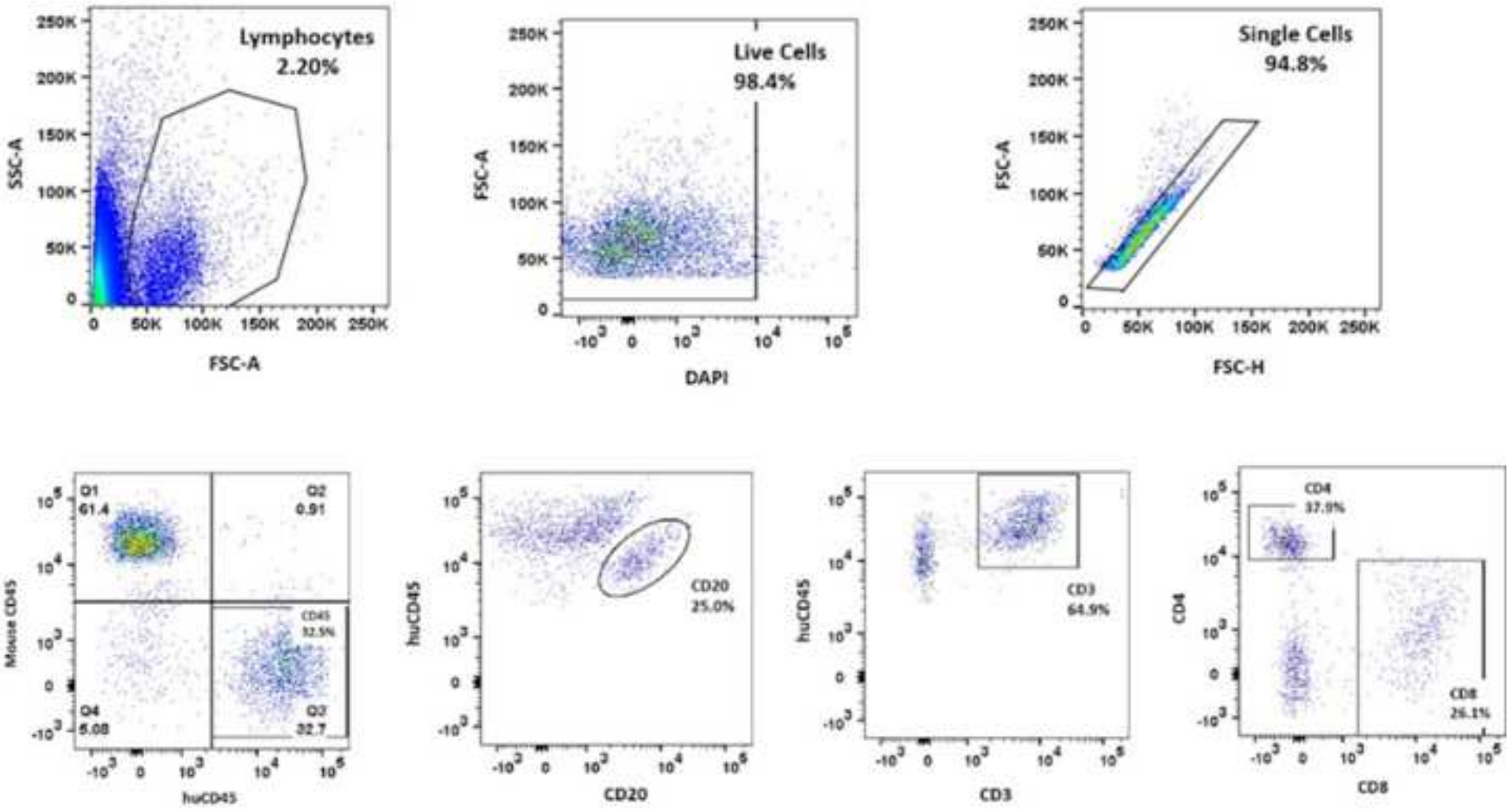

Philadelphia, PA, USA

25 August 2021

Dear Editor,

Please find the revised version of the manuscript, “Production of Humanized Mouse via Thymic Renal Capsule Grafting, $CD34^+$ Cells Injection and Cytokine Delivery.” (ID JoVE62906R1) by Gregorio *et al.* We appreciate the reviewer’s comments and suggestions. Please find a point-by-point response, description of the modifications and a detailed engagement with the comments as requested. I hope the reviewers and journal find the answers satisfactory.

Best,

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Editorial comments:

1. Please note that the manuscript has been formatted to fit the journal standard. Comments to be addressed are included in the manuscript itself. Please review and revise accordingly.

RESPONSE: We have reviewed accordingly.

2. Please revise the following lines to avoid previously published work: 77-79. Please refer to the iThenticate report attached.

RESPONSE: We have made changes to improve this.

3. Please confirm whether the article is a JPV. Please note that the videos uploaded in the drive cannot be used as Jove produces videos based on the script developed after the article is finalized. We would like to know the reason why the videos updated in the drive cannot be used for the production?

4. Please ensure that the comments addressed in the rebuttal (Reviewer 3) are included in the appropriate sections of the manuscript.

We have added the comments in the sections of the manuscript.

For example:

(i) The yield is dependent on the age, size, and quality of the tissue received. Purity is assessed via flow cytometry using CD34 specific antibodies.

(ii)The gating strategy used for the flow cytometry is outlined in detail in the Nature Communications paper referenced (Supplementary Figure 12), as well as a list of antibodies (Supplementary Table 1). (Please include a statement regarding the gating strategy in the representative results and cite the Nature communication paper as reference)

5. Please consider including the cytokine delivery also for filming to match the title of the manuscript.

RESPONSE: We can include it in the video if is needed.

Reviewers' comments:

Reviewer #1:

The manuscript is much improved. All of my concerns have been addressed in this last revision.

RESPONSE: We appreciate the comments of the reviewer.

Reviewer #3:

Manuscript Summary:

This is a re-review of an original submission in regards to the authors' response to my original comments. In general, the authors have addressed my comments thoroughly, with a few exceptions

below. While the authors did address my main points clearly in the responses, many of these comments were not included in the revised manuscript, and I do still feel that these are questions that other readers/viewers will have regarding the protocol.

Major Concerns:
To that end, I feel that two points could be added to the manuscript that would cover most of these points all together in a general way:
First: the authors should specify that veterinarian and IACUC approval are required and individuals may have variations in the specific surgical techniques and animal handling compared to the protocol (or some other equivalent wording).
RESPONSE: Thank you for this comment. We have added to the disclaimer prior to surgery to address that specifically.

Second: Some of the details as to variation could also be included in a general comments, especially as regard to tissue quality/cell number/pieces used. This is definitely important especially as the authors state, in regards to my original comment for line 276-278 that "there is no published data that supports this". Since there is no available reference, the authors are not showing any data to support these points, and they have elected not to address these points specifically, again a general comment at the beginning covering all these points (original points for 3.7, lines 276-278, 1.26, 3.13 etc) instructing the reader/viewer to evaluate the quality of the tissues & cell yield to determine number of animals (obviously in more specific detail) would greatly improve the usability of the protocol.
RESPONSE: We have added more details to address the reviewer's comments.

Original comment for 3.6 (now 4.5) has not been addressed. As written, one person is "position[ing] the hand under the mouse's side opposite the incision and push to expose the kidney" this (as written) says this person is touching the non-sterile mouse with their hand. The instructions then go on to say "Utilize the fingers on the same hand to stabilize the exposed kidney" - same hand implying also the same person and unless using different fingers the person is now touching the exposed kidney with fingers that have come in contact with the mouse's side, that according to the previous instructions and the authors' rebuttal is the "opposite side" and not sterile as "just the shaved area is sterile". This is in direct violation of sterile surgical technique regardless of whether

gloves are changed between mice, gloves are not changed between touching the non-sterile area of this mouse and its internal organs. For, this point, I still strongly disagree with the authors' wording - these protocols would not be approved by our veterinarians or IACUC. Since the authors have approval at their institution and this is their protocol, that is clearly acceptable at some places. But since this is intended to be a protocol usable by others, at minimum the whole protocol/step 1 should include a note/critical point at the beginning advising readers to get their own veterinary approval for the procedures prior to beginning any work.
The point in bold above would also cover my comments for 3.9, 3.13 etc which the authors have also not elected to update for a more general audience in their protocol.
RESPONSE: Thank you for the observation. We have adjusted the wording to make it more specific that there are different fingers being used to contact the mouse's side and that of the kidney - that is an important detail and we appreciate the reviewer highlighting that. Additionally, we have added the note that there should be veterinary training and approval for this procedure.

Minor Concerns:
3.10 I do not object that the authors use a dose approved by the veterinarian - this is fine and completely understandable; my comment was solely that the math as listed in the original (and subsequent unchanged protocol) does not add up with the information given. The authors' explanation also does not help me understand their reasoning as they now (rebuttal for line 3.10) say "0.5mL total volume" yet line 4.9 still says "25uL of sustained-release buprenorphine (1 mg/kg)".
RESPONSE: We apology for the mistake, here are the correct calculations and explanation about it. In our hands.5-7 weeks old mice are around 20-25 gr. Accordingly, we use 25 μL of buprenorphine at 1 mg/mL in order to use 1mg of drug per kilogram of weigh.
We have edited the point to address this.

Figure. Since the gating strategies and necessary flow cytometry information are already published, this should be referenced in the figure legend so that it can be found and/or replicated.
RESPONSE: We have added the reference in the figure legend.