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A Mouse Model of Vascularized Heterotopic Spleen Transplantation for Studying Spleen Cell Biology and Transplant Immunity

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TITLE:

A Mouse Model of Vascularized Heterotopic Spleen Transplantation for Studying Spleen Cell Biology and Transplant Immunity

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Spleen, transplantation, mouse, model, microsurgery, cell biology

SUMMARY:

This protocol details the surgical steps of a mouse model of vascularized heterotopic spleen transplantation, a technically challenging model that can serve as a powerful tool in studying the fate and longevity of spleen cells, the mechanisms of distinct spleen cell populations in disease progression, and transplant immunity.

ABSTRACT:

The spleen is a unique lymphoid organ that plays a critical role in the homeostasis of the immune and hematopoietic systems. Patients that have undergone splenectomy regardless of precipitating causes are prone to develop an overwhelming post-splenectomy infection and experience increased risks of deep venous thrombosis and malignancies. Recently, epidemiological studies indicated that splenectomy might be associated with the occurrence of cardiovascular diseases, suggesting that physiological functions of the spleen have not yet been fully recognized. Here, we introduce a mouse model of vascularized heterotopic spleen transplantation, which not only can be utilized to study the function and behavioral activity of splenic immune cell subsets in different biologic processes, but also can be a powerful tool to test the therapeutic potential of spleen transplantation in certain diseases. The main surgical

steps of this model include donor spleen harvest, the removal of recipient native spleen, and spleen graft revascularization. Using congenic mouse strains (*e.g.*, mice with CD45.1/CD45.2 backgrounds), we observed that after syngeneic transplantation, both donor-derived splenic lymphocytes and myeloid cells migrated out of the graft as early as post-operative day 1, concomitant with the influx of multiple types of recipient cells, thus generating a unique chimera. Despite relatively challenging techniques, this procedure can be performed with >90% success rate. This model allows tracking the fate, longevity, and function of splenocytes during steady state and in a disease setting following a spleen transplantation, thereby offering a great opportunity to discover the distinct role for spleen-derived immune cells in different disease processes.

INTRODUCTION:

The spleen is the largest secondary lymphoid organ in the body and is critical in the immune and hematopoietic systems. Its functions are primarily carried out by two morphologically distinct compartments, the red pulp and the white pulp[1]. The red pulp is a three-dimensional meshwork of venous sinuses and splenic cords that consist of reticular fibers, reticular cells, and associated macrophages. This unique structure allows the red pulp to act as an effective blood filter that removes foreign materials and old or damaged erythrocytes. The white pulp includes follicles, marginal zone, and the periarteriolar lymphoid sheaths (PALS) and is an important site for antigen trapping and processing, lymphocyte homing, transformation, proliferation, and maturation[2]. Nevertheless, the spleen has commonly been considered as a dispensable organ because other lymphatic organs, such as lymph nodes, can also carry out some of its functions and the loss of spleen does not usually lead to death. Splenectomy has therefore been widely performed as a therapeutic method for patients with splenic injury or benign hematologic diseases[3]. However, patients with splenectomy face a number of long-term complications. Bacterial infections are the best-recognized complications of splenectomy[4,5]. Recently, the overwhelming post-splenectomy sepsis has been recognized as an intensive complication of splenectomy associated with a high mortality[6]. Moreover, recent epidemiological studies indicate that splenectomy may be associated with the occurrence of cardiovascular diseases, suggesting that further physiological functions of the spleen remain to be explored[7,8].

Both spleen autotransplantation and spleen allotransplantation have been utilized in the clinic. Currently, spleen autotransplantation by implanting sections of splenic tissue into pouches created in the greater omentum is considered as the only possibility for preserving splenic function after traumatic splenectomy[9,10]. However, the efficacy of this surgery is debatable as post-surgery complications like aseptic necrosis of the splenic tissue and small bowel obstruction due to postoperative adhesions could occur[11]. Spleen allotransplantation is involved in multivisceral transplantation[12]. Clinical evidence from multivisceral transplantation suggests that spleen allotransplantation may play a protective role in small bowel allograft rejection without causing graft-versus-host disease (GVHD)[12]. Yet literature regarding the beneficial effect of spleen allotransplantation as a component of multivisceral transplantation is still limited and the underlying mechanisms remain to be defined. In 2006, Yair Reisner et al. reported that transplanting pig embryonic spleen tissue that has no T cells to mice could cure hemophilia A, a genetic disease without causing GVHD[13], supporting that spleen transplantation holds

therapeutic promise in certain diseases. Therefore, there is a need for further investigations on the therapeutic potential of spleen transplantation.

Animal models of spleen transplantation are valuable to explore the unappreciated function of the spleen-derived immune cells in disease progression as well as to test the potential therapeutic effect of spleen transplantation. Experimental whole spleen transplant models have been documented since early 1900s, as reviewed by Cohen[14]. In 1969, Coburn Richard J. and Lee et al. detailed the technique of spleen transplantation in rats[15,16]. More recently, Swirski FK et al. described a mouse model of spleen transplantation[17]. Compared to rat models, mouse models of spleen transplantation are more attractive due to its several inherent advantages. For example, by utilizing a mouse model, we can access an expansive variety of reagents unavailable to that of rat models. Moreover, by using congenic mice (e.g., mice with CD45.1/CD45.2 background), a syngeneic spleen transplantation makes it possible to track the fate, longevity, and function of splenocytes[18]. Based on the work by Swirski FK et al.[17], we further established this simplified and enhanced protocol of spleen transplantation in mice. The protocol described below combines both reliability and feasibility in a standardized manner and can be utilized as a tool to study spleen biology and transplant immunity.

PROTOCOL:

All procedures and animal use in this study were performed according to protocols approved by the Northwestern University Internal Animal Care and Use Committee (IACUC). In this study, 8 to 10-week old male CD45.2 and CD45.1 mice (both on BALB/c background, from Jackson laboratory) were used as spleen donors and recipients, respectively, to create syngeneic spleen transplantation models. All animals were housed in the sterile environment in the animal facilities of Northwestern University. The eye lubricant was applied to all mice post-anesthetization to prevent dryness.

1. Surgical preparation, anesthetization, and analgesia regimen.

1.1. Place a sterile disposable drape (45.7 cm x 66 cm) on the surgical platform. Gently grab the mouse, inject ketamine (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p) for anesthesia, and inject 0.05 mg/kg buprenorphine subcutaneously for analgesia.

1.2. Ensure the depth of anesthesia by toe-pinch, shave the hair in the whole abdomen area with a razor and place the mouse on the sterile surgical platform under the operating microscope at 6-10X magnification.

2. Donor spleen harvest

2.1. Sterilize the abdomen with an alcohol prep pad, secure the limbs with surgical tape, and make a 3-4 cm midline vertical skin incision from the pubis to the xiphoid process with scissors.

2.2. Retract the abdominal wall with sterile retractors made from paperclips. Move the intestines to the right flank of the abdomen (surgeon's left) side with a sterile cotton swab to

expose the spleen. Cauterize the short gastric vein attached to the spleen with a sterile low temperature cautery (**Figure 1A**). Place a piece of sterile gauze soaked with 37 °C saline over the spleen to keep it moist (**Figure 1B**).

2.3. Separate and mobilize the portal vein from the pancreatic tissue (**Figure 1C**) by ligating the portal vein branches (superior pancreaticoduodenal vein and right gastric vein); place a suture around the portal vein distal from the splenic vein (**Figure 1D**).

2.4. Flip the spleen to the right side to expose the aorta and celiac trunk with the splenic artery (**Figure 1E**). Dissect and mobilize aortic-celiac-splenic artery by ligating the hepatic artery and gastric artery; place a suture around the aorta proximal to the celiac artery (**Figure 1F-G**).

2.5. Inject 100 international units (IU) heparin into the inferior vena cava (IVC) to heparinize the whole body and wait 3 min to ensure the heparin take effects. Ligate the aorta proximal to celiac artery, transect the portal vein, and then perfuse the whole body using 10 mL of heparinized cold (4 °C) saline (10 mL/20 s) from the abdominal aorta distal to celiac trunk (**Figure 1H**).

2.6. Collect the spleen graft *en bloc* with the associated aortic-celiac-splenic segment and the portal vein along with a segment of splenic vein and a small portion of pancreatic tissue. Preserve the graft in 5 mL of 4 °C saline before transplant. Euthanize the mouse by cervical dislocation.

3. Recipient splenectomy and spleen graft implantation

3.1. Place a heating pad on the surgical platform and adjust the temperature to 37 °C. Place a sterile drape (45.7 cm x 66 cm) on top of the heating pad to create a sterile surgical platform. Repeat steps 2.1 and 2.2 for surgical preparation and anesthetization. Make a 3-4 cm midline incision and retract the abdominal wall as described in step 2.1 and step 2.2.

3.2. Carefully move the intestine to right side of the mouse with a sterile cotton swab to expose the recipient's spleen. Ligate the splenic vein and artery and remove the spleen.

3.3. Carefully move the intestine to left side of the mouse and cover the intestines with wet gauze (soaked with sterile 37 °C saline). Dissect and ligate the lumbar branches of the infrarenal aorta and IVC; cross-clamp the infrarenal aorta and IVC by using two 4-mm microvascular clamps.

3.4. Place an 11-0 nylon suture through the infrarenal aorta (a full thickness) and retract to create an elliptical aortotomy by a single cut with microscissors (the length should match the diameter of the donor aorta, **Figure 2A**). Pierce the IVC using a 30G needle to create an elliptical venotomy and extend the opening to donor portal vein-matched length using microscissors (**Figure 2A**).

3.5. Clear the intraluminal blood or blood clot (in the aorta and IVC) with 500 µL of heparinized saline (10 units/mL).

3.6. Place the spleen graft in the right flank of the recipient mouse abdomen; carefully identify the donor's aortic cuff and the donor's portal vein. After making sure that the vessels are not twisted, cover the spleen graft with gauze soaked with cold (4 °C) saline.

3.7. Connect the donor's aortic cuff to the proximal and distal apex of the recipient's aortaotomy with two stay sutures (11-0 nylon suture, same as below) (**Figure 2B,C**). Make an anastomosis with 2-3 bites of continuous 11-0 nylon sutures between the donor's aortic cuff and the recipient's aortaotomy (anterior wall) (**Figure 2D**). Turn the spleen graft over to the left side of the recipient; make the anastomosis between the donor's aortic cuff and the recipient's aortotomy (posterior wall) (**Figure 2E**).

3.8. Perform an anastomosis to connect the donor's portal vein to the posterior wall of the recipient's IVC, using 4 to 5 bites of continuous sutures on the inside of the IVC and then close the suture on the outside of the IVC (**Figure 2F,G**).

3.9. Release the vessel clamps and use sterile cotton swab to tamponade bleeding until the spleen color is recovered (**Figure 2H**).

3.10. Close the abdomen with a 5-0 synthetic absorbable vicryl suture in a continuous pattern. Close the skin layer with a 5-0 nylon suture in an interrupted pattern.

NOTE: For the steps 4.7-4.8, alternatively, make an anastomosis between the donor's portal vein and the recipient's IVC first (step 4.8); then make an anastomosis between the donor's aortic cuff and the posterior wall of the recipient's aortotomy, using 2 to 3 continuous sutures in the inside of the aorta and close the suture on the outside of the aorta.

4. Animal recovery

4.1. Inject 1 mL of warm saline subcutaneously via 4 separate locations (0.25 mL/location) after closing the abdomen.

4.2. Keep the mouse in a temperature-controlled incubator (30 °C) for the first few hours post-operation, monitor the mouse until it has regained sufficient consciousness, and then transfer the mouse to a new clean cage with regular food and water, with a heating pad (30 °C) underneath the cage. Keep the mouse post-surgery in a separate cage.

5. Post-surgical pain management

5.1. Inject 0.05 mg/kg buprenorphine subcutaneously 24 h and 48 h post-surgery to maintain analgesia regimen.

REPRESENTATIVE RESULTS:

The entire procedure of mouse spleen transplant can be completed within 90 min by experienced microsurgeons. Our laboratory has performed over 100 spleen transplants in mice. The success rate is over 90%, as defined by the survival of both recipient mouse and the spleen graft to post-operative day (POD) 1 or POD 7 (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. The technical challenges of this procedure, common complications, and their troubleshooting are summarized in **Table 1**.

To test the graft morphology post-transplantation, Haemotoxylin and Eosin (H&E) staining was performed in spleen isografts of BALB/c mice at POD 1 and POD 7. The representative pictures are shown in **Figure 3**. The architecture of the spleen isografts remained intact during the first postoperative week. The red pulp, white pulp, and the marginal zone were still clear and distinguishable. To investigate the cell migration after spleen transplantation, flow cytometry was performed at POD 1 and POD 7 to examine the phenotypes of leukocytes in the spleen isografts, lymph nodes, blood, and bone marrow. As shown in **Figure 4A**, at POD 1, 51 ± 7% (mean ± SD, same as below) of the spleen cells were donor-derived and 46 ± 3% were recipient-derived. At POD 7, donor-derived leukocytes accounted for 32 ± 10 % of total spleen cells, and recipient-derived cells were up to 56 ± 13%. We also observed that spleen leukocytes migrated into the lymph nodes, blood, and bone marrow as early at day 1 and maintained at day 7 (**Figure 4B**), generating a unique chimera valuable for splenocyte trafficking research.

TABLE AND FIGURE LEGENDS

Table 1. Troubleshooting methods.

Figure 1. Donor spleen harvest. (A) Cauterize the short gastric vein attached to the spleen. **(B)** Place a small piece of sterile warm wet gauze over the spleen to keep it moist. **(C)** Dissect and isolate the portal vein behind the pancreas. **(D)** Ligate the side branches of the portal vein and place a suture round the portal vein distal to the splenic vein. The dashed lines represent the location transecting later for the anastomosis with the recipient IVC. **(E)** Flip the spleen over to the right side of the abdomen (surgeon's left) to expose the aorta and celiac trunk with its branches including splenic artery. **(F)** Dissect and mobilize aortic -celiac -splenic artery by ligating the two other branches. **(G)** Place a suture around the aorta proximal to the celiac artery. The dashed lines represent the location transecting later used for the anastomosis with recipient abdominal aorta. **(H)** After ligating the aorta and transecting the portal vein, perfuse the spleen graft with 10 mL of heparinized saline through the aorta.

Figure 2. Spleen graft implantation. (A) After isolation and cross clamping the aorta and IVC, make a longitudinal aortotomy and venotomy in the aorta and IVC, respectively. **(B-D)** Place the spleen graft on the right side of the abdomen (surgeon's left side). Make the end-to-side anastomosis between donor aortic cuff and the anterior wall of the recipient's aortotomy, using 2-3 bites of continuing suture. **(E)** Turn the spleen graft over to the left flank of the recipient; repeat the previous procedure between the donor aortic cuff and the posterior wall of the

recipient's aortotomy and close the suture on the outside of aorta. **(F-G)** Make an end-to-side anastomosis between the donor portal vein and the posterior wall of the recipient's IVC, using 4 to 5 bites of continuous 11-0 nylon suture in the inside of the IVC and then close the suture on the outside of the IVC. **(H)** After completing the anastomosis, release the vessel clamps and place some cotton buds to help stop the bleeding.

Figure 3. Representative histology of spleen isografts on day 1 and day 7 post-operation. Syngeneic spleen transplantations were performed using BALB/c mice. Spleen isografts were harvested on day 1 and day 7 post-transplantation and fixed in 10% formalin for 48 h. H&E staining was performed using paraffin-embedded tissue section. The representative histology shows that the spleen isografts remain intact at 1-week post-transplantation. Scale bar is 250 μm. POD, post-operative day.

Figure 4. The chimera created by syngeneic spleen transplantation. Three syngeneic spleen transplantations were performed using BALB/c CD45.2 mice as donors and BALB/c CD45.1 mice as recipients. The spleen grafts, blood, lymph node (LN), and bone marrow (BM) in recipient mice were harvested on day 1 and day 7 post-transplantation. Single cell isolation and flow cytometry analysis were performed to analyze the phenotype of the cells. **(A)** Representative dot plots (gating from live singlets) showing the percentages of donor or recipient - derived leukocytes in the indicated samples. **(B)** Percentages (mean ± SEM) of the indicated populations in donor-derived cells in the indicated samples at day 1 and day 7. POD, post-operative day.

Supplementary Figure 1. Representative plots (n = 3) showing the percentages of donor-derived versus recipient-derived lymphocytes in the transplanted spleen, lymph node, blood, and bone marrow.

Supplementary Figure 2. Representative plots (n = 3) showing the percentages of donor-derived versus recipient-derived CD11b+Ly6C+monocytes in the transplanted spleen, lymph node, blood, and bone marrow.

DISCUSSION:

Compelling evidence suggests that spleen-derived monocytes play an important role in sterile inflammatory processes such as atherosclerosis[19], acute ischemic brain[20] or lung injury[18], as well as myocardial I/R injury and remodeling[21-23]. These reports highlight the under-recognition role of the spleen in many chronic diseases, of which cardiovascular disease is an important one (especially given it is the number one killer globally). The mouse model of spleen transplantation offers a great opportunity to discover the role of spleen-derived immune cells in various diseases as well as how they are primed in the spleen. 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[17]. Furthermore, this model is useful to address the longevities of mature immune cells in the spleen and underlying mechanisms and to explore therapeutic potentials of spleen transplantation.

Several aspects should be taken into consideration to improve the success of this protocol. First, it is critical to choose the proper experimental animals during the design process since the mouse weight, strain, and health condition could affect the difficulty of the surgical steps and experimental results. Our laboratory recommends using 8 to 12-week old mice with over 25 g weight to decrease the mortality that might be caused by bleeding. Second, during the spleen harvest procedure, too much manipulation of the spleen vessels could easily lead to breeding or vasospasm and potentially result in micro thrombosis in the spleen graft. Getting familiar with the anatomy of the mouse abdomen before surgery would be helpful to accelerate the learning process. Third, during the recipient surgery, the spleen graft should be always maintained moist and cool. Appropriate protection of spleen grafts could reduce the transplant ischemia/reperfusion (I/R) injury and prevent graft failure after transplantation. In addition, considering that the donor vessels for anastomosis are relatively long, positioning the spleen grafts properly before the anastomosis is critical to prevent twisting of the vessels. Moreover, the diameters of the recipient aortotomy and venotomy of IVC should be always comparable to those of the donor aorta and portal vein to ensure the proper blood flow and prevent thrombosis.

The average storage time of spleen grafts in 4 °C saline is around 10 min. The total ischemic time should be limited to less than 50 min to ensure minimum transplant failure. We recommend using the University of Wisconsin (UW) cold storage solution to preserve the spleen graft, if over 1 h cold, ischemic time is needed in studies. It should be noted that a small portion of the pancreas intimately attach with the spleen, and attempt to remove the whole from the spleen grafts would easily lead to graft or vascular complications and markedly increase the operation time. We observed that the pancreas tissue attached to the spleen graft would undergo atrophy at POD 7 though some remained viable with the spleen grafts. Whether or not to remove the pancreas tissue attached with spleen grafts depends on the research purposes.

Availability of congenic mice has made it possible to track the origin of the spleen cells, donor versus recipient after transplantation. In this study, we used BALB/c CD45.2 and BALB/c CD45.1 congenic mice as spleen donors and recipients to create a syngeneic spleen transplantation model. Organ or tissue transplantation between these congenic mouse strains has been widely used to track the origin and the development of immune system. Despite the recent report regarding a point mutation associated with CD45.1 that influences NK cell response[24], no transplant rejection was reported between these strain combinations. We observed a substantial influx of recipient cells into spleen grafts that occurred as soon as at POD 1. It is likely that recipient cells responded to the transplant I/R injury as the majority of the recipient cells were granulocytes. 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 (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 (**Figure 4**, **Supplementary Figure 1** and **Supplementary Figure 2**).

The major limitation of this protocol is that it requires extensive microsurgical training for individuals with limited microsurgical experience to master this technique. 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. Compared to mouse models of heart, or kidney transplantation, this model could be more challenging since it involves additional tissue dissection steps to isolate the spleen graft during the donor procedures. Moreover, the diameter of the donor portal vein (around 0.6 mm) is smaller than the IVC, which makes it more difficult for the anastomosis. Another limitation is that it is not clear if the grafted spleen would be massively invaded by recipient' cells at later transplant phase (e.g., POD 60). However, this model is also very attractive for its several inherent advantages. Firstly, mice and humans share a substantial range of similar genomes, thereby allowing for a relatively accurate representation of a realistic application. Moreover, comparing to the mouse model of non-vascularized spleen autotransplantation using the sections of splenic tissue, this vascularized spleen transplant model is less likely to develop complications such as aseptic necrosis of the splenic tissue and small bowel obstruction due to postoperative adhesions.

The mouse model of spleen transplantation has been previously reported by Swirski FK et al. However, no detailed information was not described. Our study provides a comprehensive step-by-step protocol of mouse spleen transplantation for interested researchers to follow and to mater this technique. Moreover, this protocol eliminates several unnecessary steps described in the report by Swirski FK et al. (e.g., the bile duct ligation) and introduces the 11-0 suture for anastomosis, which would help shorten the surgical time and prevent the bleeding.

In conclusion, this model could be a powerful tool to explore the mechanisms of the splenic cell population in responses to pathogens, injury, inflammation, or transplant rejection and is valuable for the test of the therapeutic potential of the spleen transplantation. With proper training and practice, this procedure can be performed with >90% success.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Cesta, M. F. Normal structure, function, and histology of the spleen. *Toxicologic Pathology.* **34** (5), 455-465, doi:10.1080/01926230600867743, (2006).

2 Mebius, R. E. & Kraal, G. Structure and function of the spleen. *Nature Reviews Immunology.* **5** (8), 606-616, doi:10.1038/nri1669, (2005).

3 Misiakos, E. P., Bagias, G., Liakakos, T. & Machairas, A. Laparoscopic splenectomy: Current concepts. *World Journal of Gastrointestinal Endoscopy.* **9** (9), 428-437, doi:10.4253/wjge.v9.i9.428, (2017).
4 Kristinsson, S. Y., Gridley, G., Hoover, R. N., Check, D. & Landgren, O. Long-term risks after splenectomy among 8,149 cancer-free American veterans: a cohort study with up to 27 years follow-up. *Haematologica.* **99** (2), 392-398, doi:10.3324/haematol.2013.092460, (2014).
5 Thai, L. H. *et al.* Long-term complications of splenectomy in adult immune thrombocytopenia. *Medicine (Baltimore).* **95** (48), e5098, doi:10.1097/MD.0000000000005098, (2016).
6 Sinwar, P. D. Overwhelming post splenectomy infection syndrome - review study. *International Journal of Surgery.* **12** (12), 1314-1316, doi:10.1016/j.ijsu.2014.11.005, (2014).
7 Rorholt, M., Ghanima, W., Farkas, D. K. & Norgaard, M. Risk of cardiovascular events and pulmonary hypertension following splenectomy - a Danish population-based cohort study from 1996-2012. *Haematologica.* **102** (8), 1333-1341, doi:10.3324/haematol.2016.157008, (2017).
8 Crary, S. E. & Buchanan, G. R. Vascular complications after splenectomy for hematologic disorders. *Blood.* **114** (14), 2861-2868, doi:10.1182/blood-2009-04-210112, (2009).
9 Di Carlo, I., Pulvirenti, E. & Toro, A. A new technique for spleen autotransplantation. *Surgical Innovation.* **19** (2), 156-161, doi:10.1177/1553350611419867, (2012).
10 Holdsworth, R. J. Regeneration of the spleen and splenic autotransplantation. *British Journal of Surgery.* **78** (3), 270-278 (1991).
11 Tzoracoleftherakis, E., Alivizatos, V., Kalfarentzos, F. & Androulakis, J. Complications of splenic tissue reimplantation. *Annals of the Royal College of Surgeons of England.* **73** (2), 83-86 (1991).
12 Kato, T. *et al.* Transplantation of the spleen: effect of splenic allograft in human multivisceral transplantation. *Annals of Surgery.* **246** (3), 436-444; discussion 445-436, doi:10.1097/SLA.0b013e3181485124, (2007).
13 Aronovich, A. *et al.* Correction of hemophilia as a proof of concept for treatment of monogenic diseases by fetal spleen transplantation. *Proceedings of the National Academy of Sciences of the United States of America.* **103** (50), 19075-19080, doi:10.1073/pnas.0607012103, (2006).
14 Cohen, E. A. Splenosis; review and report of subcutaneous splenic implant. *Archives of surgery.* **69** (6), 777-784 (1954).
15 Coburn, R. J. Spleen transplantation in the rat. *Transplantation.* **8** (1), 86-88 (1969).
16 Lee, S. & Orloff, M. J. A technique for splenic transplantation in the rat. *Surgery.* **65** (3), 436-439 (1969).
17 Swirski, F. K. *et al.* Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* **325** (5940), 612-616, doi:10.1126/science.1175202, (2009).
18 Hsiao, H. M. *et al.* Spleen-derived classical monocytes mediate lung ischemia-reperfusion injury through IL-1beta. *Journal of Clinical Investigation.* **128** (7), 2833-2847, doi:10.1172/JCI98436, (2018).
19 Robbins, C. S. *et al.* Extramedullary hematopoiesis generates Ly-6C(high) monocytes that infiltrate atherosclerotic lesions. *Circulation.* **125** (2), 364-374, doi:10.1161/CIRCULATIONAHA.111.061986, (2012).

20 Kim, E., Yang, J., Beltran, C. D. & Cho, S. Role of spleen-derived monocytes/macrophages in acute ischemic brain injury. *Journal of Cerebral Blood Flow & Metabolism.* **34** (8), 1411-1419, doi:10.1038/jcbfm.2014.101, (2014).
21 Bronte, V. & Pittet, M. J. The spleen in local and systemic regulation of immunity. *Immunity.* **39** (5), 806-818, doi:10.1016/j.immuni.2013.10.010, (2013).
22 Wang, N. P. *et al.* Recruitment of macrophages from the spleen contributes to myocardial fibrosis and hypertension induced by angiotensin II. *Journal of the Renin-Angiotensin-Aldosterone System.* **18** (2), 1470320317706653, doi:10.1177/1470320317706653, (2017).
23 Tian, Y. *et al.* The spleen contributes importantly to myocardial infarct exacerbation during post-ischemic reperfusion in mice via signaling between cardiac HMGB1 and splenic RAGE. *Basic Research in Cardiology.* **111** (6), 62, doi:10.1007/s00395-016-0583-0, (2016).
24 Jang, Y. *et al.* Cutting Edge: Check Your Mice-A Point Mutation in the Ncr1 Locus Identified in CD45.1 Congenic Mice with Consequences in Mouse Susceptibility to Infection. *Journal of Immunology.* **200** (6), 1982-1987, doi:10.4049/jimmunol.1701676, (2018).

Figure 1

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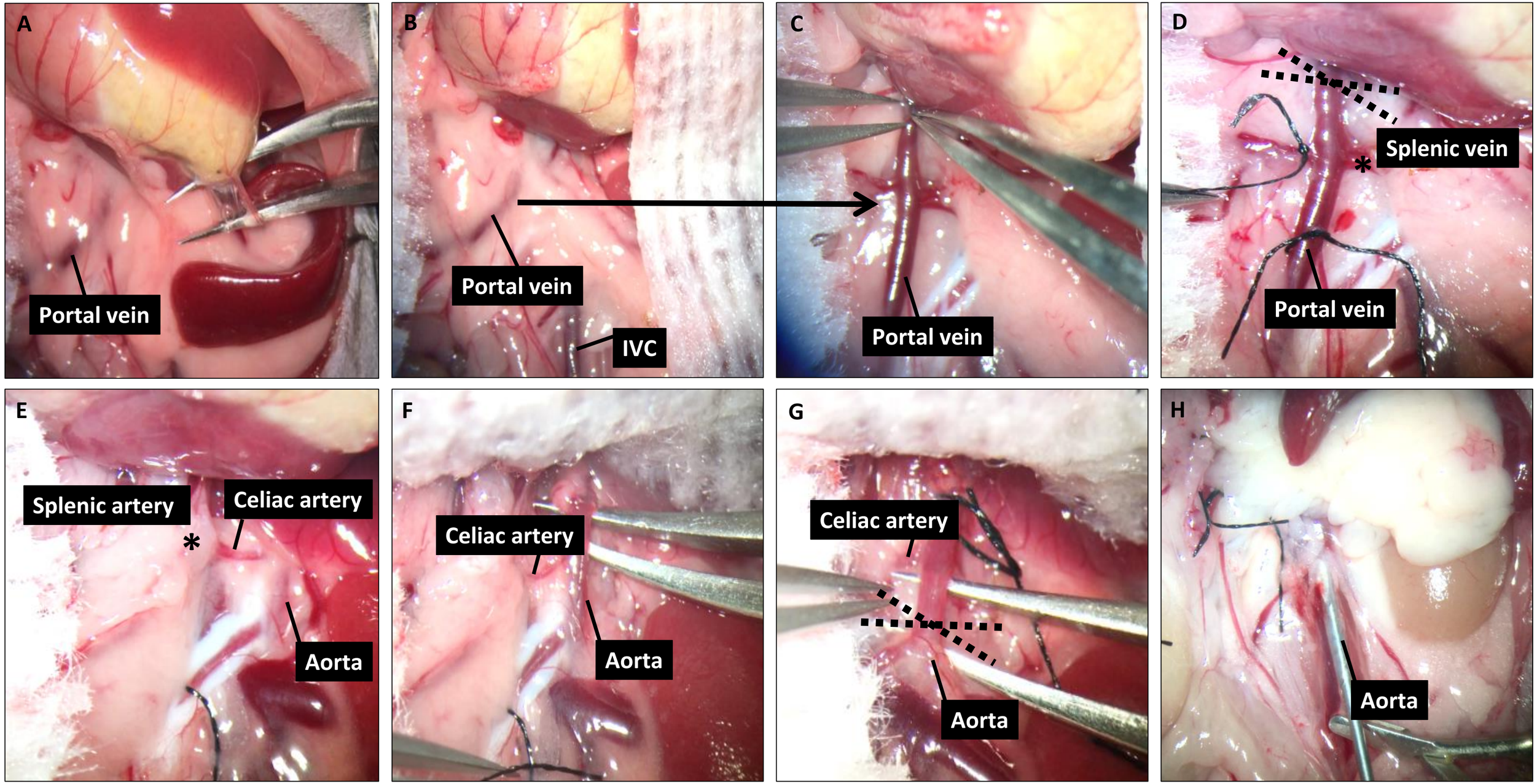

Figure 2

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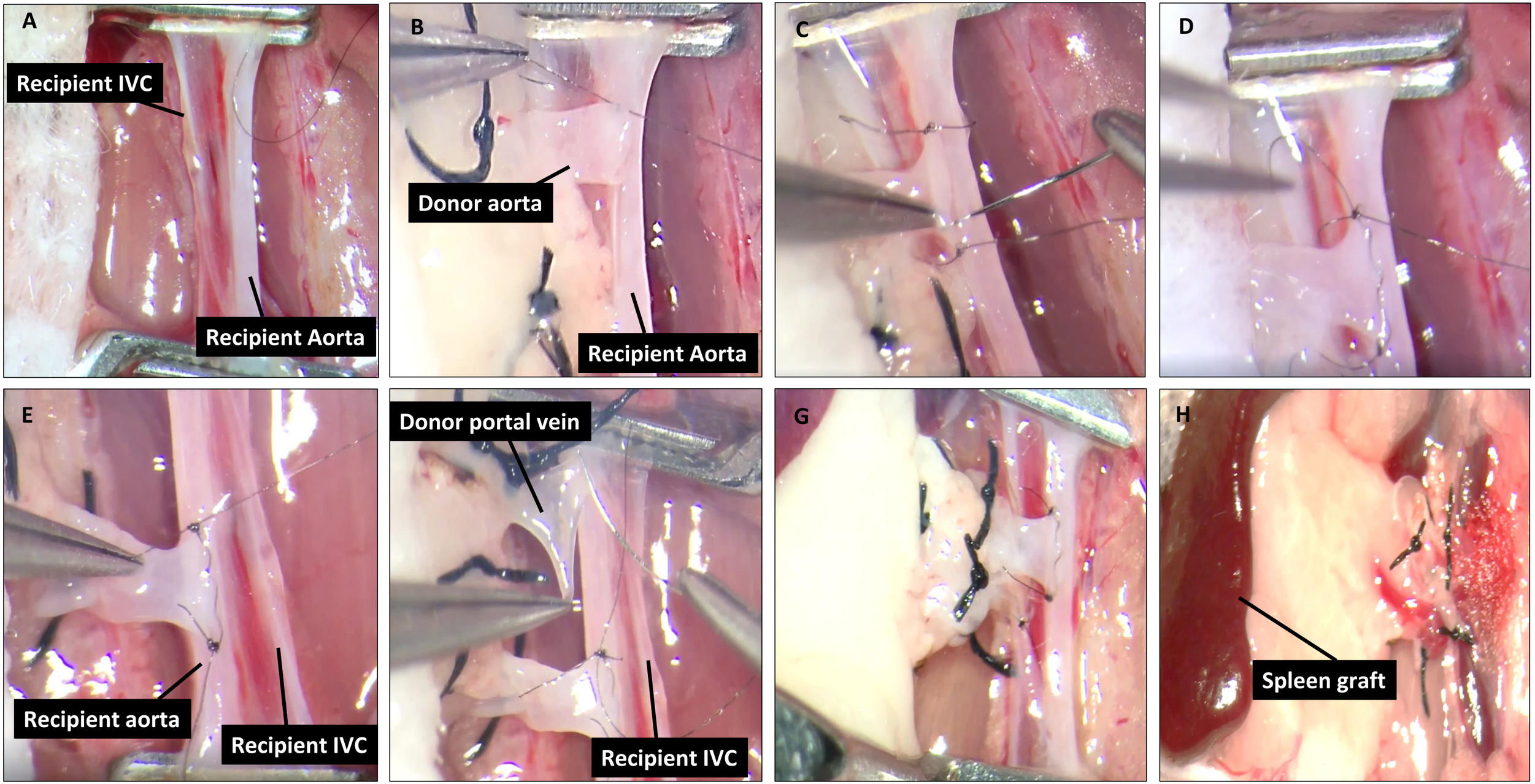


Figure 3

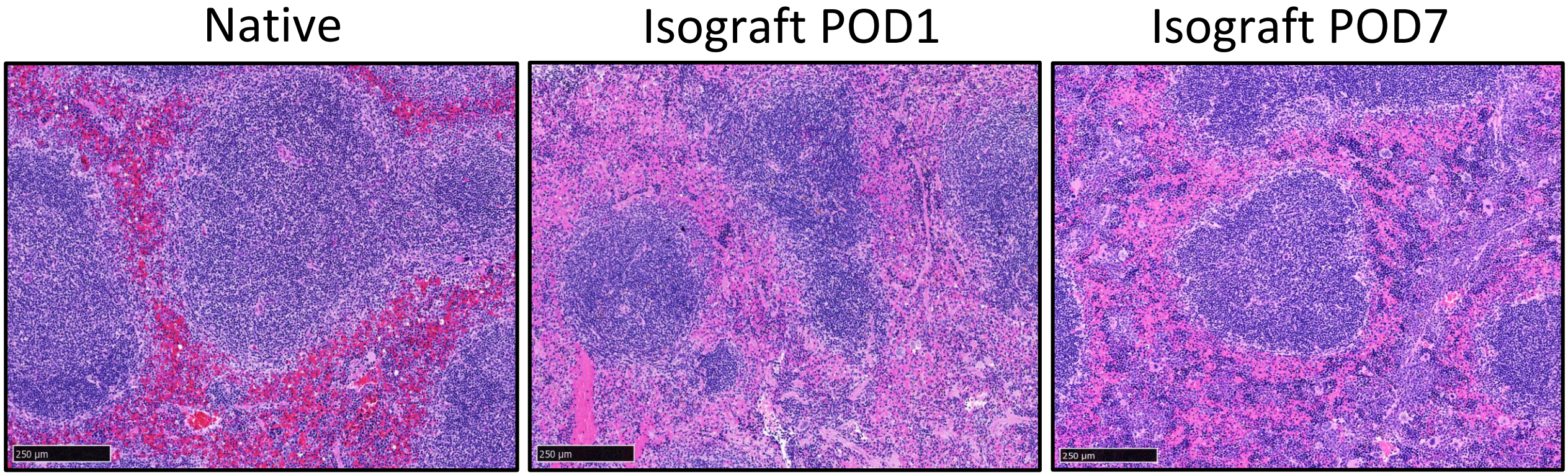
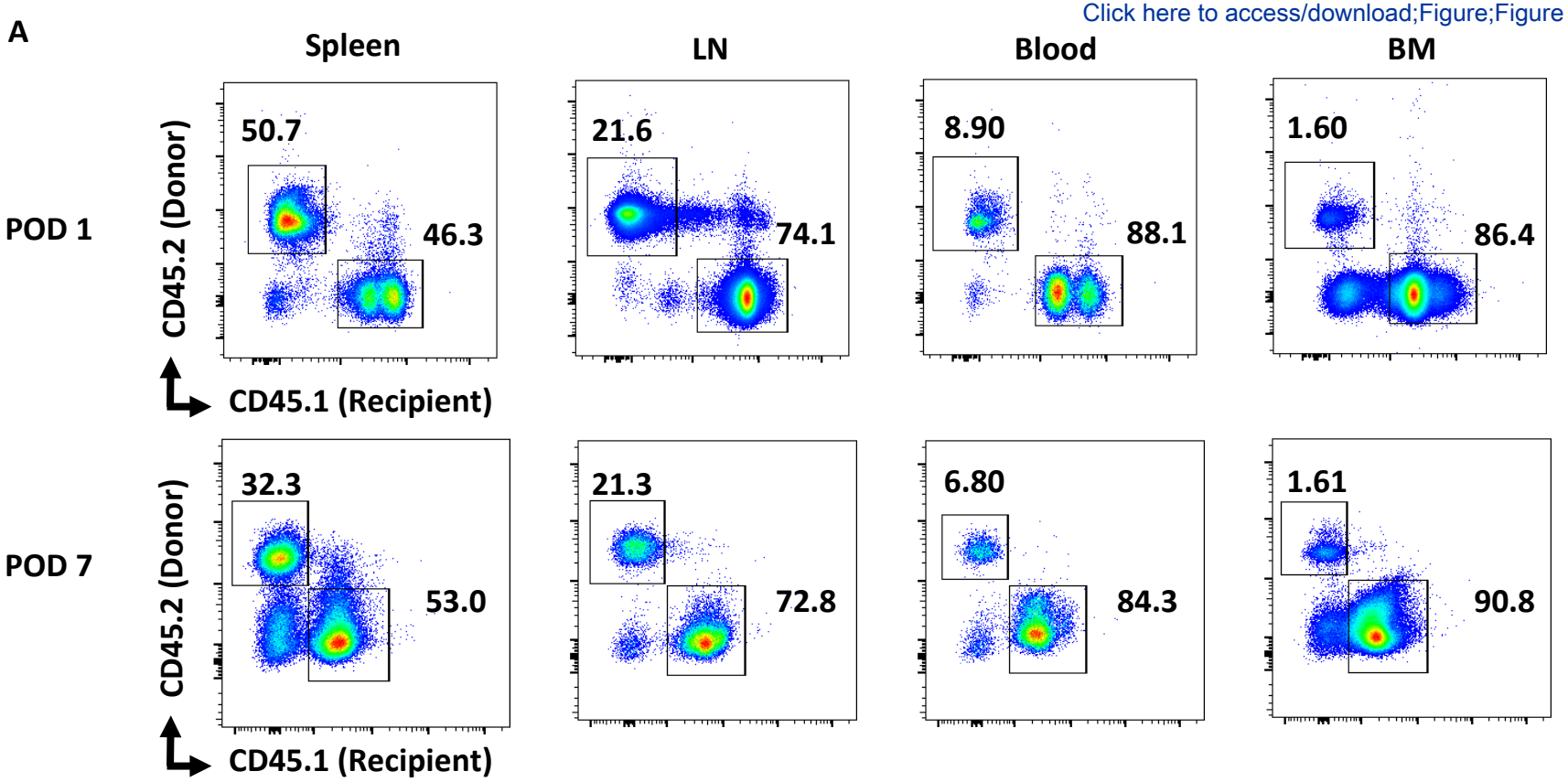


Figure 4



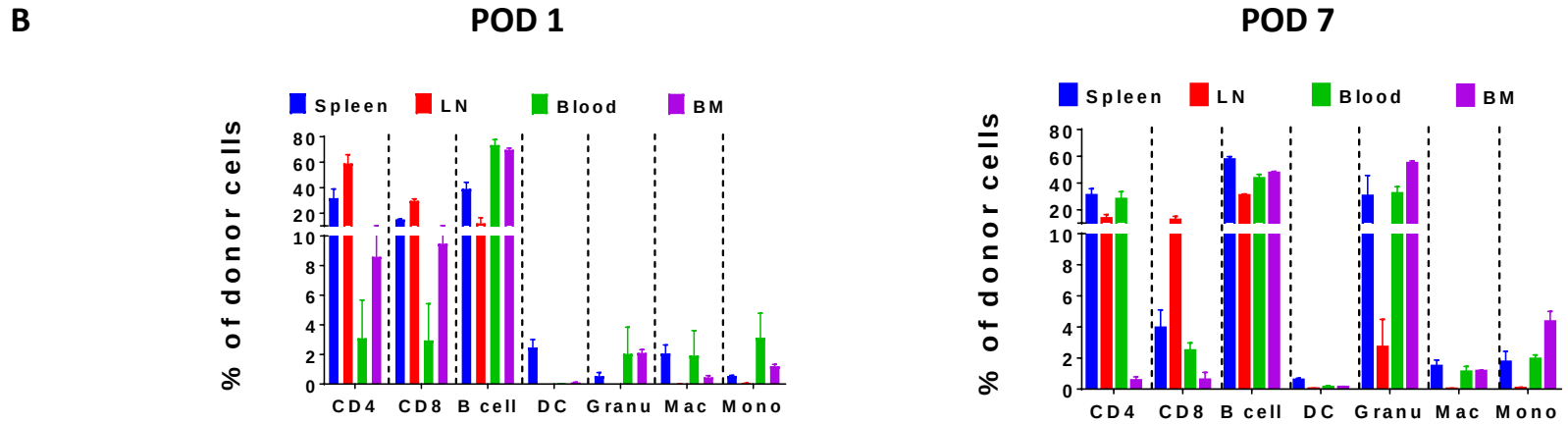


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_Spleen Tx.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
Ketamine	Wyeth	206205-01
Xylazine	Lloyd Laboratories	139-236
Heparin solution	Abraxis Pharmaceutical Products	504031
Injection grade normal saline	Hospira Inc.	NDC 0409-4888-20
70% Ethanol	Pharmco Products Inc.	111000140
ThermoCare Small Animal ICU System	Thermocare, Inc.	
Adson Forceps	Roboz Surgical Instruments	RS-5230
Derf Needle Holder	Roboz Surgical Instruments	RS-7822
Extra Fine Micro Dissecting Scissors	Roboz Surgical Instruments	RS-5881
Micro-clip	Roboz Surgical Instruments	RS-5420
7-0 silk	Braintree Scientific	SUT-S 103
11-0 nylon on 4-mm (3/8) needle	Sharpoint DR4	AK-2119
Ms CD45.2 antibody	BD Bioscience	553772
Ms CD45.1 antibody	BD Bioscience	553776
Ms CD11b antibody	BD Bioscience	557657
Ms B220 antibody	BD Bioscience	553089
Ms Ly6C antibody	eBioscience	48-5932-80
Ms Ly6G antibody	BD Bioscience	561236
Ms F4/80 antibody	BD Bioscience	565614
Ms CD11c antibody	BD Bioscience	558079
Ms CD3 antibody	eBioscience	48-0032-82
Ms CD4 antibody	BD Bioscience	552051
Ms CD8 antibody	BD Bioscience	563786
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit	Thermo Fisher	L34955

Comments/Description

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Title of Article: A mouse model of vascularized heterotopic spleen transplantation for studying spleen biology and transplant immunity

Author(s): Jiao-Jing Wang, Longhui Qiu, Ramiro Fernandez, Xin Yi Yeap, Charlie Xiaoying Lin, Zheng Jenny Zhang

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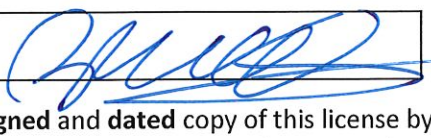
Signature:	 Date: 12/20/2018

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NORTHWESTERN UNIVERSITY

February 10, 2019

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.

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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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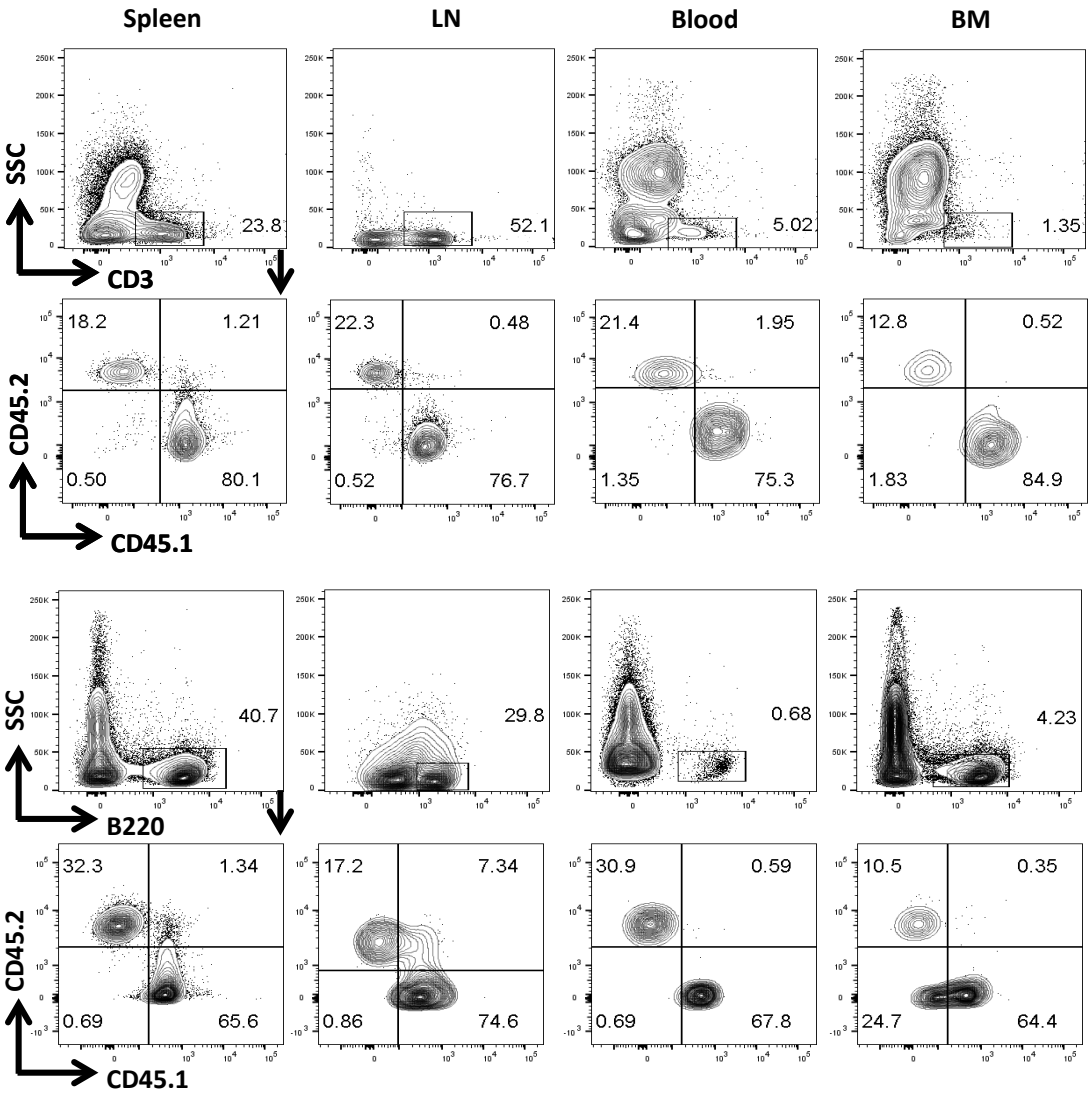
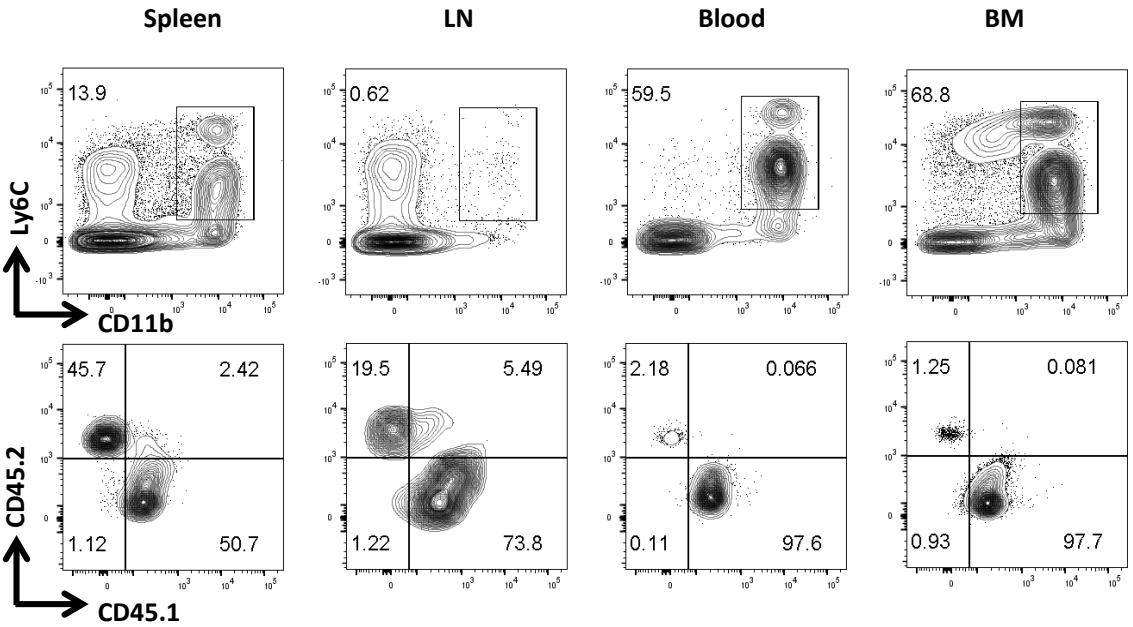


Supplementary Figure 1. Representative plots (n=3) showing the percentages of donor-derived versus recipient-derived lymphocytes in the transplanted spleen, lymph node, blood, and bone marrow. CD3+ cells and B220+ cells were gated from live single cells isolated from transplanted spleen, lymph node (LN), blood, and bone marrow (BM) at day 7 post-transplantation. The percentages of CD45.2+ (donor) and CD45.1+ (recipient) cells are indicated.



Supplementary Figure 2. Representative plots (n=3) showing the percentages of donor-derived versus recipient-derived CD11b+Ly6C+monocytes in the transplanted spleen, lymph node, blood, and bone marrow. CD11b+Ly6C+monocytes were gated from live single cells isolated from transplanted spleen, lymph node (LN), blood, and bone marrow (BM) at day 7 post-transplantation. The percentages of CD45.2+ (donor) and CD45.1+ (recipient) cells are indicated.