Video Article

A Novel Microsurgical Model for Heterotopic, En Bloc Chest Wall, Thymus, and Heart Transplantation in Mice

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Abstract

Exploration of novel strategies in organ transplantation to prolong allograft survival and minimizing the need for long-term maintenance immunosuppression must be pursued. Employing vascularized bone marrow transplantation and co-transplantation of the thymus have shown promise in this regard in various animal models.[1-11] Vascularized bone marrow transplantation allows for the uninterrupted transfer of donor bone marrow cells within the preserved donor microenvironment, and the incorporation of thymus tissue with vascularized bone marrow transplantation has shown to increase T-cell chimerism ultimately playing a supportive role in the induction of immune regulation. The combination of solid organ and vascularized composite allotransplantation can uniquely combine these strategies in the form of a novel transplant model. Murine models serve as an excellent paradigm to explore the mechanisms of acute and chronic rejection, chimerism, and tolerance induction, thus providing the foundation to propagate superior allograft survival strategies for larger animal models and future clinical application. Herein, we developed a novel heterotopic en bloc chest wall, thymus, and heart transplant model in mice using a cervical non-suture cuff technique. The experience in syngeneic and allogeneic transplant settings is described for future broader immunological investigations via an instructional manuscript and video supplement.

Video Link

The video component of this article can be found at https://www.jove.com/video/53442/

Introduction

Cardiac transplantation is the treatment of choice for end-stage heart failure. Both technical advancements and pharmacological innovations have propelled the field to early graft acceptance rates above 90%.[12,13] Despite this, 60-80% 5-year graft survival is at a standstill and chronic rejection, characterized by transplant vasculopathy, remains inevitable.[14-16] Furthermore, patients are subjected to multiple surgical procedures and lifelong immunosuppression, which are associated with chest wall deformities and medical sequelae and toxicities, respectively. The need for innovative approaches to extend allograft survival, minimize the immunosuppressive requirements, and offer reconstructive options for anatomical deformities is pressing.

Vascularized composite allotransplantation offers a unique strategy for improving heart transplant outcomes both from an immunological aspect as well as a reconstructive perspective.[17] Vascularized composite allografts are also unique in a way that they have an inherent source of donor-derived hematopoietic stem cells which has shown a favorable ability to reduce immunosuppression and induce and sustain mixed chimerism.[1-8] Additionally, co-transplantation of the thymus has shown to prolong survival of both, solid organ transplants and vascularized composite allografts.[2,9-11] Combining these strategies with heart transplantation offers a novel solution to the aforementioned challenges facing heart transplantation.[18]

Murine models serve as excellent platforms for mechanistic *in vivo* investigation because of the availability of antibodies and well-defined inbred and knockout strains.[19-21] Although heart transplantation in mice is commonly studied using a heterotopic intraabdominal microsurgical suture transplant model[22-25], a heterotopic, cervical, non-suture cuff technique model has shown to be extremely replicable, reliable, and carries fewer rates of thrombosis.[19,26,27] The goal of this study is to develop a heterotopic en bloc osteomyocutaneous chest wall, thymus, and heart transplant

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technique in mice to study the immunological mechanisms of combined solid organ and vascularized composite allotransplantation using a cervical non-suture cuff technique. This cluster allograft is perfused through the anastomosis of the donor descending aorta to the right common carotid artery and the donor pulmonary artery to the right external jugular vein. Preservation of the internal thoracic vessels and associated thymus branches is paramount to perfusing the chest wall (sternum, ribs, muscles, and skin) and thymus.

Protocol

All operative procedures were completed in compliance with Johns Hopkins University and the United States Department of Agriculture and Public Health Service requirements. This protocol follows the Johns Hopkins University Animal Care and Use Committee, institutional review board approved guidelines (protocol number M013M490). Final survival data was recorded for the surgical procedures described below. Both donor and recipient animals receive pre-emptive anesthesia using buprenorphine at 0.1 mg/kg s.c. one hour prior to surgery and in the recipient animal buprenorphine is re-administered at the same dose after transplant and re-dosed as needed in the first 48 hours after surgery.

1. Donor Allograft Recovery

Note: Begin the donor portion of the transplant 40 min earlier than the recipient transplant to minimize recipient anesthesia time and to facilitate a simultaneous end time or slightly earlier end time versus the recipient preparation.

1. Use standard sterile microsurgical instruments and sterile gloves for the procedure. Our laboratory uses autoclave sterilization of microsurgical instruments.
2. Anesthetize the donor mouse (male) using isoflurane induction vaporizer at 4%. Using atraumatic mechanical clippers remove the hair from the cervical, thoracic, and abdominal region. Place the animal in the supine position and maintain isoflurane on 1-2% through a nose cone. Ensure adequate anesthesia throughout the procedure by periodically evaluating the toe pinch withdrawal reflex.
3. Prior to the skin incision, widely prepare the operative by applying povidone iodine antiseptic followed by isopropyl alcohol using a sterile cotton swab.
4. Begin with a superficial transverse skin incision with scissors across the cervical and abdominal skin. Connect both incisions bilaterally along the midaxillary lines.
5. Using microsurgical forceps dissect the cervical region bilaterally to identify, ligate and divide the external jugular veins with 6-0 silk suture and scissors. Then using electrocautery divide the sternocleidomastoid muscles to expose the internal jugular veins and common carotid arteries, bilaterally. Pass a 6-0 silk suture under the left-sided and the right-sided common carotid and internal jugular veins in bulk fashion. NOTE: They will be tied and divided later in Step 1.9.
6. Sharply divide the strap muscles and associated loose areolar tissue, located anterior to the trachea, using scissors to free the remaining attachments of the cervical region.
7. Using bipolar electrocautery and sharp dissection, divide the pectoralis major muscles and clavicles to expose the subclavian vessels and ligate (6-0 silk suture) and divide proximally.
8. Next, gently, grasp and withdraw the animal's penis. Along the dorsum of the penis visualize the dorsal vein of the penis, and disinfect the region with isopropyl alcohol. Using a 30 G needle, inject 30,000 units of heparin intravenously through the dorsal vein and allow the penis to recoil back to its original position. Partial leakage of the heparin solution into the surrounding tissue may occur.
9. Using the previously placed bulk ties around common carotid artery and internal jugular vein, ligate and divide the structures, bilaterally.
10. Next use scissors to make a transverse intrabdominal incision. Eviscerate the intestines to expose the infrahepatic inferior vena cava and inject 2 ml of cold Euro-Collins cardioplegia solution into the infrahepatic inferior vena cava. Ensure proper injection by visualizing liver discoloration and cessation of the heartbeat prior to advancing to the following step.
    NOTE: Euro-Collins solution is prepared in our laboratory, see table of specific reagents and instruments.
11. Using scissors access the intrathoracic cavity via a bilateral diaphragmatic incision from the exposed abdomen. Extend the incision cephalad through the intercostals muscles and ribs. Reflect chest wall exposing the heart, thymus, and great vessels while simultaneously ensuring preservation of the internal thoracic vessels along the chest wall.
12. Inject the suprahepatic inferior vena cava with 4 ml of cold Euro-Collins cardioplegia solution.
13. Identify the root of the aorta and trace distally to the descending aorta. Sharply cut the descending aorta (preserving maximal length).
14. Identify the pulmonary trunk and divide just proximal to its branch point (preserving maximal length). Then using 2 ml of cold Euro-Collins cardioplegia solution, flush the pulmonary trunk and heart by placing a soft plastic tip catheter into the lumen of the pulmonary trunk.
15. Using a 6-0 silk suture, ligate and divide the inferior vena cava, confluence of pulmonary veins, and accessory branches of the bilateral superior vena cava. Then elevate and dissect the heart cephalad from the attachments along the main stem bronchi and trachea with care not to enter the airway. Using sharp and bipolar electrocautery dissect the chest wall, thymus, and heart completely liberating it from the donor mouse.
16. Finally, trim the allograft chest wall *ex vivo* to a smaller size, using scissors, along the sternum and lateral costae, with care not to disrupt the internal thoracic vessels (**Figure 1A**). To minimize hemorrhage following revascularization, use bipolar electrocauterization along the borders of the osteomusculocutaneous sternum.
17. Place the allograft in 10 ml of cold (4^{o} Celcius) Euro-Collins solution if the recipient is not prepared for inset. However, if the recipient is ready for inset, transfer the allograft straight to the recipient operative field.

2. Recipient Preparation

Note: To minimize recipient anesthesia time, begin the recipient preparation at a separate operative station approximately 40 min prior to completion of the donor allograft harvest.

1. Use a separate set of standard sterile microsurgical instruments and sterile gloves for the procedure.
2. Anesthetize the recipient mouse (male or female) using isoflurane induction vaporizer at 4%. Using atraumatic mechanical clippers remove the hair from the right cervical and thoracic region.

3. Place the mouse in the supine position and angle the right upper limb slightly inferiorly forming a 110 degree angle between the head and right upper limb. Maintain anesthesia on 1-2% isoflurane through a nose cone.
4. Place petroleum ophthalmic ointment on the mouse eyes using a cotton tip applicator. Prior to the skin incision, widely prepare the operative site using povidone iodine antiseptic followed by isopropyl alcohol.
5. Using scissors, make a skin incision from the midline along the right inferior border of the mandible and extend the incision infero-laterally to the right thoracic region. Using blunt dissection with microvascular forceps, mobilize the external jugular vein by circumferentially free the vessel from soft tissue and adventitia. Divide all branches using electrocautery, and remove the right lobe of the submandibular gland using sharp dissection and electrocautery to free space for the allograft.
6. Ensure enough length of the external jugular vein to evert over a cuff, and ligate the external jugular vein using a 6-0 silk suture. Insert the vein through the lumen of a precut polyimide cuff and use a bulldog microvascular clamp to fix the vessel-cuff complex in place. Then using scissors, proximally divide the external jugular vein, evert over the cuff, and fix in place with a 10-0 nylon suture. (**Figure 1B**)
7. Divide the right sternocleidomastoid muscle with bipolar electrocautery to expose the common carotid artery. Circumferentially mobilize the artery cephalad to the distal most point within the cervical region. This is accomplished using blunt dissection of the vessel with forceps to remove soft tissue and surrounding adventitia.
8. Using 6-0 silk suture, ligate and divide the common carotid artery. Pass the artery through the lumen of a precut polyimide cuff and fix it in place with a bulldog microvascular clamp as close to the thoracic inlet as possible. Divide the vessel distally, gently dilate the vessel using a microsurgical dilator, evert over the cuff, and fix in place with a 10-0 nylon suture. (**Figure 1B**)
   NOTE: The specific microsurgical dilator is described in the table of specific reagents and instruments.

3. Allograft Inset

1. Maintain standard sterile instrumentation and sterile gloves to place the allograft within the recipient cervical region in an upside down and oblique position.
2. Next, place the donor descending aortic lumen over the arterial cuff construct of the recipient and fix it in place it with a 10-0 nylon suture (**Figure 1C and 1D**).
3. Fashion the same anastomosis as in step 3.2 between the donor pulmonary artery and the everted external jugular vein-cuff construct of the recipient mouse (**Figure 1C and 1D**).
4. First remove venous microvascular clamp (external jugular vein clamp) and then release the arterial clamp (common carotid artery clamp). During arterial reperfusion, inspect the entirety of the allograft to address any hemorrhage. If hemorrhage is visualized, reapply the arterial clamp to minimize blood loss and mitigate the source of bleeding using bipolar electrocautery.
5. Inspect the graft and ensure hemostasis. Release and completely remove the arterial microvascular clamp. Observe the heart to show signs of reperfusion, which will be instantaneously apparent with rapid volume expansion of the heart chambers, and wait for beating to begin within 0.5-1 min. Use warm saline (35° Celsius) to moisten the heart.
6. Drape the chest wall into an anatomical position so as not to induce any kinking or tensions on the anastomoses. Close the skin of the surgical wound is using 6-0 continuous nylon sutures (**Figure 1E**).

4. Postoperative Care

1. Administer a 0.3 ml normal saline intraperitoneal fluid bolus immediately postoperatively for fluid replacement.
2. Then subcutaneously inject buprenorphine (0.1mg/kg) and enrofloxacin (5mg/kg) for pain and infection prophylaxis, respectively.
3. Place the animal under a heat lamp until awaking from anesthesia and return to sternal recumbency. During recovery, inspect the neck to visualize the fibrillating heartbeat of the allograft ensuring adequate allograft perfusion.
4. Once awake and in the recumbent position, return the mouse to a separate cage (without the company of other mice) where it can receive food and water ad libitum. Due to any temporary minor restrictive motion of the right upper limb, leave a gelatin food source on the floor of the cage.
5. Observe the recipient mouse for 1 hr postoperatively and then return it to the cage facility where it can receive food and water ad libitum and is inspected three times a day for the first 24 hr for activity and nutritional intake. Monitor mice for signs of pain and distress and re-dose with buprenorphine (0.1mg/kg) subcutaneously twice a day as needed for the first 72 hr. Examine the animals daily thereafter and weigh them every week.
6. Consult with a veterinary staff member if any mice display signs of pain, distress, or decreased feed intake. Consider early euthanasia (in our protocol the euthanasia technique employs CO_2 overdose for 7 min, followed by cervical dislocation).
7. Cessation of the allograft heart beat is defined as a specific endpoint prompting the mouse to be sacrificed.

Representative Results

Syngeneic C57BL/6 transplants achieved long term survival. The design of the allograft (**Figure 1**) proved to be successful from an animal survival perspective and the ability to evaluate ongoing allograft survival. This was demonstrated through overlying skin remaining viable, active ongoing allograft hair growth, and heartbeats were able to be evaluated with visualization and finger palpation. Survival data is represented in **Figure 2** for syngeneic transplanted mice. The mean survival time was greater than 109 days. Based on the survival data, it is reasonable to infer that the technical aspect of the transplanted allograft is designed to perfuse the entirety of the chest wall, thymus, and heart. Furthermore, the ability of the syngeneic animals to survive long term further supports that this mouse model is not only feasible but can be replicated. This proof-of-concept en bloc chest wall, thymus, and heart transplant validates the murine model to study combined solid organ and vascularized composite allotransplantation.

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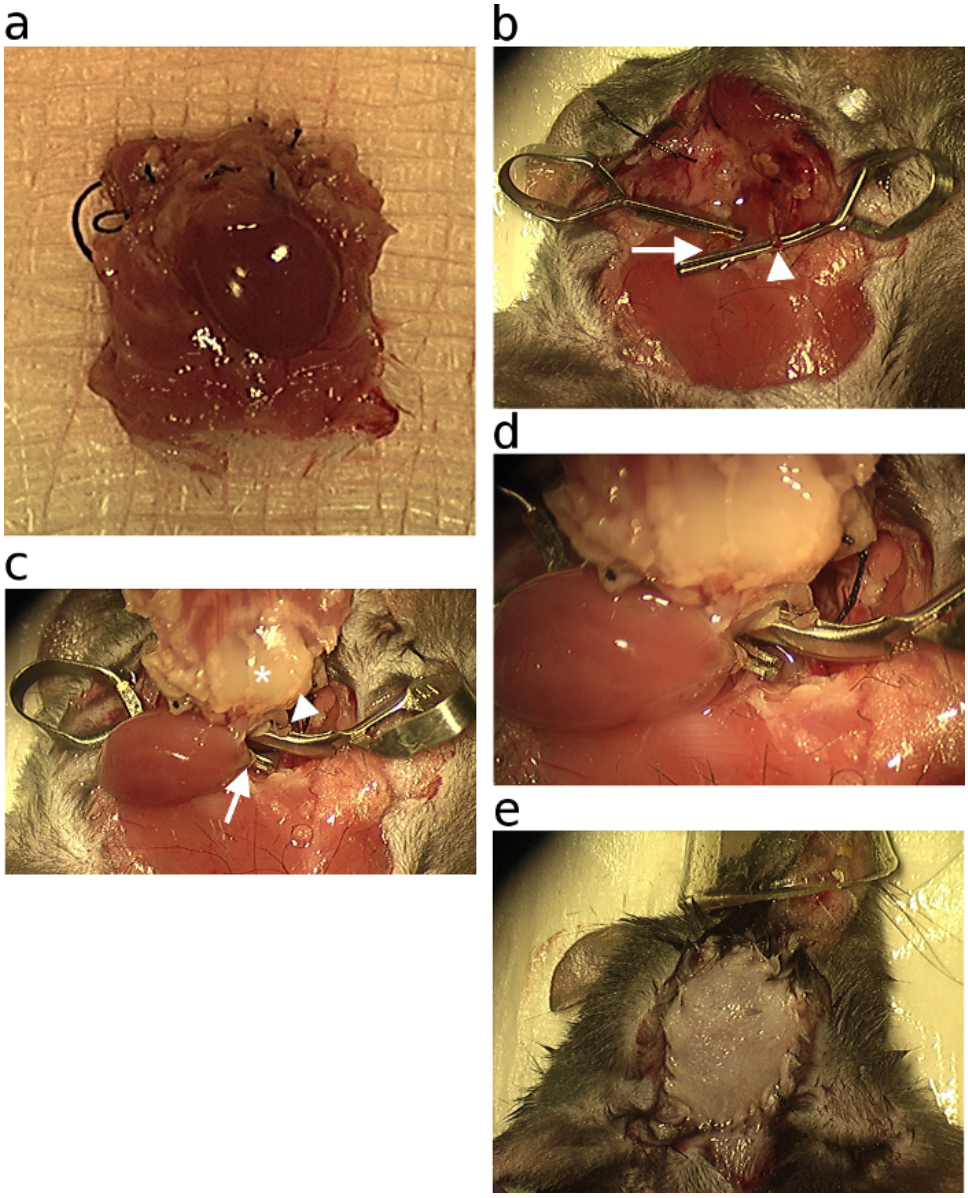

Figure 1. Intraoperative photos. (A) The chest wall, thymus, and heart allograft is successfully recovered, trimmed, and visualized *ex vivo* from the posterior aspect. The bilateral internal thoracic vessels are preserved. **(B)** The recipient external jugular vein (arrow) and common carotid artery (arrowhead) are everted fixed over polyimide cuffs in preparation for vascular anastomosis. **(C)** Allograft vascular anastomoses are completed. The arrow shows the anastomosis between the donor pulmonary artery and the recipient external jugular vein. The arrowhead shows the anastomosis between the donor descending aorta and the recipient common carotid artery. The asterisk identifies the thymus and the reflected chest wall is visualized overlying the thymus. (d) A higher magnification shows the microvascular-cuff anastomoses. (e) Complete allograft inset. Please click here to view a larger version of this figure.

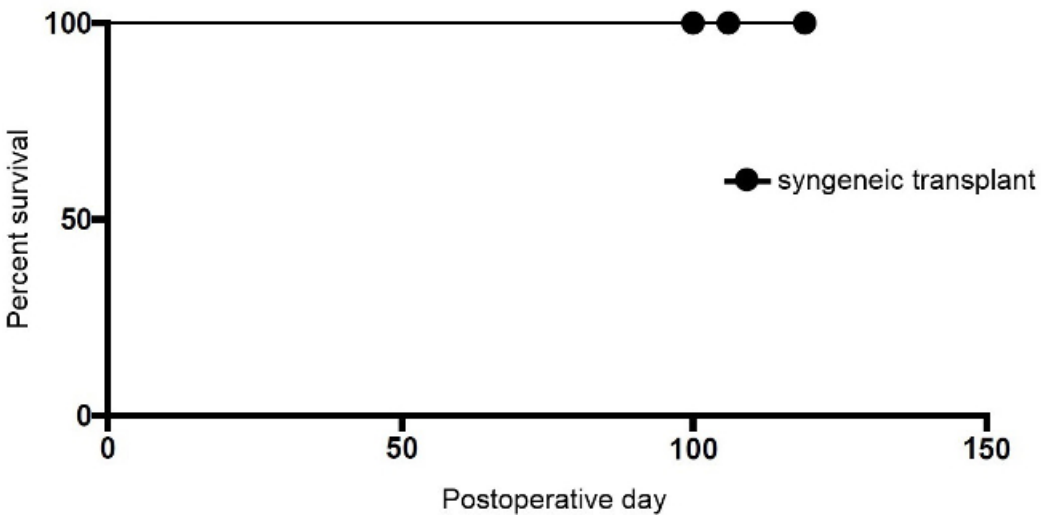

Figure 2. En bloc chest wall, thymus, and heart allograft survival. Kaplan-Meier survival curves of the en bloc chest wall, thymus, and heart allotransplant in syngeneic C57BL/6 mice (n = 3; mean survival time was greater than 109 days). Please click here to view a larger version of this figure.

Discussion

There are a multitude of phenomena that factor into the immunological investigation of allotransplantation, which include but are not limited to mechanisms of acute and chronic rejection, direct and indirect antigen presentation, recipient sensitization, or the induction of mixed chimerism.[19] Animal models have become the gold standard for the study of transplant immunology, and mouse models are popularly implemented due to their low cost, availability of transgenic and gene knockout mice, commercially available monoclonal antibodies, relative decreased veterinary and housing demands, and the ease of replication. To date, multiple heart transplant models have been designed to study solid organ transplantation.[19,22-27] Similarly, a plethora of mouse models have been developed to study vascularized composite allotransplantation.[28] However, the study of combined solid organ and vascularized composite allotransplantation is limited, and techniques have yet to be established in mice. The en bloc chest wall, thymus, and heart transplant murine model presented here is a reliable and replicable tool to study the effects and immunological mechanisms of combined solid organ and vascularized composite allotransplantation.

To further advance the field of transplantation, the promise of prolonging allograft survival and minimization of immunosuppression through novel treatment modalities must be pursued. One such approach is through the induction of mixed chimerism (partial engraftment of donor hematopoietic cells in the recipient), which can lead to immunosuppression-free donor specific tolerance, even if in some instances chimerism is not sustained.[29,30] Bone marrow transfusion/transplantation paired with solid organ[31] or vascularized composite allotransplanation[32,33] requires extensive preconditioning posing a significant challenge. Vascularized bone marrow, as part of a vascularized composite allograft construct, can circumvent this problem. Vascularized bone marrow transplantation allows for the uninterrupted transfer of donor bone marrow cells within the preserved donor microenvironment, and is considered to be superior to cellular bone marrow transplantation alone in the induction of tolerance and reduction of immunosuppression requirements.[34-36] Moreover, the incorporation of thymus tissue with vascularized bone marrow transplantation has shown to increase T-cell chimerism of donor origin ultimately playing a supportive role in the induction and maintenance of chimerism.[2,9] The aforementioned strategies of prolonging allograft survival and minimizing immunosuppression was the foundation to conceptualize a combined solid organ, thymus, and vascularized composite allograft mouse model.

The heterotopic en bloc chest wall, thymus, and heart transplantation is an amalgamation of multiple historical animal models. Heterotopic cervical heart transplantation using a non-suture cuff in mice has been well established and considered less technically demanding than a heterotopic abdominal microvascular heart transplant.[19] In fact, the cuff technique has been implemented in multiple other animal transplant models.[37-44] Heterotopic sternal transplantation in rats was introduced in 1999 by Santiago *et al.* as an alternative method to study vascularized bone marrow transplantation.[1] They were able to show long term peripheral chimerism, tolerance, and survival following cessation of immunosuppression on postoperative day 30.[1] Bozkurt *et al.* subsequently developed a rat model in 2013 to incorporate the thymus and the full extent of the osteomyocutaneous portion of the chest wall. It should be noted, however, that this model differs from our model in multiple aspects. This includes: (1) their model being devoid of any solid organs, (2) being completed in rats using traditional microsurgical techniques, (3) ligation of the internal thoracic vessels during donor harvest, (4) implementation of a unilateral, single pedicle via a common carotid artery and external jugular vein, and (5) transplantation of the allograft into the inguinal region.[2] Nevertheless, their model was able to demonstrate that the thymus of donor-origin plays a significant role not only for chimerism augmentation but also for chimerism maintenance as compared with vascularized bone marrow transplantation alone.[2] Subsequent swine models of co-transplantation of the thymus and heart have shown a superior effect on heart allograft survival.[10,11] The advantages of each the animal models, but lack of mechanistic *in vivo* studies pertaining to combined solid organ, thymus, and vascularized composite allotransplantation prompted our group to design this model.

The experience executing this novel model exhibited certain lessons requiring our team to institute modifications in order to achieve better animal survival. This model was attempted with one operator, which ultimately prolonged the operative and anesthesia time to over 3-4 hr and also prolonged cold ischemia time. Animals would not awake following termination of the procedure. Implementation of a two-team approach cut the total operative and anesthesia time to 90 min. This reflected in 60 min anesthesia time of the recipient mouse, and 0-10 min allograft cold ischemia time. During allograft reperfusion, the mouse is susceptible to hemorrhage, which can limit its survivability in the immediate peri-operative. We advocate meticulous inspection of the allograft *ex vivo* for potential sources of hemorrhage, as well as gentle release of the

bulldog microvascular clamp during allograft reperfusion. By placing the allograft in a reflected position it is easier to identify specific sites of bleeding. Furthermore, this graft inset position fosters the most ergonomic lay of vessels minimizes the risk of vessel kinking. Lastly, with the first 48 hr of recovery, the recipient mouse's right upper extremity range of motion may be hindered with regards to climbing the cage to obtain food and water. Therefore, we recommend placement of gelatin nutritional sources along the cage to facilitate nutritional intake. Typically by postoperative day 3, full range of motion is returned within the right upper extremity.

Although there are limitations to this model, which include the need for technical skill in microsurgery, availability of two simultaneous microscopes, and the requirement of a two-team approach, it has nevertheless shown to be a successful approach to perform mechanistic immunological studies related to combined solid organ and vascularized composite allotransplantation. Its broader application may further contribute to develop novel immunosuppressive protocols, studying mechanics of acute and chronic rejection, and implementation of potential strategies to induce and sustain chimerism, and prolong allograft survival.

Disclosures

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