

Vascularized Thymosternal Composite Tissue Allo- and Xenotransplantation in Nonhuman Primates: Initial Experience

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Background: Vascularized composite allotransplantation is constrained by complications associated with standard immunosuppressive strategies. Vascularized thymus and bone marrow have been shown to promote prolonged graft survival in composite organ and soft-tissue vascularized composite allotransplantation models. We report development of a nonhuman primate vascularized thymosternal composite tissue transplant model as a platform to address donor-specific immune tolerance induction strategies. **Methods:** Vascularized thymosternal allograft (skin, muscle, thymus, sternal bone) was transplanted between MHC-mismatched rhesus monkeys (feasibility studies) and baboons (long-term survival studies), with end-to-side anastomoses of the donor aorta and SVC to the recipient common femoral vessels. A male allograft was transplanted to a female's lower abdominal wall, and clinically applicable immunosuppression was given. Skin biopsies and immunological assays were completed at regular intervals, and chimerism was quantified using polymerase chain reaction specific for baboon Y chromosome.

Results: Four allo- and 2 xenotransplants were performed, demonstrating consistent technical feasibility. In 1 baboon thymosternal allograft recipient treated with anti-CD40-based immunosuppression, loss of peripheral blood microchimerism after day 5 was observed and anticipated graft rejection at 13 days. In the second allograft, when cutaneous erythema and ecchymosis with allograft swelling was treated with anti-thymocyte globulin starting on day 6, microchimerism persisted until immunosuppression was reduced after the first month, and the allograft survived to 87 days, 1 month after cessation of immunosuppression treatment.

Conclusions: We established both allo- and xeno- composite vascularized thymosternal transplant preclinical models, which will be useful to investigate the role of primarily vascularized donor bone marrow and thymus. (*Plast Reconstr Surg Glob Open* 2017;6:e1538; doi: 10.1097/GOX.0000000000001538; Published online 22 December 2017.)

INTRODUCTION

Vascularized composite allografts are increasingly considered as an option for reconstructing bony and soft-tissue defects when conventional surgical techniques are inadequate or unsuccessful. The use of vascularized composite

allografts (VCAs) are currently limited primarily by concerns regarding morbidities associated with the need for life-long immunosuppression with its attendant side effects and toxicities (infection, malignancy, renal insufficiency, diabetes mellitus, and poor wound healing).¹⁻³

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To prevent immunologic VCA failure and eliminate the need for long-term immunosuppression, donor-specific immune tolerance needs to be achieved.

Donor-specific tolerance has been achieved clinically by transiently inducing mixed hematopoietic chimerism with perioperative infusion of donor bone marrow (BM) in preclinical organ allograft models⁴⁻⁶ and in kidney transplant patients.⁷ The use of vascularized BM increases allograft survival and promotes mixed chimerism in VCA models,⁸⁻¹¹ perhaps by providing a continuous source of donor stem cells. In addition to BM stem cells, a primarily vascularized donor thymus plays an essential, nonredundant role,¹² because its addition to heart, kidney, limb, and islet cell allotransplantation models has facilitated indefinite composite graft survival and is associated with donor-specific T-cell hyporesponsiveness after withdrawal of immunosuppression in thymectomized recipients.¹³⁻¹⁷ In principle, the addition of vascularized thymus to vascularized BM in a VCA model may facilitate induction of durable mixed chimerism, enhance deletion or anergy among donor-reactive recipient T-cells that migrate through the donor thymus, and perhaps thereby promote tolerance.

Although rodent transplant models have provided mechanistic insights at a comparatively low cost, they do not faithfully model the complexities of the human immune response. Consequently, treatments developed in only rodents often fail in transition to large animals or humans. The molecular homology of human and non-human primate (NHP) immune system molecules allow informative, predictive testing of monoclonal antibodies intended for translation into the clinic. For these reasons, we used NHP for the development of our VCA model.

In this study, we report establishment of an NHP composite thymosternal VCA model as a platform to explore how co-transplantation of primarily vascularized thymus and BM modules host-donor immune interactions. This model is important because, to the best of our knowledge, en-bloc transplantation of a vascularized composite tissue including thymus, BM, muscle, skin has never been investigated before. This preliminary report focuses on the technical aspects and provides a “proof of principle” demonstrating feasibility of the model in 2 different recipient primate species, and in a cross-species “xeno” context, illustrating our vision for future use of this model.

MATERIALS AND METHODS

All animal care and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, School of Medicine and were conducted in compliance with NIH Guidelines for the Care and Use of Laboratory Animals. Two pairs of rhesus macaque (*Macaca mulatta*, mean age, 10.8 years; weight, 7.3–14.9 kg) were used for nonsurvival studies to demonstrate technical feasibility of alloTS. Two baboons (*Papio Anubis*, mean age, 6.5 years) were used first for survival alloTS studies and subsequently received a xenoTS without emergence from anesthesia, to establish procedure feasibility. Two GalTKO.hCD46 genetically engineered pigs (which lack galactose-a(1,3)-galactose epitope and express

human CD46, a complement regulatory protein; mean age, 5–6 weeks; weight, 8–9 kg) were used as donors in xenoTS model. Baboon and rhesus macaque donors were male and recipients were female, to facilitate tracking of donor chimerism in peripheral blood by detection of the Y chromosome by polymerase chain reaction (PCR). Donor–recipient pairs were matched by ABO blood typing.

Three to 5 days before transplantation, recipients were anesthetized with 10–15 mg/kg IM ketamine and inhaled isoflurane (1–4%) via endotracheal intubation with continuous electrocardiogram (EKG), blood pressure, pulse oximetry, and rectal temperature monitoring. A 7-Fr, heparin-coated, polyurethane catheter (CBAS-C70; Instech Solomon, Plymouth Meeting, Pa.) was inserted into the right internal jugular vein and tunneled over the shoulder and out the upper back skin. The animal was maintained in a jacket and tether system for up to 10 days to facilitate IV administration of medications.

Thymosternal Vascularized Composite Issue Harvest

The donor and recipient NHPs were anesthetized with 10–15 mg/kg IM ketamine and inhaled isoflurane (1–4%) via endotracheal intubation, with continuous EKG, blood pressure, pulse oximetry, and rectal temperature monitoring. In donor pigs, 1–2 mg/kg IM xylazine was additionally given for sedation, and tracheostomy was electively performed for mechanical ventilation. After positioning supine, bilateral paramedian anterior thoracotomy skin incisions were connected below xiphoid process and above the sternal notch (Fig. 1). Preserving a wide donor skin component facilitated tension-free coverage of the recipient implant site. Costal cartilages were divided bilaterally, and intercostal vessels were ligated and divided. A segment of clavicle was removed bilaterally, then the exposed subclavian vessels were ligated and divided distal to the internal thoracic vessels. The pericardium was then incised starting at the diaphragm to expose the heart and great vessels and continued cephalad posterior to the phrenic nerves. Using the phrenic nerves as landmarks, the retrosternal soft tissue in continuity with the anterior pericardium and thymus protected the internal thoracic and phrenic vessels bilaterally and thus preserved all the vasculature of the thymus (Fig. 1). Mobilization of the graft was completed by dividing all secondary vessels to the posterior neck and posterior thoracic mediastinum and chest apices.

Bilateral internal thoracic and subclavian vessels, ascending aorta, and SVC along with the anterior mediastinal and neck structures were removed en-bloc with the TS graft, ligating all visible vascular branches (Fig. 2). The TS grafts were flushed with University of Wisconsin solution. The ascending aorta was selected as the arterial inflow, and SVC was selected as the venous outflow, oversewing the subclavian and jugular veins distally. The graft was stored on aseptic saline ice slush until transplantation.

Unlike the NHP thymus, the swine thymus has additional cervical lobes bilaterally connecting to the mediastinal lobe. The cervical lobes that extend to the level of mandibular angle are exposed via a median neck incision. The common carotid artery (the main artery supplying the

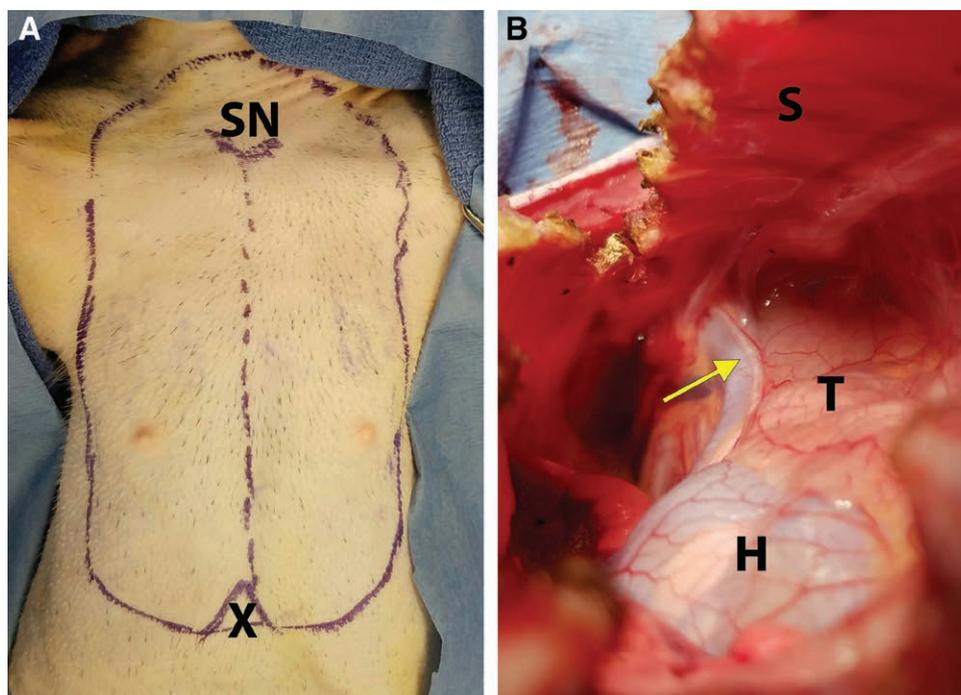


Fig. 1. A, Skin marking of TS allograft. B, Elevating the sternum (S) allows visualization of phrenic nerve and vessels (yellow arrow), which are included in the graft to ensure vascular supply to the thymus (T). H, heart; SN, sternal notch; X, xiphoid.

cervical lobes) as well as other tributary arteries, including superficial cervical artery, were preserved in the harvest. Numerous thymic veins that drain into the internal/external jugular veins and superficial cervical veins were preserved as well¹⁹ (Fig. 3). After the harvest procedure, donor animals were killed according to the protocol.

TS Transplant Procedure

The common femoral vessels were chosen as recipient vessels, preferentially using the right side for convenience of donor and recipient vessel orientation. Heparin (300–2,000 IU/kg) was administered intravenously before femoral vessel clamping. Donor aorta and SVC of the en-bloc thymus-sternum composite graft were anastomosed to the common femoral artery and vein, respectively, in an end-to-side fashion using the operative microscope (Leica Microsystems, Inc., Bannockburn, Ill.). The anastomoses were performed using 7-0 or 8-0 prolene sutures (Fig. 4). The graft was inset to the lower abdominal wall fascia, and the skin was closed in multiple layers with interrupted sutures. Penrose drains were sutured to the skin and left in-situ for 2–5 days. The graft was protected by the jacket and a custom-fitted plastic “skirt” shaped like an inverted Elizabethan collar. Anticoagulant therapy was not routinely administered postoperatively.

Postoperative Care and Follow-Up

The graft was assessed regularly (on day 1 and every 2–3 days thereafter) under chemical restraint with ketamine. Protocol blood draws (for routine hematology, serum biochemistry, chimerism analysis, serum archiving for drug level, and anti-donor antibody measurement) and

skin punch biopsies were performed on POD 3, 7, 14, 21, 35, and 56. Additional blood draws or biopsies were taken as deemed clinically necessary.

Immunosuppressive Therapy

Two pairs of rhesus monkeys were used for technical feasibility studies and did not receive any immunosuppressive agents. AlloTS recipient baboons received 2C10R4 (α CD40, 10 mg/kg at POD 0, 3; 5 mg/kg at POD 7, 10, 14, 21, 28, 35, 45, and 56), methylprednisolone (250 mg IV on POD 0, tapered to 125 mg daily IV/SQ; Table 1). Baboon 1010 also received Mycophenolate Mofetil (MMF, 40 mg/kg/d IV/PO on POD 0–13). For these technical feasibility xenoTS pilot studies, no immunosuppression was given, and recipients were electively euthanized the morning after the xenotransplant without emergence from anesthesia.

Chimerism Determination by Semi-Quantitative Real-Time PCR

DNA was isolated from peripheral whole blood after lysing red blood cells. A semi-quantitative real-time PCR for baboon SRY (sex-determining region Y) was performed using 100 ng of DNA per 25 μ l reaction volume on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using Roche FastStart Universal qPCR master mix (Roche Life Science) with the following Rh-SRY primers and probe: (forward primer: 5'-GCAAAGCAGTTAATGCCAGCT-3', reverse primer: 5'-ACTTTCTTGACTGTTGCATTATATTA-3', probe: 5'-/56-FAM/ACAGATCCA/ZEN/ACACTATTA-3'). Human ribosomal protein L32 (RPL32, forward primer: 5'-GTTCGTAGAAGATTCAAGGCC-3', reverse

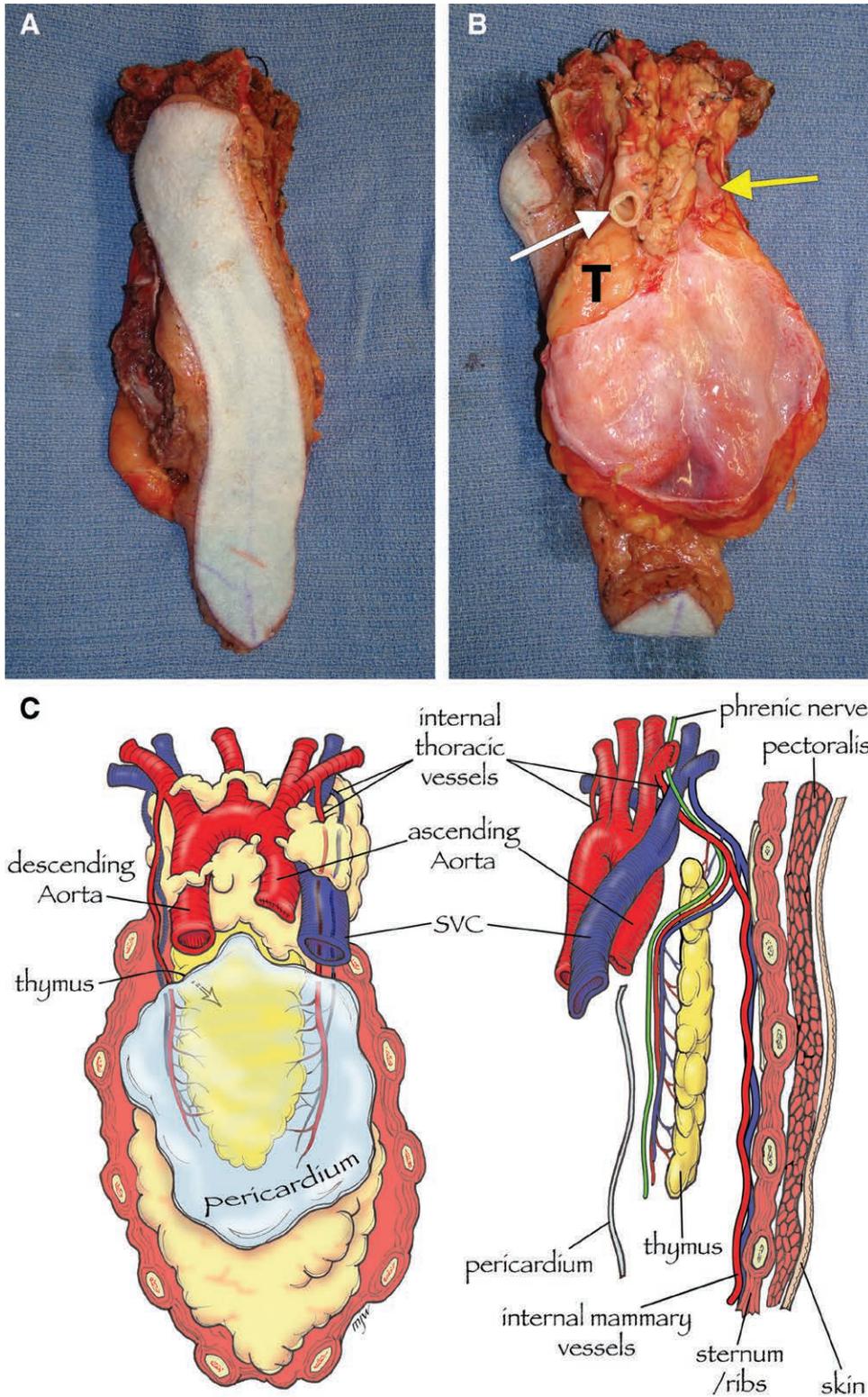


Fig. 2. Explanted baboon thymosternal allograft. A, Anterior view. B, Posterior view. Aorta (white arrow) and SVC (yellow arrow) were used as donor vessels. Thymus (T) is partially obscured by pericardium. C, Illustration depicting key anatomical features of the thymosternal allograft.

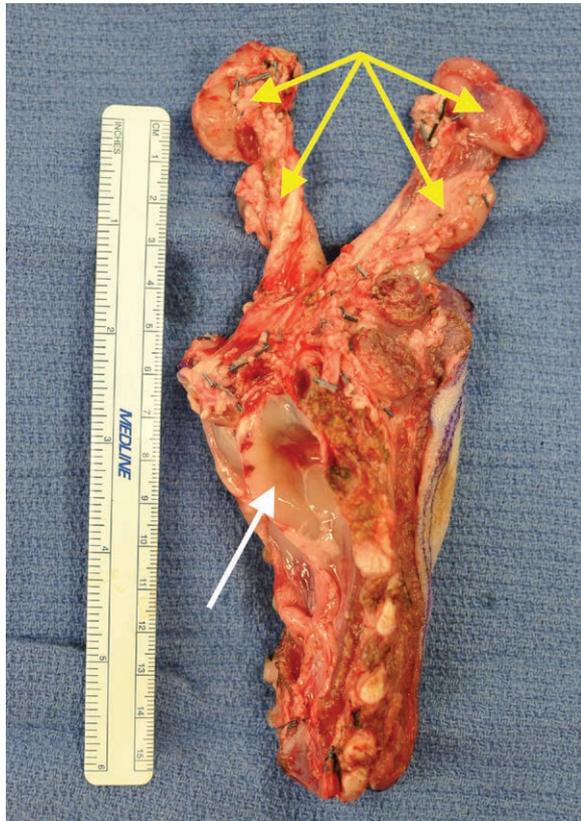


Fig. 3. Posterior view of explanted swine thymosternal graft showing the presence of cervical lobes (yellow arrows) in addition to the mediastinal lobe (white arrow).

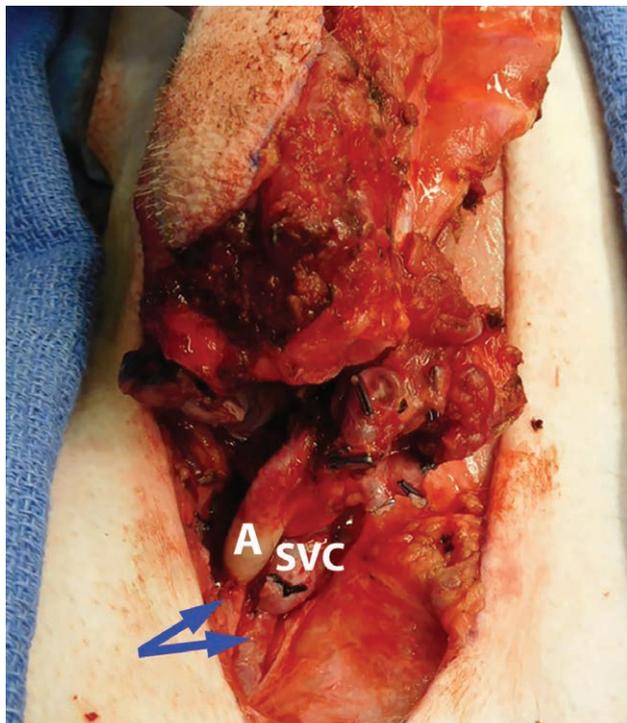


Fig. 4. Anastomoses of allograft aorta (A) and SVC to recipient's femoral vessels (blue arrows).

Table 1. Animal Characteristics, Immunosuppression, Clinical Outcomes and Graft Survival

Donor ID	Donor Species	Recipient ID	Recipient Species	Immunosuppression	Chimerism	Chimerism Last Detected (POD)	Rejection (POD)	Graft Loss (POD)
Nonsurvival allotransplant (Rheus) 97C098	Macaca Mulatta	DL2X	Macaca Mulatta	None	N/A	N/A	N/A	> 12 h
M256	Macaca Mulatta	M285	Macaca Mulatta	None	N/A	N/A	N/A	> 12 h
Survival allotransplant (Baboon) 13709	Papio Anubis	30433	Papio Anubis	2C10R4 Methylprednisolone	Transient, micro	10, 34	6, 77	87
7212	Papio Anubis	1010	Papio Anubis	2C10R4 Methylprednisolone MMF	Transient, micro	5	8	13
Nonsurvival xenotransplant (Baboon) 850-13	GalTKO.hCD46 pig	30433	Papio Anubis	Methylprednisolone	N/A	N/A	N/A	> 12 h
852-02	GalTKO.hCD46 pig	1010	Papio Anubis	Methylprednisolone	N/A	N/A	N/A	< 12 h

2C10R4, anti-CD40; MMF, mycophenolate mofetil; GalTKO.hCD46, lacks galactose- α (1,3)-galactose epitope and expresses human CD46 protein.

primer: 5'-CAGAGTAAGATTTGTTGCACATCA-3', probe: 5'-/5TAT/GTTCCTGGTCCACAACGTCAACGA-3'), with complete homology to baboon ortholog, was used as house-keeping gene control. Expression levels of SRY gene were normalized to RPL32 and expressed as $\Delta\Delta CT$. Percent chimerism was determined based on the standard curve plotted with mixtures of known quantities of male and female baboon DNAs.

Flow Cytometry Analysis

For assessing receptor coverage in 2 baboons treated with $\alpha CD40$, blood samples were lysed with Ammonium-Chloride-Potassium Lysing Buffer and stained with CD40-PE (Biolegend, San Diego, Calif.), CD20-PerCP (BD Biosciences, San Jose, Calif.) on the day of blood collection. Data were acquired with BD-FACSVERSE (BD Biosciences, San Jose, Calif.) and analyzed by FlowJo Software (FlowJo, LLC, Ashland, Ore.).

RESULTS

Surgical Outcomes and Clinical Course

Four allo- and 2 xenoTS VCA transplantations were technically successful (Table 1).

One baboon TS allograft (1010) demonstrated epithelial inflammation on POD 3 skin biopsy, which was diagnosed as mild rejection only on retrospective review. Mild erythematous skin discoloration progressed by POD 8, and marked swelling of the graft was associated with dehiscence along the inferior aspect of the wound closure (Fig. 5). Because the dehiscence was felt clinically to be technical rather than immunologic and in light of the biopsy interpretation, anti-rejection treatment was not initiated. The wound was thoroughly cleaned, debrided, and closed primarily on POD 11. However, graft swelling progressed and necessitated explant on POD 13 due to severe soft-tissue edema and histology consistent with acute cellular rejection.

The other baboon TS allograft (30433) exhibited diffuse skin erythema and focal ecchymosis raising the clinical suspicion of acute rejection on POD 6 (Fig. 6). Skin biopsy demonstrated venous congestion and no clear evidence of acute cellular rejection. Despite the "bland" histology, clinical findings resolved with anti-thymocyte globulin (ATG; 75 mg/d infusion on POD 6) and methylprednisolone bolus. Skin discoloration significantly improved within 7 days after immunosuppression (IS) augmentation, leaving small areas of skin necrosis. On POD 21, a small, superficial dehiscence occurred at the inferior aspect of the allograft attributable to increasing mobility of the animal and the relatively large size of the allograft. Repair sutures were placed, prophylactic antibiotics were initiated, and daily wound care was provided. The graft was otherwise well incorporated, and there was a robust hair growth over the graft. $\alpha CD40$ dosing was reduced by 50% at 1 month and discontinued at 2 months. Prominent, diffuse erythematous color changes were observed starting on POD 77, which progressed to diffuse graft erythema and edema by POD 87 when the

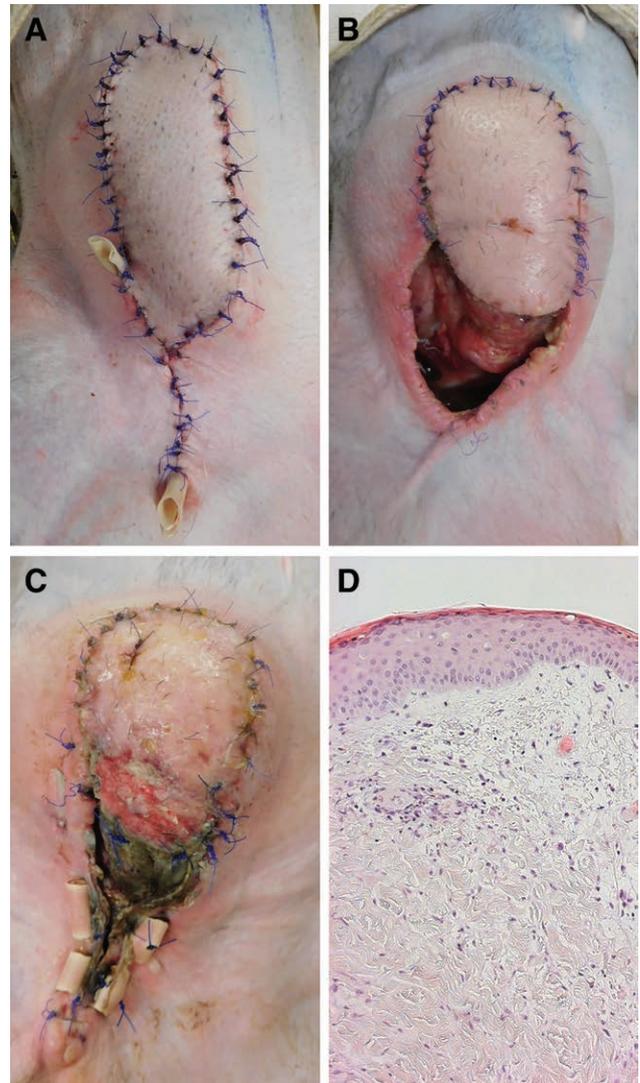


Fig. 5. Clinical course and histology of the allograft skin from recipient Baboon 1010. A, TS allograft on POD 1. B, Mild erythema and marked graft edema with enlargement and resultant sterile wound separation on POD 8. C, Partial necrosis of inferior graft, progressive skin rejection before explant on POD 13. D, Histology of explanted graft skin on POD 13.

TS graft was explanted with clinically and histologically severe acute rejection (Fig. 7).

Reciprocal Chimerism by qPCR

Donor DNA was detected at a low and steadily declining level in the peripheral blood from both allograft baboons by PCR (Fig. 8). Microchimerism was detected in the peripheral blood of Baboon 30433 by POD 3 (0.37%) and in decreasing amounts on POD 14 (0.02%) through POD 34 (0.004%) but not subsequently. Microchimerism was present at 0.09% in the peripheral blood of Baboon 1010 on POD 3, but not on POD 7.

Flow Cytometry

During the administration of $\alpha CD40$, flow cytometry analysis demonstrated complete receptor coverage (CD40; data not shown).

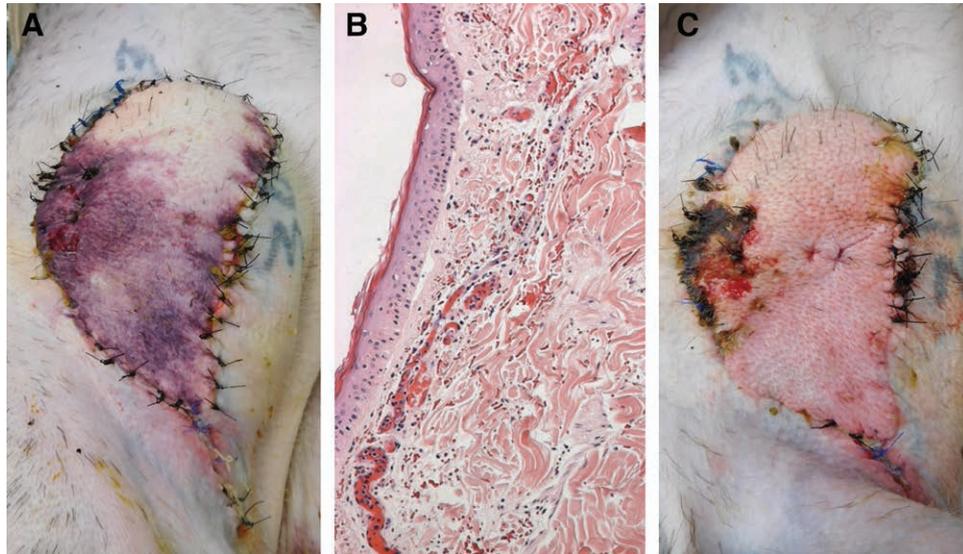


Fig. 6. Clinical course and histology of the allograft skin from recipient Baboon 30433. A, Significant cutaneous ecchymosis of skin on POD 6. B, Histologic evidence of deep dermal vascular congestion and sparse perivascular cellular infiltration, lacking classical cellular features associated with acute organ allograft rejection. C, Improvement of graft edema and skin discoloration by POD 14 after ATG and steroid administration starting on POD 6, with residual blanching skin erythema and focal full-thickness skin necrosis laterally.

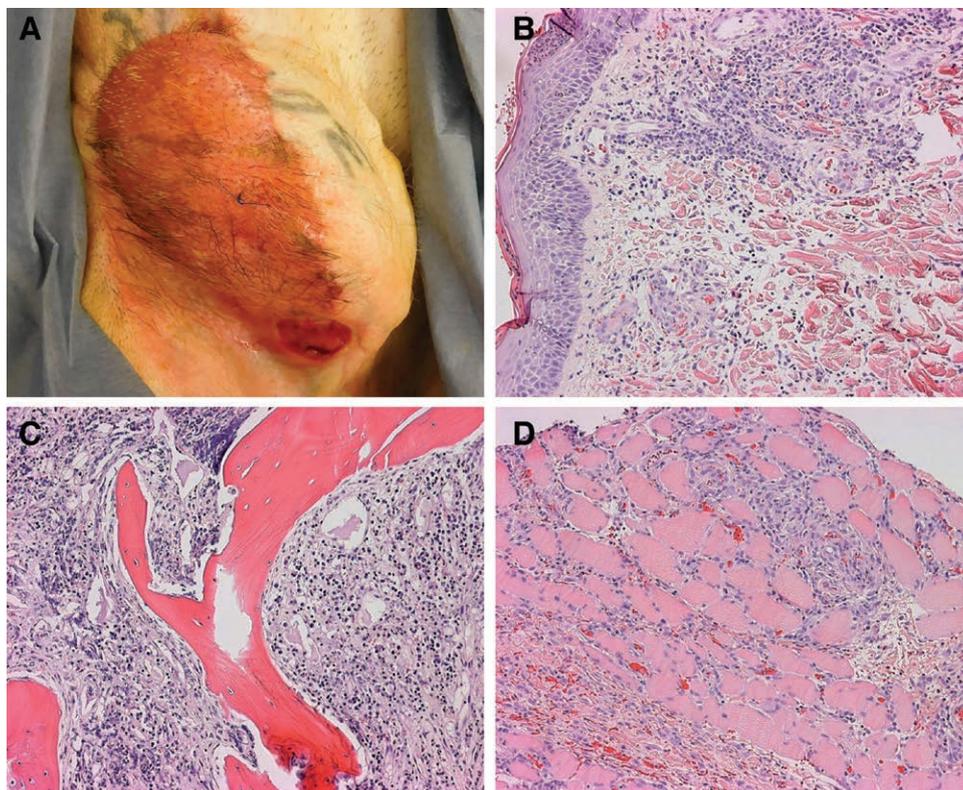


Fig. 7. Baboon 30433 allograft on the day of explantation on POD 87, after cessation of IS on POD 60. A, Severe graft edema and skin erythema are present, and acute cellular rejection is seen in skin (B), BM (C), and muscle (D).

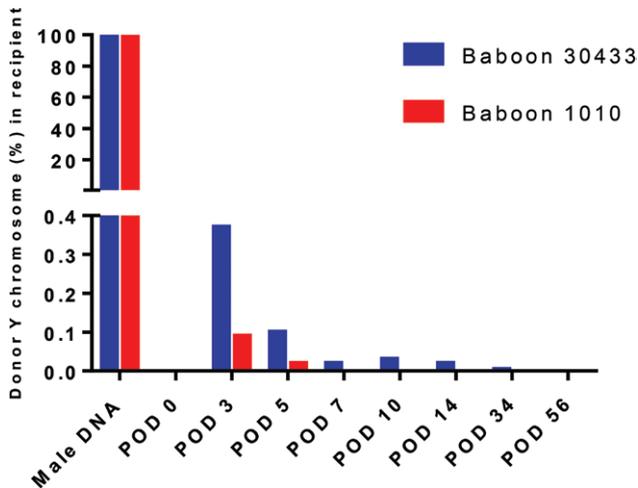


Fig. 8. Microchimerism was detectable in the peripheral blood of Baboon 30433 by POD 3 (0.37%) and in decreasing amounts on POD 14 (0.02%) through POD 34 (0.004%) but not subsequently. Microchimerism was present at 0.09% in the peripheral blood of Baboon 1010 on POD 3, but not on POD 7.

DISCUSSION

Achieving donor-specific tolerance is one of the major goals in the field of transplantation, to prolong graft survival and decrease or even eliminate the need for chronic immunosuppression, which are often associated with postoperative infections, malignancies, renal failure, and metabolic disorders.¹⁻³ Mixed hematopoietic chimerism is a state in which host and donor-derived lymphohematopoietic cells coexist within the recipient after transplantation.²⁰⁻²² Extensive evidence in animal models and in clinical setting suggests that achieving stable macrochimerism is generally associated with donor-specific acceptance of an allograft in the unusual circumstances when this state is achieved. BM or stem cell co-transplantation in large animal models have been applied to various preclinical experimental organ models and proven successful at prolonging graft survival and promoting durable graft acceptance.^{23,24} Similarly, a recent report in xenogeneic pig-to-NHP model showed that BM transplantation from GalTKO pigs to baboons resulted in detectable chimerism and prolonged cellular hyporesponsiveness, but neither durable chimerism nor long-term engraftment were observed.²⁵ Vascularized BM allograft transplantation in NHPs is necessary to prolong graft survival using conventional IS,^{8,10,26-28} but it was not sufficient to induce tolerance, since graft rejection occurred after IS cessation.

In the absence of donor thymic tissue, the recipient's immunocompetence of donor BM-derived stem cells (whether infused or primarily vascularized) depends on bi-directional interactions between donor and recipient T-cells educated in the recipient thymus and BM-derived antigen presenting cells in the periphery.²⁹ Immunology dogma dictates that educating the recipient's immune system to accept donor antigen as "self" should be facilitated by transferring vascularized donor thymus, particularly after recipient thymectomy and may induce donor-specific tolerance for kidneys and hearts.^{30,31} Transplanted thymic stromal cells induce central tolerance primarily by dele-

tion of T-cells that have T-cell receptors of high affinity with thymic epithelium, although anergy or regulatory mechanisms may also play a role.^{32,33}

Importantly, presence of residual recipient thymus may constitute a major barrier to induction of tolerance by vascularized donor thymosternal VCA, because vascularized thymic lobe transplantation has been shown to induce tolerance in thymectomized pigs with short-term tacrolimus use (12 days) but not in euthymic pigs.¹⁵ However, this conclusion was based on few observations, leaving the question unanswered as to whether conditions exist where "cross-tolerance" and stable chimerism may be induced in the presence of both donor and recipient thymus. As a logical first step, it will be important to explore whether native thymectomy is necessary or sufficient to promote durable graft acceptance during treatment, or robust allograft tolerance after IS withdrawal.

Our preclinical NHP platforms to study the roles of thymus and vascularized marrow on tolerance induction will allow us and others to test novel immunosuppressive treatment modalities and provide new, unique perspectives on VCA immunology.

In this article, we demonstrate the consistent technical feasibility of both allo- and xeno thymosternum transplantation for the first time. In one alloTS case, graft edema likely caused by acute rejection contributed to wound dehiscence, because the lymphocyte-predominant infiltrate classically associated with allograft rejection was not observed on the day 3 biopsy, ATG, and intensified IS were not implemented. In the second case, reversal of clinical acute rejection was observed with ATG administration and an intensified (but clinically applicable) IS regimen despite similar histologic findings on POD 6; this regimen was associated with prolonged subsequent allograft acceptance until IS withdrawal. Based on this initial experience in this model, we remark that clinical acute "rejection" of TS grafts appears to be relatively acellular at least in the skin but appears to be responsive to treatments traditionally used to reverse T-cell-driven acute cellular rejection. These preliminary observations suggest that this new model is likely to provide potentially valuable new insights into the role of the thymus and vascularized BM in allotransplantation.

Peripheral blood donor chimerism was detected for the first month in 1 alloTS VCA recipient, albeit at declining levels. Detection of chimerism is not a trivial problem in NHP allotransplant experiments, where monoclonal antibody reagents specific for donor or recipient are generally not available. We detected microchimerism in 2 alloTS recipient's peripheral blood by PCR, based on quantification of donor Y chromosome when male donors were used for female recipients and have explored short tandem repeat polymorphisms for this purpose. Antibodies specific for baboon transplant antigens would be a powerful tool to more sensitively detect donor cells in the circulation, or resident in recipient tissues, and engraftment of recipient cells in donor thymus, marrow, and myocutaneous compartments. Tolerance was not achieved in any recipient in this small pilot study, which may be due to an intact native thymus or rapid withdrawal of IS. The relative importance of these factors remains to be established.

Our initial experience using GalKTO.hCD46 porcine TS xenografts, hoping to induce transplant tolerance across the xenogeneic barrier, reveals a procoagulant phenotype xenografts similar to that observed for other xenografts.^{34–36} Although we cannot formally exclude a technical failure (anastomotic narrowing, inadequate organ preservation), in our estimation it is more likely that dysregulated coagulation, previously identified in pig-to-primate systems, causes (or at least contributes significantly to) this phenomenon.

Based on our experience to date, we offer several technical suggestions to other investigators interested in using this model. (1) Both the right and left recipient femoral vessels were used as the implant site in this initial experience. We generally prefer to use the right side as a recipient site when the donor's proximal ascending aorta and SVC are used. (2) With advanced planning and careful coordination, procurement of other allografts, including the heart, lungs, and abdominal organs, can be safely accomplished. (3) For experiments where regular blood sampling or drug administration are desirable, we find that a jacket and tethered swivel connector system are useful.

In conclusion, we describe development of new clinically relevant, preclinical models for studying basic principles of transplant immunology in NHPs. The composite thymosternal VCA model creates for the first time the ability to explore how co-transplantation of primarily vascularized thymus and BM modulates host-donor immune interactions. Inclusion of the overlying skin provides a "sentinel" tissue to permit early diagnosis of rejection phenomena. Demonstrated feasibility in 2 different recipient primate species, and in a cross-species "xeno" context, illustrates our vision for future use of this model to study how simultaneous engraftment of vascularized thymus with sternal BM, influences the prevalence of donor-specific tolerance, and the efficacy of candidate induction immunosuppression strategies. Future studies will help elucidate the optimal IS strategy for the thymosternal VCA model and further exploration into the T-cell-mediated effects of native and donor vascularized thymus.

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