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VASCULARIZED COMPOSITE ALLOGRAFT TRANSPLANT SURVIVAL IN MINIATURE SWINE: IS MHC TOLERANCE SUFFICIENT FOR ACCEPTANCE OF EPIDERMIS?

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Abstract

Background—We have previously reported that MGH miniature swine which had accepted class-I mismatched kidneys long-term (LT) following 12 days of high dose Cyclosporine (CyA), uniformly accepted donor-MHC matched kidneys without immunosuppression but rejected donor-MHC matched split-thickness skin grafts (STSG) by day 25, without changes in renal graft function or anti-donor *in vitro* responses. We have now tested whether this “split tolerance” would also be observed for the primarily-vascularized skin of vascularized composite allografts (VCAs).

Methods—Group 1 animals (n=3) received donor-MHC matched VCAs <70 days following primary kidney transplant (KTx). Group 2 animals (n=3) received a second donor-matched t KTx followed by a donor-matched VCA >200 days after primary KTx.

Results—Animals in Group 1 lost the epidermis on days 28, 30, and 40, with all other components of the VCAs remaining viable. Histology showed cellular infiltration localized to dermal-epidermal junction. One of 3 recipients of VCAs including epidermis in Group 2 accepted all components of the VCAs (>200 days). The other two recipients lost only the epidermis at day 45 and 85, with survival of the remainder of the VCA long-term.

Conclusions—All tissues of a VCA are accepted long-term on animals tolerant of class-I mismatched kidneys, with the exception of epidermis, the survival of which is markedly prolonged

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⁸Participated in data analysis.

⁹Participated in the writing of the paper

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compared to STSG, but not indefinite. Exposure of tolerant animals to second donor-matched kidneys prior to VCA increases the longevity of the VCA epidermis, suggesting an increase in the immunomodulatory mechanisms associated with tolerance of the kidney.

Keywords

MGH miniature swine; vascularized composite allograft; skin specific antigens; vascularized skin

Introduction

Interest in clinical vascularized composite allograft (VCA) transplantation has increased significantly in the past two decades. As of 2012, over 100 patients have received VCAs, including hand and face transplantation (1). Indications for VCA transplantation have included loss of specialized anatomic structures (nose, lips, and hands) due to both military and civilian-related trauma, burns, and surgery (2). In order to prevent rejection of VCAs, these patients require intensive induction immunosuppression regimens as well as lifelong immunosuppression, placing VCA recipients at risk for post-transplantation infection and metabolic complications (3–8), often leading to patient noncompliance with immunosuppression medications, the most common reason for graft loss in VCA recipients. These risk factors often deter otherwise young, healthy patients from electing VCA transplantation, despite the remarkable functional and cosmetic reconstructions possible with VCAs (2). Clinical tolerance protocols which offer the potential to eliminate the need for long-term immunosuppression, may allow more widespread application of VCA transplantation.

Our laboratory has studied such protocols in a unique preclinical large animal model, the MGH miniature swine, in which MHC genetics can be reproducibly controlled (9–12). These animals have been particularly useful in assessing the effects of MHC matching on rejection and/or tolerance induction. We have studied mechanisms of tolerance of kidneys (13–17), heart (18–20), islets (21), thymus (17, 22, 23) and bone marrow (24, 25) using the MGH miniature swine model.

We have previously reported that 12 days of high-dose CyA facilitates the induction of stable tolerance of renal allografts across a two-haplotype class-I disparity in euthymic miniature swine (15). Once tolerant, these animals accepted donor MHC-matched kidney retransplants without the need for further immunosuppression (15,16). However, animals tolerant of renal grafts uniformly rejected donor-matched split-thickness skin grafts (STSG) by day 25, without changes in immunologic status, as indicated by anti-donor MHC cell mediated lympholysis (CML) responses, or renal graft function, suggesting a role for skin specific antigens (SSA) (15, 16) in the immunosurveillance of skin.

We have also previously demonstrated the importance of vascularization of allograft tissue for the induction of tolerance, using models of thymic transplantation (22, 26, 27) and more recently islet transplantation (21). We therefore hypothesized that tolerance of kidneys would allow donor-matched skin to be accepted if transplanted as a primarily-vascularized component of a myocutaneous VCA with its own vascular pedicle and angiosome. To test this hypothesis, we performed a study in which primarily-vascularized donor skin (as a component of a myocutaneous VCA) was transplanted onto animals that were already tolerant of a donor-matched renal allograft.

Results

Primary class-I mismatched kidneys were accepted following a short course of CyA, as were second, donor-matched kidneys without further immunosuppression

Six MGH miniature swine received class-I mismatched kidneys followed by 12 days of CyA that uniformly facilitates to induce tolerance of kidneys (15, 16). Renal function was within normal range (creatinine <2.0mg/dL) for all animals except for animal #19842 (Group 1) which had interstitial nephritis on biopsy, but no signs of rejection. All animals showed donor-specific hyporesponsiveness by CML assays at the time of VCA transplantation.

Recipients in Group 1 received donor-matched VCAs without immunosuppression <70 days after primary KTx. Recipients in Group 2, at least 100 days after primary KTx, underwent nephrectomy of the primary KTx and received a second donor-matched KTx without immunosuppression to confirm maintenance of renal tolerance, followed by VCAs >100 days after the second KTx.

Early epidermal loss was observed when VCAs were transplanted early after primary KTx (Group 1); however, VCA survival, including the epidermis, was markedly prolonged in long-term acceptors of kidneys (Group 2)

(i) Gross findings—Animals in Group 2 had significantly prolonged epidermal survival in comparison to Group 1 (Fig 1A, $p=0.0246$) with a median survival of 85 days compared to 30 days. A VCA was transplanted across the same Class-I mismatch in one naïve control animal, followed by 12 days of CyA. This animal rejected all components of the VCA by day 20.

Recipients in Group 1 received a VCA <70 days after primary kidney transplants. Group 1 animals (#19842, #19941, #20652) lost the epidermal component of the VCA at 40, 28, and 30 days after VCA, respectively. However, all Group 1 animals accepted the dermal and muscle layers of the VCA by clinical assessment. Animals #19842 and #19941 did not demonstrate histologic evidence of rejection; however, animal #20652, exhibited moderate cellular infiltrates in the dermis despite clinically viable dermis and muscle (details of histologic findings below).

Group 2 animals received a donor-matched VCA at least 100 days after kidney retransplantation, without further immunosuppression (i.e. >200 days after primary transplantation). Animal #18954 accepted *all* components of the VCA (muscle, dermis and epidermis) with no signs of rejection, grossly (Fig. 1C) or histologically, for greater than 200 days following VCA transplantation (see next section). Animals #18958 and #18955 rejected the epidermis 85 and 45 days after VCA, respectively (Fig. 1D and 1E). Despite loss of the epidermis, both animals #18958 and #18955 accepted the dermal and muscle components of the VCA, both clinically and histologically (details of histologic findings below).

(ii) Histologic findings—VCA biopsy of Animal #18954 at 150 days post VCA was representative of the histological appearance of all animals in Groups 1 and 2 before the start of epidermal rejection (Fig. 2A). This biopsy demonstrated viable, muscle, dermis, and epidermis without evidence of rejection. Minimal cell infiltrates were seen in the epidermis.

Biopsies were obtained when Animals #18958 and #18955 developed darkening of the skin approximately 10 and 5 weeks after VCA transplantation, respectively. Histologically, both showed obvious cellular infiltrates localized to the epidermis (Fig. 2B). In contrast, cellular infiltrates spread through the epidermal layer to the dermal layer in Animal #19941 of Group 1 (Fig. 2C).

Complete histologic examination performed at graftectomy revealed that muscle layers were free from signs of rejection in all animals (Fig. 2D). Myocyte atrophy was observed in all animals, presumably due to denervation. At all biopsy time points, transplanted renal grafts demonstrated only a minimal, mononuclear cell infiltrate (Fig. 2E).

A donor-matched split thickness skin graft (STSG) was accepted in an acceptor of a VCA, including epidermis, but rejection of a third party STSG abrogated acceptance of the donor-epidermis

The above data suggested that (1) immunologic responses to epidermis, likely associated with a skin specific antigen (SSA), differed from those to the donor MHC; and (2) responses to SSA may be inhibited, at least partially, when skin is transplanted as primarily-vascularized skin (i.e. as a flap) during the maintenance phase of tolerance.

We next tested if immunologic responses were epidermis-specific (i.e. associated with SSA). To test this hypothesis, donor-matched (SLAgg) STSG were placed on animals from Group 2 >100 days following VCA transplantation.

Animal #18954, which had accepted all components of the VCA, accepted the donor-matched STSG for greater than 60 days (see below), whereas animals #18958 and #18955 rejected donor-matched STSGs by day 12 (Fig. 3A). As previously published, no change in creatinine levels were observed after STSG grafting, indicating stable tolerance of the donor MHC.

To confirm immunocompetence, Animal #18954 received a third-party STSG (SLAaa) 64 days after the donor-matched STSG. The third party STSG was rejected by day 14 with development of anti-SLA class-Ia IgG. Interestingly, the previously transplanted *donor-matched STSG* (which had been fully viable for 75 days) became hyperemic during the time that the *third-party* (SLAaa) STSG rejected. These inflammatory changes to the donor-matched SLAgg STSG subsided spontaneously over the subsequent 2 weeks. However, 43 days after third-party skin grafting (107 days after donor-matched STSG, 239 days following VCA), both the donor-matched STSG and the epidermis of the VCA were rejected simultaneously (Fig. 3B and C). The muscle and dermis remained viable and no change in renal graft function was induced by loss of STSG (Fig. 3D). No anti-donor (SLA Class-Ic) antibody developed (See next section).

Both donor MHC specific hyporesponsiveness in vitro and renal graft function in vivo were maintained even after loss of epidermis of VCA

CML assays were performed to assess anti-donor and anti-third party responses before each VCA, 100 days after VCA, and after rejection of STSG. All animals displayed donor-specific hypo or unresponsiveness in CML at all time points (Fig. 4A) except for Animal #18955 (Fig. 4B). Animal #18955 displayed weak anti-donor responsiveness after rejection of a donor-matched STSG, but maintained normal renal function. To ensure tolerance of donor MHC, 18955 received a donor-matched kidney without immunosuppression 60 days after the STSG. The donor-matched kidney maintained normal renal function with no signs of rejection for greater than 150 days before termination of the experiment. Additionally, despite loss of the epidermis, neither anti-donor (SLA class-Ic) IgM nor IgG were detected in any of the 6 recipients (Fig. 4C and D).

Taken together, both *in-vivo* and *in-vitro* data indicate that loss of the epidermis/STSG was not due to loss of tolerance of the donor MHC.

Enhanced survival of VCAs was observed in the setting of augmented levels of CD4+/CD25+/FoxP3+ T-regs

In order to further elucidate the mechanism of acceptance or rejection of VCAs, phenotypic analysis of animals' PBMCs was performed.

Animals in Group 2, which demonstrated enhanced survival of VCAs, all had higher ratio of CD4+/CD25+/FoxP3+: CD4+/CD25+/FoxP3– prior to VCA transplant, when compared to animals in Group 1 (Fig. 5). Moreover, Animal #18954 which accepted all components of VCA for > 200 days, maintained the highest absolute number of CD4+/CD25+/Foxp3+ T cells in the periphery, during the first 12 weeks following VCA transplantation, at which time all other animals had rejected epidermis (Fig. 5A, 5B and 5C). The average absolute number of CD4+/CD25+/FoxP3+ T cells in the periphery was lower at the time of epidermal rejection for Animals #18958, #18955, #19842, #19941, and #20652 (mean=27.4, SD=8.6), when compared to a nadir level of this population in Animal #18954 (Fig. 5B). More strikingly, Animal #18954 maintained a higher ratio of CD4+/CD25+/Foxp3+: CD4+/CD25+/Foxp3– cells following VCA transplantation while others (Animals 2–6) demonstrated a markedly decreased ratio at the time of epidermal rejection (Fig. 5C).

Cells in the peripheral circulation may not represent the deposition of cells in the graft. For this reason, we performed immunofluorescence staining of the VCA for FoxP3 and CD25. Animal #18954 showed an increased number of CD25+/FoxP3+ cells in superficial and deep dermis (Fig. 5D) compared to other animals in the study (Fig. 5E), before the rejection of epidermis. Many cells stained positive with FoxP3 staining (red), in animal #18954, while other animals displayed some yellow staining with FoxP3, suggesting non-specific macrophage staining.

Discussion

Previous reports have shown that 12 days of CyA facilitates the induction of tolerance of class-I disparate kidneys (15, 16). These animals uniformly accepted second donor matched kidneys without immunosuppression, indicating systemic tolerance of donor kidneys. However, these animals rejected donor-matched STSG by day 25 despite maintenance of normal renal function. Data in the present study demonstrated that survival of donor-matched skin was extended when allogeneic skin was transplanted as a primarily-vascularized component of the VCA. The muscular and dermal components of donor-matched VCAs were all accepted throughout the experimental period (range 28–239 days), and did not depend on the timing of VCA transplant in relation to the duration of pre-existing tolerance. These data support our previous findings in thymic- and islet-kidney transplant models (26, 21) which indicated that vascularization of allografts is advantageous for graft acceptance, likely due to differences in the manner of antigen presentation. Furthermore, maintenance of the 1) kidney and 2) dermal and muscle layers of the VCA in combination with donor-specific unresponsiveness in CML in recipients of VCAs support our hypothesis that the presence of non-MHC (skin specific) antigen(s) persists beyond the tolerance induced to classical MHC.

Although the epidermal survival of each VCA exceeded that of STSG, it varied greatly (range 28–239 days) depending on the length of time and number of donor matched kidney grafts prior to VCA transplantation. One mechanism that may explain this variation could be a balance between donor-specific regulatory/suppressor cells and skin-specific T cell responses that are not tolerized by donor kidneys, associated with transplantation of the epidermis. We hypothesized that the T-regs that developed after class-I mismatched KTx potentially provided linked suppressive effects on T cell responses against skin-specific antigens that coexisted on epidermis of VCAs along with donor MHC. Data in this study

showed that i) Animal #18954 which accepted the whole VCA, as well as a subsequently transplanted donor-matched STSG, had the highest absolute number of CD4⁺/CD25⁺/Foxp3⁺ PBMCs in the periphery and in the VCA, and ii) the average absolute number of CD4⁺/CD25⁺/FoxP3⁺ T cells in the periphery was lower at the time of epidermal rejection, indicating a positive correlation between the presence of T-regs and epidermal acceptance. Furthermore, we demonstrated that animals in Group 1 which received second kidneys prior to VCAs, all had higher ratio of T-regs, CD4⁺/CD25⁺/FoxP3⁺ prior to VCA transplant and enhanced survival of the VCAs than those in animals in Group 1 that received kidneys at days 60–70. This finding suggests that re-priming by donor kidneys may have augmented donor-specific T regulatory effects. We have previously demonstrated that re-priming recipient PBMCs with donor antigens, either *in-vivo* by retransplantation of donor matched kidneys or *in-vitro* by co-culture assays, led to effective suppression of anti-donor responses (28–30). We have also reported that priming via a donor-matched transplant was required for linked suppression to be seen *in vivo* (30). Moreover, we have demonstrated that the re-priming of tolerated cells with donor antigens was required to suppress naïve cytotoxic lymphocyte responses to donor antigens in co-culture assays (28), and this suppression was lost following the depletion of CD25⁺ cells, but reestablished by addition of a small number of the CD25⁺ enriched population (29). These results demonstrate that either priming via a donor-matched kidney re-transplant (through linked suppression) or priming *in vitro* for co-culture suppression is required to augment regulatory tolerance.

Taken together, studies aimed at enhancement of Treg activity and/or number *in vivo* may allow for tolerance to extend to the epidermis layer. Strategies aimed at augmenting T-regs may allow reproducible acceptance of all components of a myocutaneous VCA, leading to wider application of these transplants. Studies to test this hypothesis are currently in progress across a MHC class-I mismatched KTx model in which tolerance is adoptively transferred through the transfer of tolerant cells with a kidney from a tolerant animal. An experiment utilizing adoptive transfer of PBMCs in combination with transplantation of a VCA is under investigation.

In summary, we have demonstrated acceptance of the muscle and dermis of a VCA when the recipient was already tolerant of a donor-matched kidney. To our knowledge, this is the first time uniform induction of tolerance to dermal and muscle components of VCAs, has been achieved in a MHC disparate, large animal study. Survival of epidermis of the VCA was markedly prolonged compared with STSG, indicating skin transplantation as a vascularized graft partially protected against anti-epidermal immune responses. Subsequent epidermal loss did not break tolerance of kidneys or restored *in-vitro* anti-donor responses, indicating that the observed epidermal loss was not related to anti-donor-MHC responses. Recent data have demonstrated that despite stable multilineage mixed chimerism, VCAs rejected the epidermis but accepted the remaining components of the VCA, across a full mismatch barrier (Curt CL et al. unpublished data). Therefore, strategies beyond tolerance of donor MHC are likely required for the induction of epidermal tolerance. This special requirement may be result of SSA which we propose to define as unique minor antigens present in the epidermis, not present in other organs or tissues. Our results suggest that regulatory T cells may be capable of supporting the viability of the epidermis of the VCAs, and warrant further investigation.

Materials and Methods

Animals

Donor animals of both kidneys and VCA were SLAgg (class-Ic/IId) MGH miniature swine. To achieve a two-haplotype MHC class-I mismatch with no MHC-II mismatch, recipient animals were 4 to 6 months of age from an inbred line of SLAdd (class-Id/IId) MGH

miniature swine. The immunogenic characteristics of MGH miniature swine and of the intra MHC recombinant haplotypes have been described previously (9–12).

Experimental Groups

Surgery

(1) Kidney transplantation and induction protocol—The surgical procedures for primary and secondary transplantation have been previously described in detail (15, 16, 17). Both native kidneys were removed on the day of the primary kidney transplant. Indwelling central venous catheters were placed surgically in the external and internal jugular veins of recipient animals to facilitate frequent blood sampling and administration of fluid, drugs, blood and PBMCs.

CyA was provided by Novartis (East Hanover, NJ, USA). Following primary kidney transplants, recipients received a 12-day course of CyA, starting on the day of transplantation, at a starting dose of 10–13mg/kg per day, adjusted to maintain blood levels between 400 and 800ng/ml. Whole blood trough levels were determined by a monoclonal radioimmunoassay. No further immunosuppression was given for second kidney transplants or VCA transplants.

(2) VCA transplantation—Gracilis musculocutaneous VCAs were harvested on saphenous artery and vein to their origin on the femoral vessels as previously described (33). Femoral artery and vein were anastomosed to recipient carotid artery and internal jugular vein, or femoral artery and vein respectively.

(3) Split thickness skin graft—Zimmer dermatome was used to harvest split thickness skin grafts (4x6 cm) from donors and to create split thickness skin defects in preparation of grafting on recipients.

Histological analysis of allografts

VTL graft biopsies were performed at days 30, 60, 100 and 150 as well as at the time that the graft changed in appearance with clinical evidence of epidermal rejection (erythema, scaling). Tissues were stained using hematoxylin-eosin and periodic acid-Schiff, and coded slides were examined by light microscopy. Graft rejection of kidneys was scored according to a standardized grading system of pathological specimens (31, 32).

Immunologic assays

(1) Cell-Mediated Lympholysis (CML) Assays

PBMCs were obtained by gradient centrifugation using HISTOPAQUE (Sigma, St Louis, MO, USA) and the procedure for CML assays has been described elsewhere (15, 17). ⁵¹Cr release was determined on a gamma counter.

(2) Assessment of anti-donor antibodies

Anti-donor IgM and IgG antibodies in the serum and in frozen biopsy samples were assessed by indirect FACS and immunohistochemistry, respectively, as previously described (17, 31).

(3) Assessment of FoxP3+, CD4+, CD25+ lymphocytes

The percentage of CD4+, CD25+ and FoxP3+ cells in PBMCs was assessed by FACS analysis using FITC-conjugated anti-CD4 (VMRD, Inc., Washington, USA), Bio-conjugated

anti-CD25 (Fitzgerald industries, MA, USA), and PE-conjugated FoxP3 (eBioscience Inc., CA, USA).

Two-color immunohistochemistry for FoxP3⁺ and CD25⁺ were performed using frozen tissue sections and standard indirect technique. Briefly, 4mm frozen sections were stained with anti-FoxP3 mAb, followed by Texas Red-labeled anti-mouse IgG Ab (DAKO, Carpinteria, CA), and were then incubated with FITC-conjugated anti-CD25 mAb.

Monitoring of Rejection

Rejection of kidney grafts was monitored by serum creatinine levels and histology of kidney biopsies. VCA grafts and split thickness skin grafts were assessed for scaling, darkening and lack of bleeding at the time of biopsy grossly, and destruction of cellular architecture histologically. In particular, gross assessment of s was performed daily by primary investigators as well as independently by several different investigators in our center at least twice a week to evaluate grafts objectively. The log rank test was used to test statistical significance in epidermal survival between the two groups.

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Abbreviations

CyA	Cyclosporine A
CML	Cell mediated lympholysis
Ig	Immunoglobulin
KTx	Kidney transplantation
MGH	Massachusetts General Hospital
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cells
SLA	Swine leukocyte antigens
SSA	Skin specific antigen
STSG	Split thickness skin graft
VCA	Vascularized composite allografts

References

1. [Accessed December 1, 2010] www.handregistry.com.
2. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. *Transplantation*. 2010; 90(12):1590. [PubMed: 21052038]
3. Klintmalm GB, Davis GL, Teperman L, et al. A randomized, multicenter study comparing steroid-free immunosuppression and standard immunosuppression for liver transplant recipients with chronic hepatitis C. *Liver Transplantation*. 2011; 17(12):1394. [PubMed: 21850690]
4. Getts DR, Shankar S, Chastain EM, et al. Current landscape for T-cell targeting in autoimmunity and transplantation. *Immunotherapy*. 2011; 3(7):853. [PubMed: 21751954]

5. Aberg F, Mäklin S, Räsänen P, et al. Cost of a quality-adjusted life year in liver transplantation: The influence of the indication and the model for end-stage liver disease score. *Liver Transplantation*. 2011; 17(11):1333. [PubMed: 21770017]
6. Ramsey SD, Patrick DL, Albert RK, et al. The cost-effectiveness of lung transplantation. A pilot study. University of Washington Medical Center Lung Transplant Study Group. *Chest*. 1995; 108(6):1594. [PubMed: 7497767]
7. Vasiliadis HM, Collet JP, Penrod JR, et al. A cost-effectiveness and cost-utility study of lung transplantation. *The Journal of Heart and Lung Transplantation*. 2005; 24(9):1275. [PubMed: 16143245]
8. Woodroffe R, Yao GL, Meads C, et al. Clinical and cost-effectiveness of newer immunosuppressive regimens in renal transplantation: a systematic review and modelling study. *Health Technology Assessment*. 2005; 9(21):1. [PubMed: 15899149]
9. Sachs DH, Leight G, Cone J, et al. Transplantation in miniature swine. I: Fixation of the major histocompatibility complex. *Transplantation*. 1976; 22(6):559. [PubMed: 137560]
10. Leight GS, Sachs DH, Rosenberg SA. Transplantation in miniature swine. II: In vitro parameters of histocompatibility in MSLA homozygous minipigs. *Transplantation*. 1977; 23(3):271. [PubMed: 140482]
11. Lunney JK, Sachs DH. Transplantation in miniature swine. IV: Chemical characterization of MSLA and Ia-like antigens. *J Immunol*. 1978; 120(2):607. [PubMed: 621396]
12. Johnson H Jr, Lunney JK, Sachs DH, et al. Preparation and characterization of an antiserum specific for T cells of pigs. *Transplantation*. 1980; 29(6):477. [PubMed: 6447396]
13. Smith CV, Nakajima K, Mixon A, et al. Successful induction of long-term specific tolerance to fully allogeneic renal allografts in miniature swine. *Transplantation*. 1992; 53(2):438. [PubMed: 1531395]
14. Gianello PR, Lorf T, Yamada K, et al. Induction of tolerance to renal allografts across single-haplotype MHC disparities in miniature swine. *Transplantation*. 1995; 59(6):884. [PubMed: 7701585]
15. Rosengard BR, Kortz EO, Ojikutu CA, et al. The failure of skin grafting to break tolerance to class-I-disparate renal allografts in miniature swine despite inducing marked antidonor cellular immunity. *Transplantation*. 1991; 52(6):1044. [PubMed: 1750067]
16. Gianello PR, Sachs DH. Effect of major histocompatibility complex matching on the development of tolerance to primarily vascularized renal allografts: a study in miniature swine. *Hum Immunol*. 1996; 50(1):1. [PubMed: 8872170]
17. Yamada K, Gianello PR, Ierino FL, et al. Role of the thymus in transplantation tolerance in miniature swine. I: Requirement of the thymus for rapid and stable induction of tolerance to class-I-mismatched renal allografts. *The Journal of Experimental Medicine*. 1997; 186(4):497. [PubMed: 9254648]
18. Madsen JC, Yamada K, Allan JS, et al. Transplantation tolerance prevents cardiac allograft vasculopathy in major histocompatibility complex class-I-disparate miniature swine. *Transplantation*. 1998; 65(3):304. [PubMed: 9484744]
19. Yamada K, Mawulawde K, Menard MT, et al. Mechanisms of tolerance induction and prevention of cardiac allograft vasculopathy in miniature swine: the effect of augmentation of donor antigen load. *The Journal of Thoracic and Cardiovascular Surgery*. 2000; 119(4):709. [PubMed: 10733759]
20. Mezrich JD, Kesselheim JA, Johnston DR, et al. The role of regulatory cells in miniature swine rendered tolerant to cardiac allografts by donor kidney cotransplantation. *American Journal of Transplantation*. 2003; 3(9):1107. [PubMed: 12919090]
21. Kumagai N, LaMattina JC, Kamano C, et al. Vascularized islet cell transplantation in miniature Swine: islet-kidney allografts correct the diabetic hyperglycemia induced by total pancreatectomy. *Diabetes*. 2002; 51(11):3220. [PubMed: 12401713]
22. Kamano C, Vagefi PA, Kumagai N, et al. Vascularized thymic lobe transplantation in miniature swine: thymopoiesis and tolerance induction across fully MHC-mismatched barriers. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(11):3827. [PubMed: 15007168]

23. Mezrich JD, Benjamin LC, Sachs JA, et al. Role of the thymus and kidney graft in the maintenance of tolerance to heart grafts in miniature swine. *Transplantation*. 2005; 79(12):1663. [PubMed: 15973167]
24. Guzzetta PC, Sundt TM, Suzuki T, et al. Induction of kidney transplantation tolerance across major histocompatibility complex barriers by bone marrow transplantation in miniature swine. *Transplantation*. 1991; 51(4):862. [PubMed: 2014544]
25. Fuchimoto Y, Yamada K, Shimizu A, et al. Relationship between chimerism and tolerance in a kidney transplantation model. *Journal of Immunology*. 1999; 162(10):5704.
26. Yamada K, Shimizu A, Utsugi R, Ierino FL, Gargollo P, Haller GW, Colvin RB, Sachs DH. Thymic transplantation in miniature swine. II: Induction of tolerance by transplantation of composite thymokidneys to thymectomized recipients. *J Immunol*. 2000 Mar 15. 164(6):3079. [PubMed: 10706697]
27. Nobori S, Samelson-Jones E, Shimizu A, et al. Long-term acceptance of fully allogeneic cardiac grafts by cotransplantation of vascularized thymus in miniature swine. *Transplantation*. 2006; 81(1):26. [PubMed: 16421473]
28. Ierino FL, Yamada K, Hatch T, et al. Peripheral tolerance to class-I mismatched renal allografts in miniature swine: donor antigen-activated peripheral blood lymphocytes from tolerant swine inhibit antidonor CTL reactivity. *Journal of Immunology*. 1999; 162(1):550.
29. Mezrich JD, Kesselheim JA, Johnston DR, Yamada K, Sachs DH, Madsen JC. The role of regulatory cells in miniature swine rendered tolerant to cardiac allografts by donor kidney cotransplantation. *Am.J.Transplant*. 2003; 3:1107. [PubMed: 12919090]
30. Griesemer AD, Lamattina JC, Okumi M, et al. Linked suppression across an MHC-mismatched barrier in a miniature swine kidney transplantation model. *Journal of Immunology*. 2008; 181(6):4027.
31. Shimizu A, Yamada K, Meehan SM, et al. Acceptance reaction: intragraft events associated with tolerance to renal allografts in miniature swine. *Journal of the American Society of Nephrology*. 2000; 11(12):2371. [PubMed: 11095661]
32. Colvin RB. The renal allograft biopsy. *Kidney International*. 1996; 50(3):1069. [PubMed: 8872985]
33. Leto Barone AA, Leonard DA, Torabi R, Mallard C, Glor T, Scalea JR, Randolph MA, Sachs DH, Cetrulo CL Jr. The gracilis myocutaneous free flap in swine: An advantageous preclinical model for vascularized composite allograft transplantation research. *Microsurgery*. 2012 Jun 18.

Figure 1.

Gross appearance of VCA. Recipients of VCA following second kidney transplantation (Group 2) showed significantly prolonged epidermal survival (A, $*p=0.0246$).

Myocutaneous VCA in preparation for transplantation (B). Gross appearance of representative VCA, at the time of full viability (C, 18954-day 210), at the time of epidermal rejection (D, 18955-day 45) and after host reepithelialization (E, 18955-day 120).

Figure 2.

Histological findings. Biopsy of VCA skin (10X) and muscle (20X) from 18954 on POD 150, after H&E staining, representative of all VCAs biopsied before gross appearance of epidermal rejection, showing no histological evidence of rejection (A). Skin biopsy from 18958 on day 100, representative of all group 2 animals at the time of epidermal rejection after H & E staining (B, 10X), showing cellular infiltrate localized to the superficial dermis. Skin biopsy from 19941, representative of all group 1 animals at the time of epidermal rejection after H & E staining (C, 10X), showing a more dense infiltrate involving the deeper dermis. Muscle biopsy from 19842 at the time of graftectomy, representative of muscle biopsies from all animals at graftectomy, after H&E staining (D, 20X), showing viability although atrophic due to denervation. Kidney biopsy from 18954, representative of all kidney biopsies after PAS staining (E, 10X), was completely viable.

Figure 3.

Results of split thickness skin grafting. Appearance of completely rejected, donor matched STSG from 18955 on day 12 (A). Appearance of donor matched STSG on 18954, on day 60 that is 100% viable (B). Gross appearance of third party STSG, donor matched STSG and VCA, 14 and 43 days following third party STSG on Animal 1 (C). No change in renal graft function was induced by loss of STSG, representative serum creatinine from 18954.

Figure 4.

In vitro assays and flow cytometry analysis of anti-donor antibodies. CML assays were performed to assess anti-donor and anti-third party responses before each VCA, 60 days after VCA, 100 days after VCA, and after rejection of STSG. All animals displayed donor-specific hypo or unresponsiveness (mean 3.25% SD 2.22 at 100:1 E/T ratio) in CML at all time points before and after rejection of epidermis (A), except for 18955 which developed in vitro responsiveness only after STSG rejection (B). Flow cytometric analysis demonstrated neither anti-donor IgM (C) or IgG (D) developed following VCA transplantation.

Figure 5.

Analysis of CD4⁺, CD25⁺, Foxp3⁺ cells. T-cell phenotyping by flow cytometry demonstrates animal one maintained a higher absolute number of circulating CD4⁺, CD25⁺ and Foxp3⁺ triple positive (CD4⁺/CD25⁺/Foxp3⁺) lymphocytes for 12 weeks following VCA transplantation, which point all other animals had rejected the epidermis (A, * Time of epidermal rejection). The average number of CD4⁺/CD25⁺/Foxp3⁺ lymphocytes at the time of rejection was comparable to naïve animals, (B) and 18954 maintained a higher number of CD4⁺/CD25⁺/Foxp3⁺ lymphocytes throughout the experiment. Ratio of CD4⁺/CD25⁺/Foxp3⁺ to CD4⁺/CD25⁺/Foxp3⁻ lymphocytes, in all animals pre-VCA transplantation, average between days 28–85 (time period animals 2–6 rejected epidermis) and at the time of epidermal rejection, in comparison to naïve animals (n=4) (C). Immunofluorescent staining for FoxP3⁺ (Red) and CD25⁺ (Green), displaying more double positive cells (white arrows) in 18954 (D, superficial and deep dermis, ×600) than other animals (E, 18958-day 45, representative of animals that rejected epidermis in the first 12 weeks, ×600).