Vascularized Composite Allograft Transplant Survival in Miniature Swine: Is MHC Tolerance Sufficient for Acceptance of Epidermis?

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Background. We have previously reported that Massachusetts General Hospital miniature swine, which had accepted class I–mismatched kidneys long-term after 12 days of high-dose cyclosporine A, uniformly accepted donor-major histocompatibility complex (MHC)–matched kidneys without immunosuppression but rejected donor MHC-matched split-thickness skin grafts by day 25, without changes in renal graft function or antidonor 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 less than 70 days after primary kidney transplant (KTx). Group 2 animals (n=3) received a second donor-matched kidney transplant followed by a donor-matched VCA more than 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 three recipients of VCAs in Group 2, accepted all components of the VCA, including epidermis (>200 days). The other two recipients lost only the epidermis on days 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 compared with split-thickness skin grafts but not indefinite. Exposure of tolerant animals to second donor-matched kidneys before VCA increases the longevity of the VCA epidermis, suggesting an increase in the immunomodulatory mechanisms associated with tolerance of the kidney.

Keywords: Massachusetts General Hospital miniature swine, Vascularized composite allograft, Skin-specific antigens, Vascularized skin.

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Interest in clinical vascularized composite allograft (VCA) transplantation has increased significantly in the past two decades. As of 2012, more than 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*). To prevent rejection of VCAs, these patients require intensive induction immunosuppression regimens as well as lifelong immunosuppression, placing VCA recipients at risk for posttransplantation 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

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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 Massachusetts General Hospital (MGH) miniature swine, in which major histocompatibility complex (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 cyclosporine A (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 antidonor 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 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

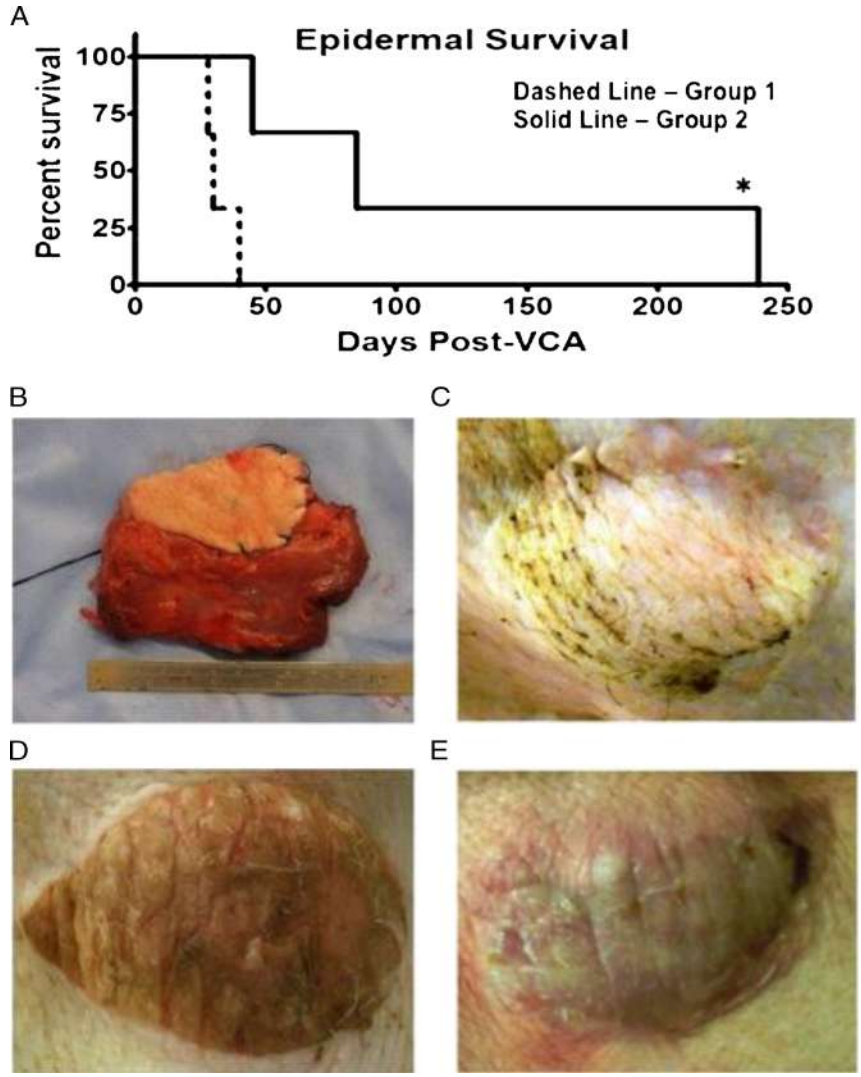

FIGURE 1. Gross appearance of VCA. A, recipients of VCA after second KTx (Group 2) showed significantly prolonged epidermal survival (*P=0.0246). B, myocutaneous VCA in preparation for transplantation. Gross appearance of representative VCA. C, at the time of full viability (animal #18954, day 210). D, at the time of epidermal rejection (animal #18955, day 45). E, After host reepithelialization (animal #18955, day 120).

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 after 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, which uniformly facilitates to induce tolerance of kidneys (*15, 16*). Renal function was within normal range (creatinine <2.0 mg/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 less than 70 days after primary kidney transplant (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 more than 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)

Gross Findings

Animals in Group 2 had significantly prolonged epidermal survival in comparison with Group 1 (P=0.0246) (Fig. 1A) with a median survival of 85 days compared with 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 less than 70 days after primary KTx. Group 1 animals (#19842, #19941, and #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).

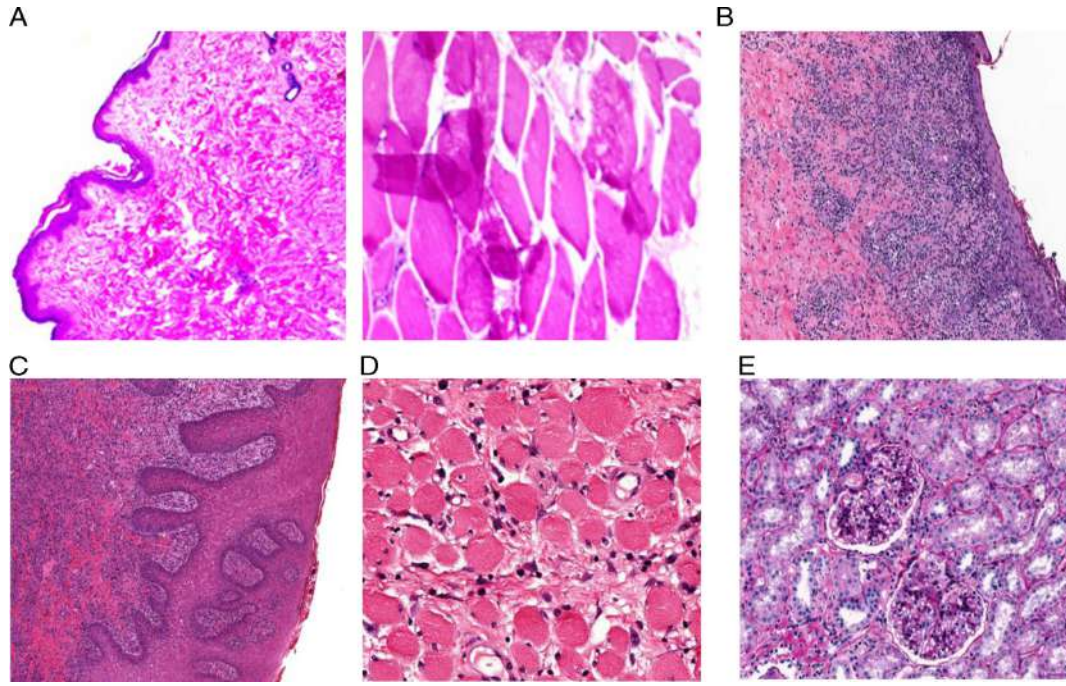

FIGURE 2. Histologic findings. A, biopsy of VCA skin (10×) and muscle (20×) from animal #18954 on postoperative day 150, after H&E staining, representative of all VCAs biopsied before gross appearance of epidermal rejection, showing no histologic evidence of rejection. B, skin biopsy from animal #18958 on day 100, representative of all Group 2 animals at the time of epidermal rejection after H&E staining (10×), showing cellular infiltrate localized to the superficial dermis. C, skin biopsy from animal #19941, representative of all Group 1 animals at the time of epidermal rejection after H&E staining (10×), showing a more dense infiltrate involving the deeper dermis. D, muscle biopsy from animal #19842 at the time of graftectomy, representative of muscle biopsies from all animals at graftectomy, after H&E staining (20×), showing viability although atrophic due to denervation. E, kidney biopsy from animal #18954, representative of all kidney biopsies after periodic acid–Schiff staining (10×), indicated that the graft was viable.

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 more than 200 days after VCA transplantation (see next section). Animals #18958 and #18955 rejected the epidermis 85 and 45 days after VCA, respectively (Fig. 1D, E). 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).

Histologic Findings

VCA biopsy of animal #18954 at 150 days after VCA was representative of the histologic 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 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 (a) immunologic responses to epidermis, likely associated with an SSA, differed from those to the donor MHC and (b) 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 more than 100 days after VCA transplantation.

Animal #18954, which had accepted all components of the VCA, accepted the donor-matched STSG for more 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 was 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 and 239 days after VCA), both the donor-matched STSG and the epidermis of the VCA were rejected simultaneously (Fig. 3B, C). The muscle and dermis remained viable and no change in renal graft function was induced by loss of STSG (Fig. 3D). No antidonor (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 preformed to assess antidonor and anti-third-party responses before each VCA, 100 days after VCA, and after rejection of STSG. All animals displayed donor-specific hyporesponsiveness or unresponsiveness in CML at all time points (Fig. 4A), except for animal #18955 (Fig. 4B). Animal #18955 displayed weak antidonor responsiveness after rejection of a donor-matched STSG but maintained normal renal function. To ensure tolerance of donor MHC, animal #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 more than 150 days before termination of the experiment. Additionally, despite loss of the epidermis, neither antidonor (SLA class Ic) IgM nor IgG were detected in any of the six recipients (Fig. 4C, 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 $CD4^+/CD25^+/FoxP3^+$ Regulatory T Cells

To further elucidate the mechanism of acceptance or rejection of VCAs, phenotypic analysis of animals' peripheral blood mononuclear cells (PBMCs) was performed.

Animals in Group 2, which demonstrated enhanced survival of VCAs, all had higher ratio of $CD4^+/CD25^+/FoxP3^+$:$CD4^+/CD25^+/FoxP3^-$ before VCA transplant when compared with animals in Group 1 (Fig. 5). Moreover, animal #18954, which accepted all components of VCA for more than 200 days, maintained the highest absolute number of $CD4^+/CD25^+/Foxp3^+$ T cells in the periphery during the first 12 weeks after VCA transplantation, at which time all other animals had rejected epidermis (Fig. 5A–C). 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±SD=27.4±8.6) when compared with 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 after VCA transplantation, whereas the others 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

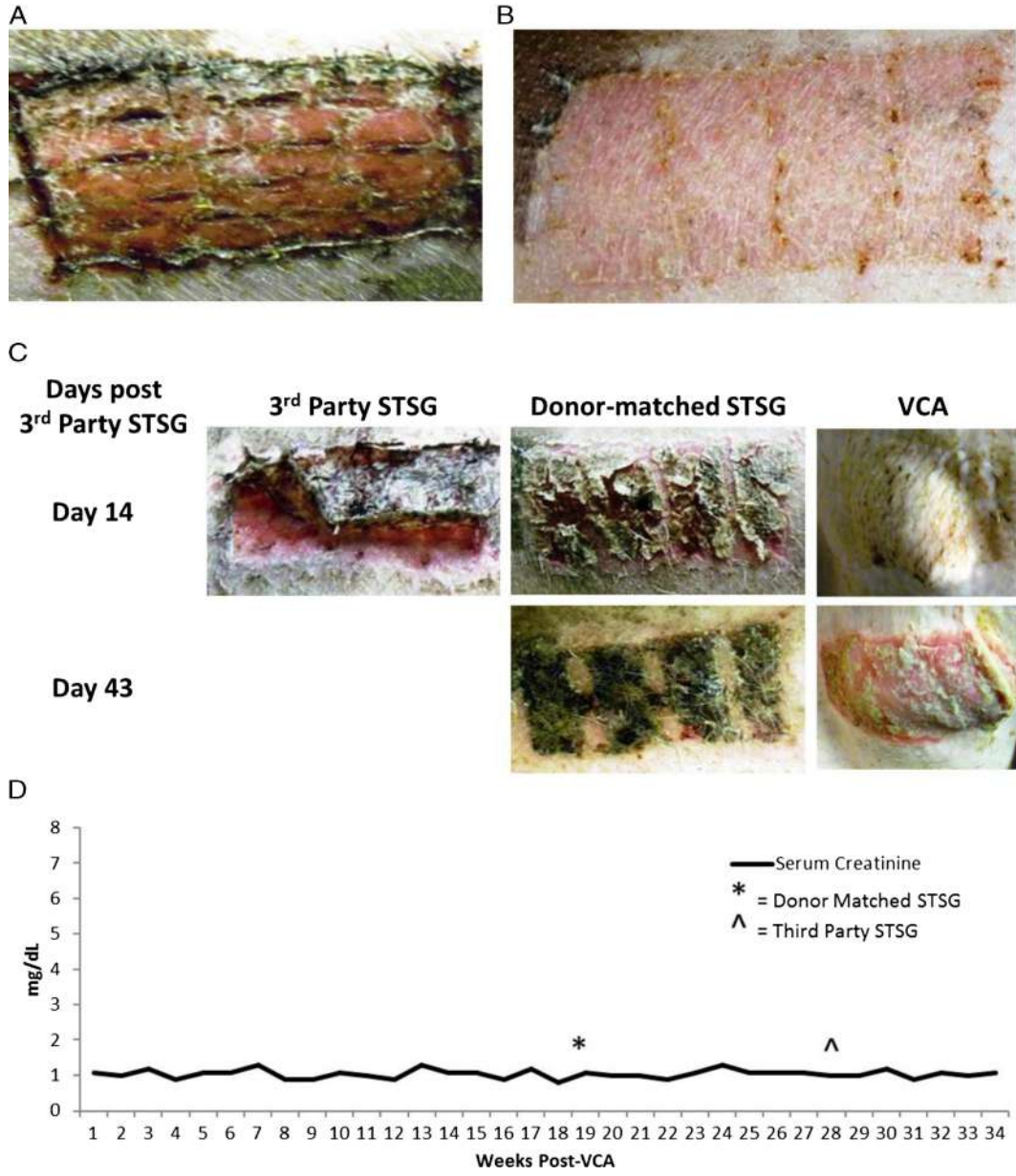

FIGURE 3. Results of STSG. A, appearance of completely rejected, donor-matched STSG from animal #18955 on day 12. B, appearance of donor-matched STSG on animal #18954 on day 60, which is 100% viable. C, gross appearance of third-party STSG, donor-matched STSG and VCA, 14 and 43 days after third-party STSG on animal #18954. D, No change in renal graft function was induced by loss of STSG, representative serum creatinine from animal #18954.

(Fig. 5D) compared with other animals in the study (Fig. 5E) before the rejection of epidermis. Many cells stained positive with FoxP3 staining (red) in animal #18954, whereas other animals displayed some yellow staining with FoxP3, suggesting nonspecific 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 preexisting tolerance. These data support our previous findings in thymic and islet KTx models (*21, 26*), which indicated that vascularization of allografts is advantageous for graft acceptance, likely due to differences in the

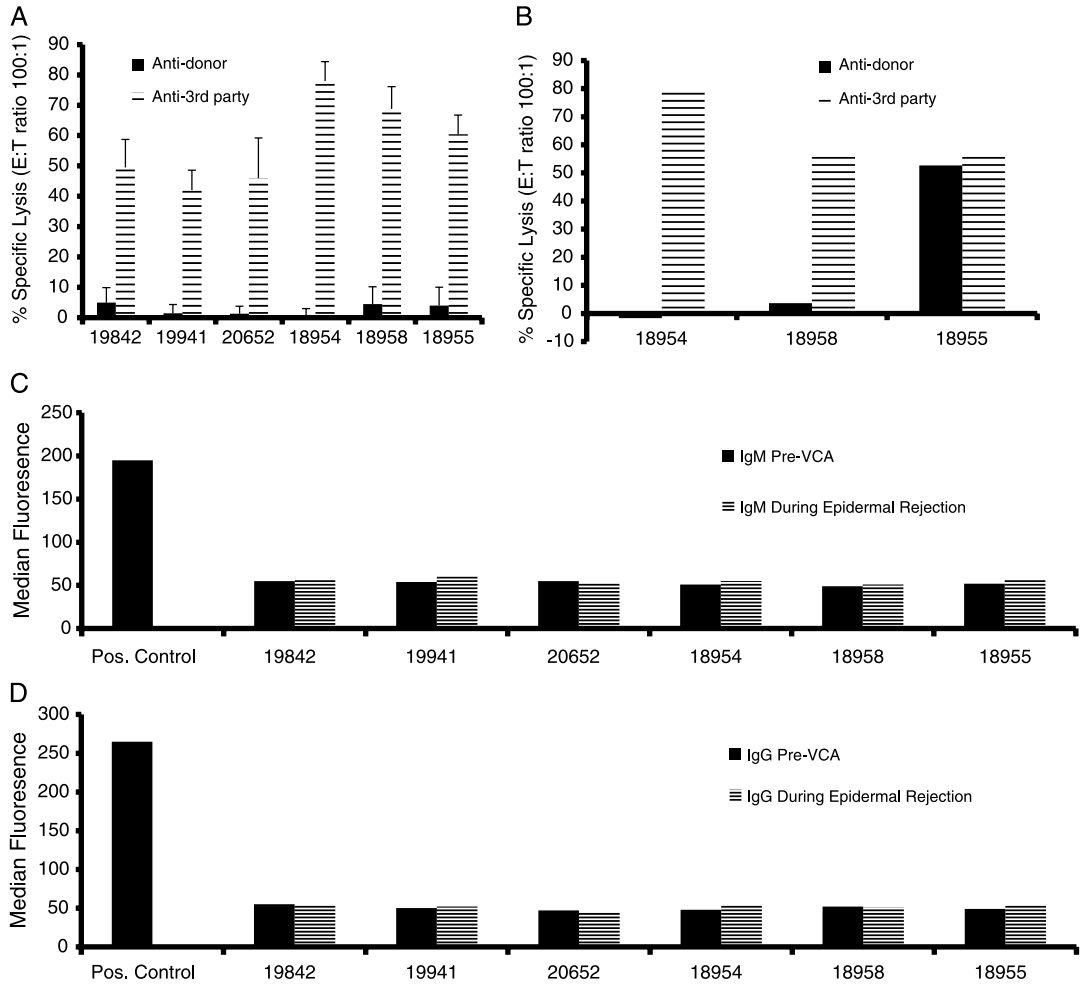

FIGURE 4. In vitro assays and flow cytometry analysis of antidonor antibodies. CML assays were preformed to assess antidonor and anti-third-party responses before each VCA, 60 days after VCA, 100 days after VCA, and after rejection of STSG. All animals displayed donor-specific hyporesponsiveness or unresponsiveness (mean±SD=3.25%±2.22% at 100: 1 E/T ratio) in CML at all time points before and after rejection of epidermis (A), except for animal #18955 that developed in vitro responsiveness only after STSG rejection (B). Flow cytometric analysis demonstrated neither ant-donor IgM (C) or IgG (D) developed after VCA transplantation.

manner of antigen presentation. Furthermore, maintenance of the (a) kidney and (b) 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 classic 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 before 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 regulatory T cells (Tregs) that developed after class I–mismatched KTx potentially provided linked suppressive effects on T-cell responses against SSAs that coexisted on epidermis of VCAs along with donor MHC. Data in this study showed that (a) 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 (b) 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 Tregs and epidermal acceptance. Furthermore, we demonstrated that animals in Group 2, which received second kidneys before VCAs, all had higher ratio of Tregs, $CD4^+/CD25^+/FoxP3^+$ before VCA transplantation, and enhanced survival of the VCAs than those in animals in Group 1 that received VCAs less than 70 days after kidney transplant. This finding suggests that repriming by donor kidneys may have augmented donor-specific T regulatory effects. We have previously demonstrated that repriming recipient PBMCs with donor antigens, either in vivo by retransplantation of donor-matched kidneys or in vitro by coculture assays, led to effective suppression of

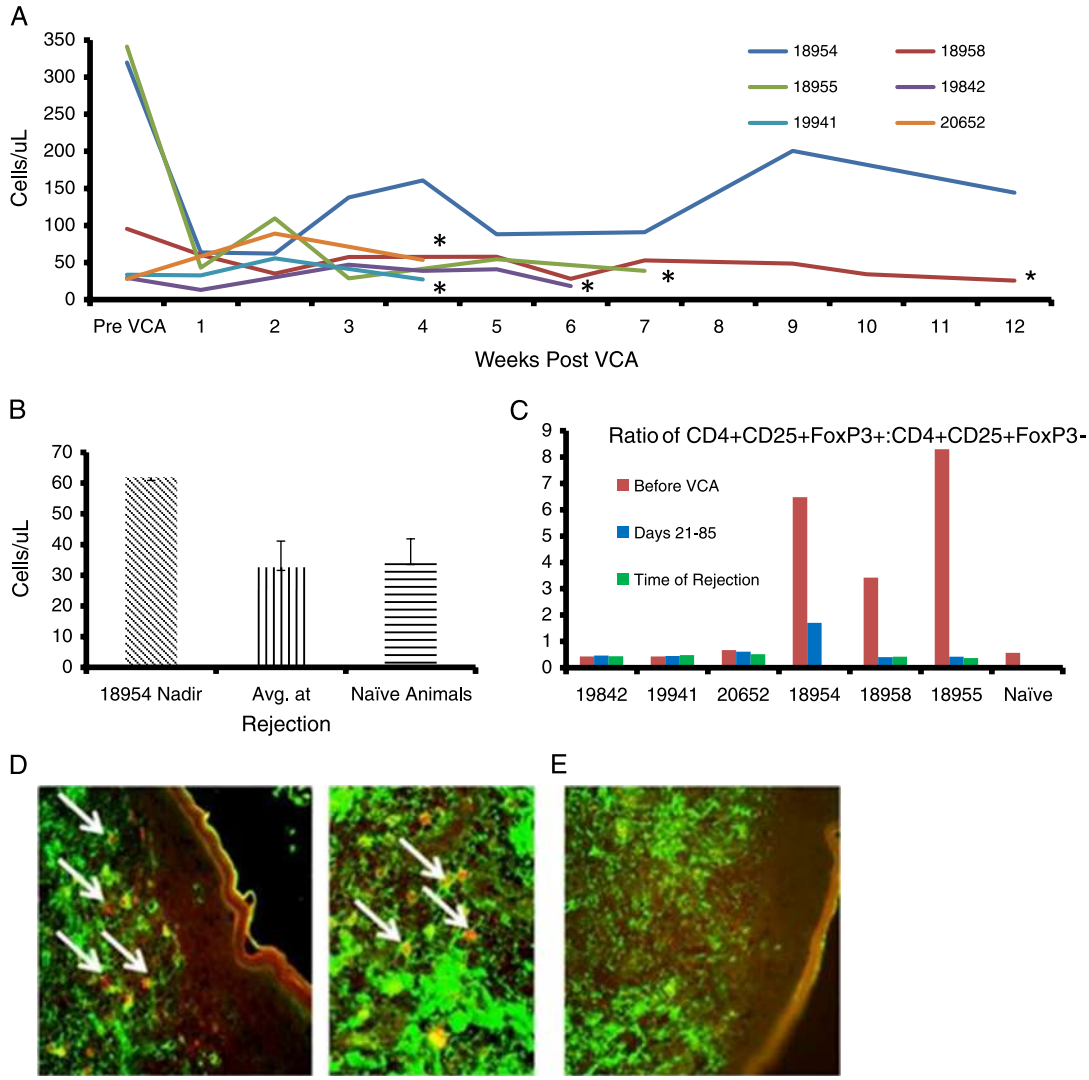

FIGURE 5. Analysis of $CD4^+$, $CD25^+$, and $Foxp3^+$ cells. T-cell phenotyping by flow cytometry demonstrates animal #18954 maintained a higher absolute number of circulating CD4, CD25, and Foxp3 triple-positive ($CD4^+/CD25^+/Foxp3^+$) lymphocytes for 12 weeks after VCA transplantation, at which point 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 with naïve animals (B) and animal #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 and 85 (time period during which the other animals rejected epidermis) and at the time of epidermal rejection in comparison with naïve animals (n=4) (C). Immunofluorescent staining for $FoxP3^+$ (red) and $CD25^+$ (green), displaying more double-positive cells (white arrows) in animal #18954 (D; superficial and deep dermis, ×600) than in other animals (E; animal #18958, day 45; representative of animals that rejected epidermis in the first 12 weeks, ×600).

antidonor responses (*20, 28, 29*). We have also reported that priming via a donor-matched transplant was required for linked suppression to be seen in vivo (*29*). Moreover, we have demonstrated that the repriming of tolerated cells with donor antigens was required to suppress naïve cytotoxic lymphocyte responses to donor antigens in coculture assays (*28*), and this suppression was lost after the depletion of $CD25^+$ cells but reestablished by addition of a small number of the $CD25^+$ enriched population (*20*). These results demonstrate that either priming via a donor-matched kidney retransplantation (through linked suppression) or priming in vitro for coculture 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 Tregs 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 using 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 that 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 antiepidermal immune responses. Subsequent epidermal loss did not break tolerance of kidneys or restored in vitro antidonor responses, indicating that the observed epidermal loss was not related to antidonor 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 (C.L. Cetrulo 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 but not present in other organs or tissues. Our results suggest that Tregs may be capable of supporting the viability of the epidermis of the VCAs and warrant further investigation.

MATERIALS AND METHODS

Animals

Donor animals of kidneys and VCA were SLAgg (class Ic/IId) MGH miniature swine. To achieve a two-haplotype MHC class I mismatch with no MHC class II mismatch, recipient animals were 4 to 6 months old from a partially inbred line of SLAdd (class Id/IId) MGH miniature swine. The immunogenic characteristics of the MGH miniature swine and the intra-MHC recombinant haplotypes have been described previously (*9–12*).

Experimental Groups

Surgery

(1) KTx and induction protocol: The surgical procedures for primary and secondary transplantation have been described previously in detail (*15–17*). Both native kidneys were removed on the day of the primary KTx. 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, and blood.

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

(2) VCA transplantation: Gracilis musculocutaneous VCAs were harvested on saphenous artery and vein to their origin on the femoral vessels as described previously (*30*). Femoral artery and vein were anastomosed to recipient carotid artery and internal jugular vein or femoral artery and vein, respectively.

(3) STSG: Zimmer dermatome was used to harvest STSGs (4×6 cm) from donors and to create split-thickness skin defects in preparation of grafting on recipients.

Histologic Analysis of Allografts

VTL graft biopsies were performed on 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 (H&E) 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 pathologic specimens (*31, 32*).

Immunologic Assays

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

(2) Assessment of antidonor antibodies: Antidonor IgM and IgG antibodies in the serum and in frozen biopsy samples were assessed by indirect fluorescence-activated cell sorting and immunohistochemistry, respectively, as described previously (*17, 31*).

(3) Assessment of FoxP3$^+$, CD4$^+$, and CD25$^+$ lymphocytes: The percentage of CD4$^+$, CD25$^+$ and FoXP3$^+$ cells in PBMCs was assessed by fluorescence-activated cell sorting analysis using fluorescein isothiocyanate–conjugated anti-CD4 (VMRD, Pullman, WA), bioconjugated anti-CD25 (Fitzgerald Industries, Acton, MA), and phycoerythrin-conjugated FoxP3 (eBioscience, San Diego, CA).

Two-color immunohistochemistry for FoxP3$^+$ and CD25$^+$ was performed using frozen tissue sections and standard indirect technique. Briefly, 4 mm frozen sections were stained with anti-FoxP3 monoclonal antibody followed by Texas red–labeled anti-mouse IgG antibody (Dako, Carpinteria, CA) and then incubated with fluorescein isothiocyanate–conjugated anti-CD25 monoclonal antibody.

Monitoring of rejection: Rejection of kidney grafts was monitored by serum creatinine levels and histology of kidney biopsies. VCA grafts and STSGs 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 skin 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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