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ORIGINAL ARTICLE

Stable mixed hematopoietic chimerism permits tolerance of vascularized composite allografts across a full major histocompatibility mismatch in swine

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Summary

This study tested the hypothesis that vascularized composite allografts (VCA) could be accepted in a robust model of hematopoietic chimerism by injecting allogeneic bone marrow cells (BMC) into swine fetuses. Outbred Yorkshire sows and boars were screened to ensure the absence of the major histocompatibility (MHC) allele SLA^{cc} of inbred MGH miniature swine and then mated. Bone marrow harvested from an SLA^{cc} swine donor was T-cell depleted and injected intravenously into the fetuses between days 50–55 of gestation. After birth, the piglets were studied with flow cytometry to detect donor cells and mixed lymphocyte reactions (MLR) and cell-mediated lympholysis (CML) assays to assess their response to donor. Donor-matched VCAs from SLA^{cc} donors were performed on four chimeric and two nonchimeric swine. The results showed donor cell engraftment and multilineage macrochimerism after the *in utero* transplantation of adult BMC, and chimeric animals were unresponsive to donor antigens *in vitro*. Both control VCAs were rejected by 21 days and were alloreactive. Chimeric animals accepted the VCAs and never developed antidonor antibodies or alloreactivity to donor. These results confirm that the intravascular, *in utero* transplantation of adult BMC leads to donor cell chimerism and donor-specific tolerance of VCAs across a *full MHC barrier* in this animal model.

Introduction

The introduction of successful hand allotransplantation in 1998 and the subsequent success of face transplantation have clearly demonstrated the clinical potential for using vascularized composite allografts (VCA) for replacing and reconstructing tissues following traumatic tissue loss or disfigurement [1–4]. Adoption of immunosuppressive regimens that have been developed for solid organ allotransplantation have permitted long-term viability of these VCAs restoring function and self-esteem to those patients receiving hands and facial tissues. There is a certain level risk, however, to taking systemic immunosuppression over many years, the long-term consequences of which are not known in VCAs as they are in solid organ transplantation. Like solid organs, achieving tolerance of VCAs is an overarching goal that would permit more widespread clinical application of allotransplantation without the risk of

immune rejection by the allograft recipient. The most robust means of achieving tolerance of organ allografts in adult large animal models relies on mixed hematopoietic chimerism [5–8]. This strategy has been successful in permitting survival of kidney and heart allografts across a single haplotype class I and II major histocompatibility (MHC) barrier in swine long after the mixed hematopoietic chimerism has been established [6,9,10]. This strategy has been successful for achieving tolerance of VCAs across this same MHC barrier in swine [11–13]. One report by Kuo *et al.* [14] has demonstrated the use of the bone marrow mesenchymal cell population in generating acceptance of a VCA in outbred swine, but no data was presented showing that the host was unresponsive immunologically to the allograft. Thus, transplantation of VCAs across a full class I and II MHC barrier in an immunologically defined large animal showing host unresponsiveness to the VCA has not been reported. The goal of this study was to employ a robust and durable means to generate mixed hematopoietic chimerism to test our hypothesis that VCAs could be transplanted across a full MHC mismatch without the need for long-term immunosuppression. One possibility was the introduction of hematopoietic stem cells (HSC) into the developing fetal immune system to generate mixed hematopoietic chimerism and subsequent acceptance of fully MHC-mismatched VCAs.

The introduction of HSC into the fetus *in utero* to influence the developing immune and hematologic systems could induce tolerance of foreign antigens. Ideally, the recipient of an *in utero* stem cell transplant would demonstrate both engraftment and proliferation of the hematologic cells as well as tolerance of the donor antigens. Investigators have shown that *in utero* transplantation of fetal liver cells into the peritoneal cavity of mice results in low levels of donor cell chimerism and donor-specific tolerance in a small percentage of those animals [15,16]. One report in sheep and one in monkeys demonstrated similar results with fetal liver cells [17,18]. A few papers have reported low levels of chimerism by PCR and tolerance in rodent models across minor and class I disparate barriers using adult marrow [19–21]. Success in large animal models using adult bone marrow cells (BMC) has been more elusive, with isolated reports of the presence of allogeneic donor cells demonstrated by PCR analysis in primates and sheep without evidence for generating tolerance [22–24], except for one report of kidney allograft prolongation in a single primate [25].

We have demonstrated previously that injection of partially T-cell depleted adult bone marrow into an intrahepatic vein of MHC-disparate swine fetuses led to donor cell macrochimerism and donor-specific tolerance [26–28]. Hematopoietic chimerism persisted in the surviving offspring postnatally without the need for immunosuppression, and there was no evidence of GVHD. The state of donor-specific hyporesponsiveness was confirmed by the use of multiple *in vitro* assays, the prolongation of donor skin graft survival, and the long-term acceptance of donor-matched kidney allografts. These experiments provided evidence that the intravascular injection of adult bone marrow into the fetal system can lead to long-term presence of donor hematopoietic cells and tolerance of donor-matched kidney allografts in this large animal, preclinical model [28–30].

A strategy to introduce HSC into the fetal immune system may be one means to generate durable mixed hematopoietic chimerism across a full MHC barrier and permit us to test our hypothesis that tolerance can be generated to fully MHC-mismatched VCAs. Secondly, we sought to determine if the transplantation of a primarily vascularized bone marrow compartment in the form of a limb allograft could provide the recipient with a functioning bone marrow microenvironment comprised of a full complement of hematopoietic and stromal cells in their natural spatial and physiologic relationship. Thus, in this study, we sought to challenge the immune system of our *in utero* injection (IUI) animals with a vascularized bone marrow compartment graft to evaluate its effect on peripheral chimerism, confirm that donor marrow continued to function within the transplant, and assess the effect on long-term tolerance.

Methods

Donor and recipient animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital under protocol number 2003N000021. Bone marrow and musculoskeletal allograft donors were from the population of MGH miniature swine (Transplantation Biology Research Center, Boston, MA, USA). These swine have well-characterized immunogenetics that permits defined transplantation barriers [31–34]. All donor swine were homozygous for swine leukocyte antigen (SLA) class I^c as evidenced by flow cytometry using monoclonal antibody 16.7.E4 directed against SLA class I^c [35,36]. The recipients were outbred Yorkshire fetuses from sows and boars whose peripheral blood lymphocytes (PBL) screened negative for SLA class I^c.

Donor bone marrow harvest, processing, and T-cell depletion, and *in utero* transplantation

These procedures have been described in detail in a previous paper [26]. Briefly, 3-month-old (SLA^{cc} in all groups) miniature swine were euthanized and exsanguinated. Donor bone marrow was collected, processed, and T-cell depleted via magnetic beads passed through a type CS separation column in the MACS magnetic cell sorting system (Miltenyi Biotec Inc., San Diego, CA, USA).

Pregnant Yorkshire sows, 50–55 days of gestation (normal gestation 115 days), underwent a midline laparotomy and the fetuses identified by ultrasound. 5×10^8 T-cell depleted BMC (fetus weighed about 250 g; dose was 2×10^9/kg) were injected through the uterine wall into the intrahepatic vein of each fetus under continuous visualization using ultrasound guidance [26].

Mixed lymphocyte reactions (MLR)

Responder (4×10^5) and irradiated (25 Gy) stimulator (4×10^5) lymphocytes were co-cultured in 96-well flat-bottomed plates. Each animal served as the responder with the following stimulators: self, donor SLA^{cc} cells, and third-party Yucatan swine cells. Cultures were incubated for 5 days and pulsed for 6 h with one μCi of ^3H thymidine per well. Proliferation of responder cells, assessed by the uptake of ^3H, was expressed as a stimulation index (SI) calculated as the ratio of average counts per minute (c.p.m.) in the experimental wells versus negative control wells.

Cell-mediated lymphocytotoxicity (CML) assays

One-way mixed lymphocyte cultures were prepared using 24-well flat-bottomed plates containing 4×10^6 responder and 4×10^6 irradiated (25 Gy) stimulator peripheral blood lymphocytes (PBLs) per well and cultured [37]. After 6 days, effector cells were harvested and tested against lymphoblast targets prepared by culturing 3×10^7 PBLs in medium containing phytohemagglutinin (M-Form) (GIBCO, Grand Island, NY, USA) for 18–24 h. Targets were labeled with ^{51}Cr (Amersham, Arlington Heights, IL, USA) and plated in 96-well round-bottomed plates with effector cells. Effector cells were tested against four targets (self, SLA^{cc}, SLA^{dd}, and Yucatan) at four effector:target ratios (100:1, 50:1, 25:1, and 12.5:1). ^{51}Cr release in the supernatant was determined using a gamma counter and compared with background and maximum release. The results were expressed as percent-specific lysis.

Tissue biopsies and flow cytometry

Peripheral blood lymphocytes and whole blood were collected, processed, and suspended in flow cytometry media for analysis by two-color flow cytometry as previously described. Single cell suspensions were prepared from biopsy samples of bone marrow (from the recipient's bone marrow and from the marrow in the vascularized composite allograft), mesenteric lymph node, thymus, spleen, and liver for flow cytometry [5]. Piglets were screened for donor cells with a mAb against SLA class I^c 16.7.E4 conjugated to fluorescein isothiocyanate (FITC). The phenotype of the donor cells were identified with MSA4 (IgG2a, antiswine CD2) [36], 898H2-6-15 (IgG2a/k, antiswine CD3) [38], 74-12-4 (IgG2b, antiswine CD4) [39], 76-2-11 (IgG2a/k, antiswine CD8) [40], 2.27.3a (IgG1/k, antiswine Class I), 76-7-4 (IgG2a/k, antiswine CD1) [39], 1038H-H-4-6 (IgM/K, anti-CD9) [41], and 74-22-15 (IgG1/k, antiswine monocyte/granulocyte) [40]. To determine multilineage chimerism, whole blood was incubated with donor-specific FITC-conjugated antiswine class I^c 16.7.E4 FITC together with the biotin-conjugated CD172 mAb 74-22-15 (BALB/c, IgG1K), which is specific for monocytes and granulocytes, followed by streptavidin phycoerythrin (PESA; PharMingen, San Diego, CA, USA). Two animals received VCA from donors that were positive for pig allelic antigen (PAA) and were analyzed by fluoresence-activated cell sorting (FACS) using the antibody 1038-H-9 (IgM, anti-PAA). Data were analyzed using WINLIST mode analysis software (Verity Software House, Topsham, ME, USA).

In vitro tolerance assessment

In vitro testing consisting of MLR assays were performed at 4, 7, 10, 14, and 20 weeks after birth, and CML assays were performed at 6, 12, and 20 weeks after birth.

Vascularized composite allograft (VCA) harvest and implantation

The allograft procedure has been previously described in detail [42]. Briefly, the VCA consisting of the distal femur and proximal tibia along with an accompanying soft tissue cuff based on the femoral vessels was harvested from the donor. The graft was placed into a subcutaneous abdominal wall pocket, and graft vessels anastomosed to the recipient's femoral vessels. Four chimeric swine (827-4, 827-7, 827-9, and 827-11) and two nonchimeric littermates (827-6 and 827-10) underwent a VCA transplant. All of the allografts were MHC donor-matched to the original bone marrow donor (SLA^{CC}) but presumably maintained minor antigen differences from that donor. To cover these minor antigen differences, each animal was treated with 12 days of cyclosporine beginning on the day of transplant.

Immunosuppression

All VCA recipient animals received cyclosporine. An intravenous preparation of cyclosporine (Sandimmune, Norvartis, E. Hannover, NJ, USA) given each morning as a single daily infusion at a dose of 10–13 mg/kg for 12 consecutive days beginning on the day of transplant. The first dose was administered to the recipient 1 h prior to transplantation. Trough levels were obtained on a daily basis beginning on day three and adjusted to maintain a blood level of 400–800 ng/ml.

Rejection monitoring

Open biopsies were performed on postoperative days 14, 28, 56, 100, and then approximately every 3 months. Long-term graft acceptors were followed for a minimum of 150 days to confirm repeated immunological and histological findings, but some were followed as long as 260 days to observe the function of the VCA marrow component. Lack of bleeding and discoloration of muscle and bone marrow were accepted as criteria of rejection. The biopsy specimens of bone, bone marrow, and muscle were stained with hematoxylin and eosin. A pathologist (G.P.N.) reviewed the histological preparations of the biopsy specimens in a blinded fashion. Rejection was graded on histologic parameters including interstitial and perivascular lymphocytic infiltration, ischemic or necrotic muscle, presence or absence of osteocytes in bone, and decreased or abnormal bone marrow cell populations.

Donor skin graft

Prior to euthanizing the bone marrow donor, skin grafts were harvested in a sterile fashion and frozen at −80 °C [43]. At 100 days post-transplantation of the VCA, skin grafts were placed on recipients with allografts. Split-thickness skin grafts of recipient, donor, and third-party major antigen mismatched swine were secured on a deep partial-thickness bed of the posterior thorax of the limb recipient. All skin grafts, including the autografts, were frozen prior to the procedure and thawed immediately prior to placement on the recipient as previously described [44]. All skin grafts were observed on a daily basis for evidence of rejection and biopsed if the graft appeared to be rejecting.

Humoral assay

Offspring were tested for antidonor IgG and IgM antibodies, before and after skin grafting. 10 μl of serum from each offspring were added to PBLs from a SLA^{cc} MGH miniature swine. Fluoresceinated goat antiswine IgG and goat antiswine IgM antibodies were used as secondary stains and analyzed by FACS. Naive Yucatan swine served as the negative control. Sera from presensitized animals were used as a positive control.

Results

Six piglets had circulating donor cells (chimerism) ranging from 1.8% to 90% by FACS analysis of the peripheral blood (Fig. 1a–c) (Table 1). Donor cells were detectable by FACS throughout the life of the animals (>42 weeks of age; Fig. 1k). Whole-blood analysis of the offspring from these two litters included the presence of donor granulocytes, monocytes, and lymphocytes (Fig. 1d–g). Animals demonstrating persistent chimerism in the peripheral blood also manifested donor cells in their lymphohematopoietic tissues for the duration of the experiment (>42 weeks). Biopsies of bone marrow (Fig. 1h), spleen (Fig. 1i), lymph nodes (Fig. 1j), thymus, and liver were positive for donor SLA^{cc} cells by FACS analysis. Analysis of the recipient's bone marrow revealed a population of donor-derived class I^c BMC (Fig. 1h). The majority of the donor cells detected in spleen stained positive for monocyte and granulocyte markers (Fig. 1i). In the lymph node and thymus, the majority of donor cells stained positive for CD3 (Fig. 1j). The persistence of the multilineage chimerism suggests donor cell engraftment and hematopoiesis.

In vitro evidence of tolerance

All chimeric swine demonstrated in vitro evidence for donor-specific tolerance of the bone marrow donor's MHC (SLA^{cc}). Multiple MLR assays demonstrated no response against donor SLA^{cc} cells (Fig. 2a) and normal responses to control, third-party cells (Yucatan miniature swine; Fig. 2b). CML assays performed on cells from the chimeric swine were also hyporesponsive to donor-type cells (Fig. 2c). Nonchimeric littermates mounted vigorous antidonor responses in both MLR (Fig. 2) and CML (CML data not shown) assays. All swine (chimeric and nonchimeric) lysed third-party targets (Yucatan miniature swine) demonstrating immunocompetence. The transplantation of the VCA did not alter the results of the MLR and CML for the chimeric swine. In addition, the placement of the skin grafts at post-transplant day 100 also did not increase the antidonor sensitivity.

Vascularized composite allografts

The nonchimeric littermates (827-6 and 827-10) rejected their donor-matched VCAs between 19 and 21 days post-transplant despite a 12-day course of cyclosporine (Table 2). These donor-matched allografts were viable at postoperative day 7 confirming vascular perfusion but demonstrated severe rejection by postoperative day 21. Each of the control allografts showed unequivocal gross changes of rejection consisting of tissue discoloration and lack of bleeding from the muscle and bone marrow. Histologic analysis of the allograft demonstrated evidence of severe acute rejection in the muscle, bone, and bone marrow (Fig. 3a–c).

All of the chimeric animals (827-4, 827-7, 827-9, and 827-11) accepted the allograft by both gross and histologic analysis for the duration of the experiment (>150 days; Fig. 3d–f). All four allografts demonstrated patent vessels, bleeding from marrow cavities, and viable bone and soft tissue on gross examination at the time of harvest (150–

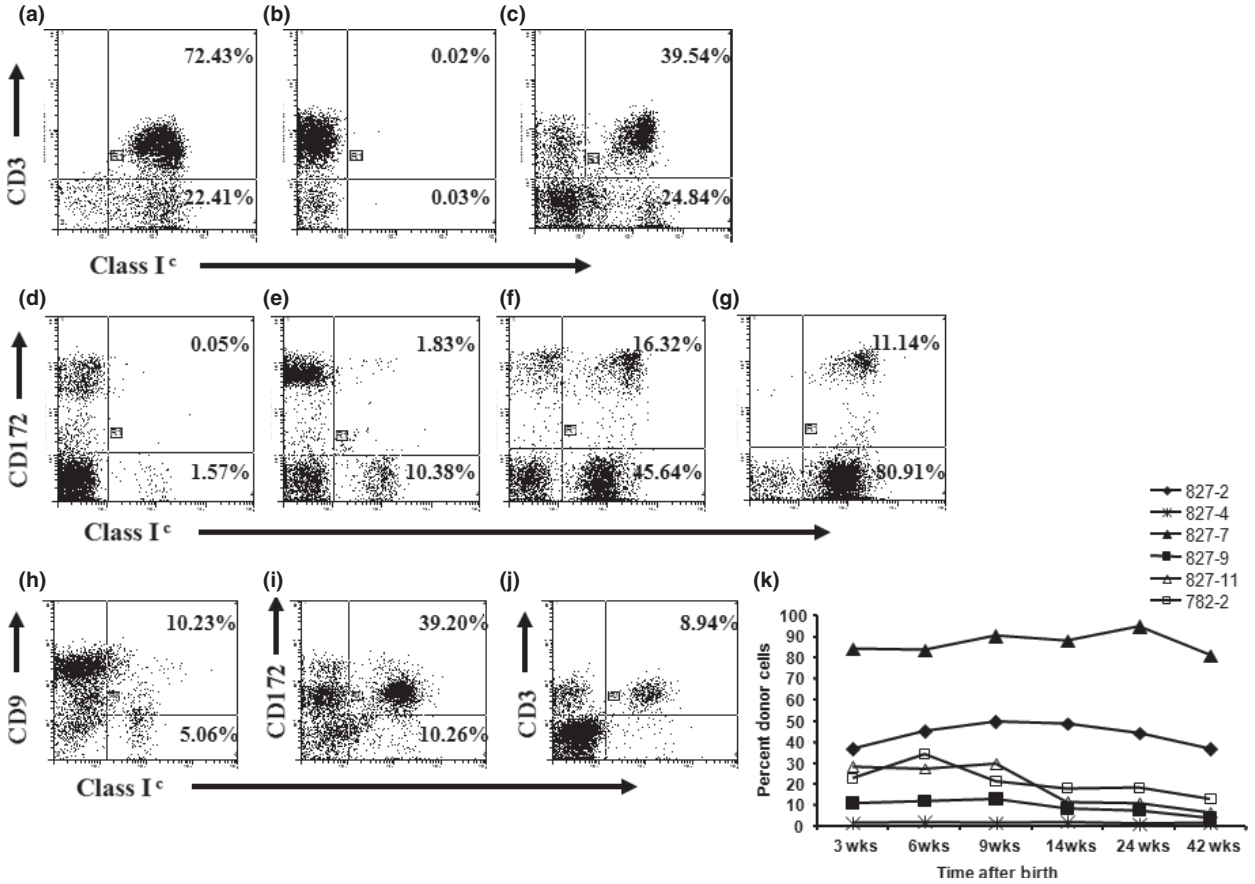

Figure 1 FACS data from offspring. (a) Peripheral blood mononuclear cells from a control SLA^{cc} swine at 4 weeks of age showing cells dual stained for class I^{c} (16.7.E4) and CD3 (898H2-6-15) to discern donor T cells. (b) Nonchimeric littermates at 4 weeks of age do not show any cells expressing the donor major histocompatibility (MHC) class I^{c}. (c) Specimen from a representative chimeric pig at 4 weeks of age showing two distinct cell populations, one staining positive for class I^{c} from the donor (>64%) and the other not staining from the host. (d) FACS analysis of whole blood from a chimeric swine at 12 weeks with no staining with the CD172 antibody which indicates that there are no detectable donor cells from the monocyte or granulocyte lineages in this animal. (e–g) Blood specimens from three other chimeric animals at 12 weeks show varying degrees of staining for monocytes and granulocytes (1.83–11.14%). (g) All of the cells from the granulocyte/monocyte lineage are donor-derived in this chimeric swine at 12 weeks, indicating full engraftment by the injected bone marrow. (h) FACS analysis of the bone marrow from swine 827-2 at 12 weeks demonstrates large percentage of cells that are class I^{c}-positive suggesting engraftment. (i) The majority of the donor cells in the spleen were positive for CD172 (monocytes and granulocytes), (j) while in the lymph node the donor cells were primarily CD3 + lymphocytes. (k) Plots of chimerism for all offspring showing levels of donor-derived cells over time. The graph plots percentage of donor cell chimerism (class I^{c} positive) over a period of 42 weeks after the birth of the swine from sow 827 and sow 782. The majority of the swine demonstrated stable chimerism during the life of the animal.

Table 1. Presence of donor cells in peripheral blood by FACS analysis.

Animal #	3 weeks	6 weeks	9 weeks	14 weeks	24 weeks	42 weeks
782-2	22.82	34.52	21.22	17.82	18.34	12.92
827-2	37.06	45.08	49.54	49	44.27	36.72
827-4	1.36	1.85	1.58	1.8	1.01	1.3
827-7	83.96	83.6	90.38	87.85	94.97	81.22
827-9	11.06	11.72	12.88	8.37	7.57	4.18
827-11	28.6	27.58	29.98	11.58	10.87	6.48
827-6	0	0	ND*	ND	ND	ND
827-10	0	0	ND	ND	ND	ND

*ND, not done after 6 weeks when confirmed animals were nonchimeric.

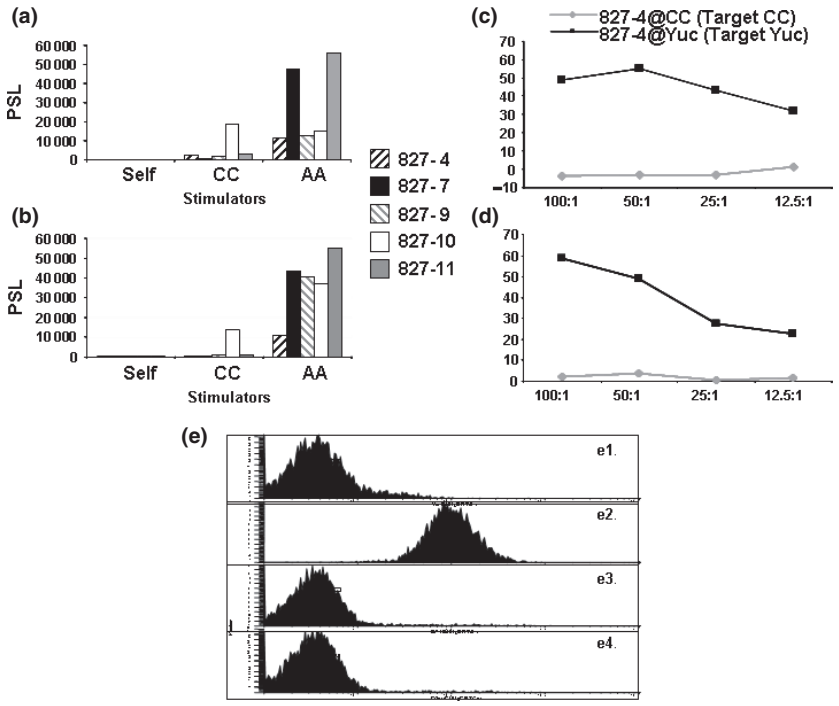

Figure 2 Mixed lymphocyte reactions (MLR), cell-mediated lympholysis (CML), and antidonor antibody production results from chimeric and nonchimeric offspring pretransplant and post-transplant. (a) Chimeric offspring from sow 827 (week 11 of age) are hyporesponsive to donor-type class I^c stimulator cells in MLR, whereas the representive nonchimeric littermate (827-10) has a significant response against the donor cells. All animals have normal responses to third-party swine stimulator cells. (b) Chimeric offspring from sow 827 post-vascularized composite allografts (VCA) (POD >60 days) continue to be hyporesponsive to donor-type class I^c stimulator cells in MLR, whereas the representive nonchimeric littermate (827-10) has a significant responses against the donor cells. All animals have normal responses to third-party swine stimulator cells. (c) CML from 827-4 at 11 weeks of age demonstrates no antidonor reactivity with a normal 3rd party response. (d) CML from 827-4 demonstrates maintenance of donor-specific tolerance 61 days after donor-matched VCA. (e) FACS analysis detection of serum antidonor IgG antibodies after the transplantation of a VCA. (e1) Control sera. (e2) All nonchimeric offspring (827-10 presented) produced antidonor antibodies following rejection of the VCA. (e3 and e4) Antidonor antibodies were never detected in any of the chimeric offspring (827-4 pod 104 and 827-11 pod 106) following VCA.

Table 2. Vacsularized composite allograft survival.

Animal number	Chimerism	12 days of cyclosporine	VCA Survival (days)	Third party skin graft	Donor skin graft	Anitdonor antibodes
827-6	No	Yes	<21	NA	NA	+
827-10	No	Yes	<19	NA	NA	+
827-4	Yes	Yes	>147	7	20	None
827-7	Yes	Yes	>150	11	42	None
827-9	Yes	Yes	>133	8	17	None
827-11	Yes	Yes	>168	8	22	None

260 days post-transplant). Allograft biopsies from tolerant animals demonstrated a mild perivascular lymphocytic infiltration beginning at POD 28. This mild lymphocytic infiltration remained constant throughout the course of serial biopsies and did not precipitate rejection. No rejection of the skeletal muscle fibers or invasion of the vessel walls was noted (Fig. 3d). The skeletal muscle fibers remained viable without evidence of rejection but demonstrated progressive disuse atrophy and fatty replacement from denervation (Fig. 3d). Sections from bone showed viable osteocytes residing in lacunae (Fig. 3e) and normal bone marrow elements (Fig. 3f).

Skin grafts

Frozen donor skin grafts were placed on all four animals with allografts surviving in excess of 100 days. All animals showed permanent acceptance of autologous frozen skin

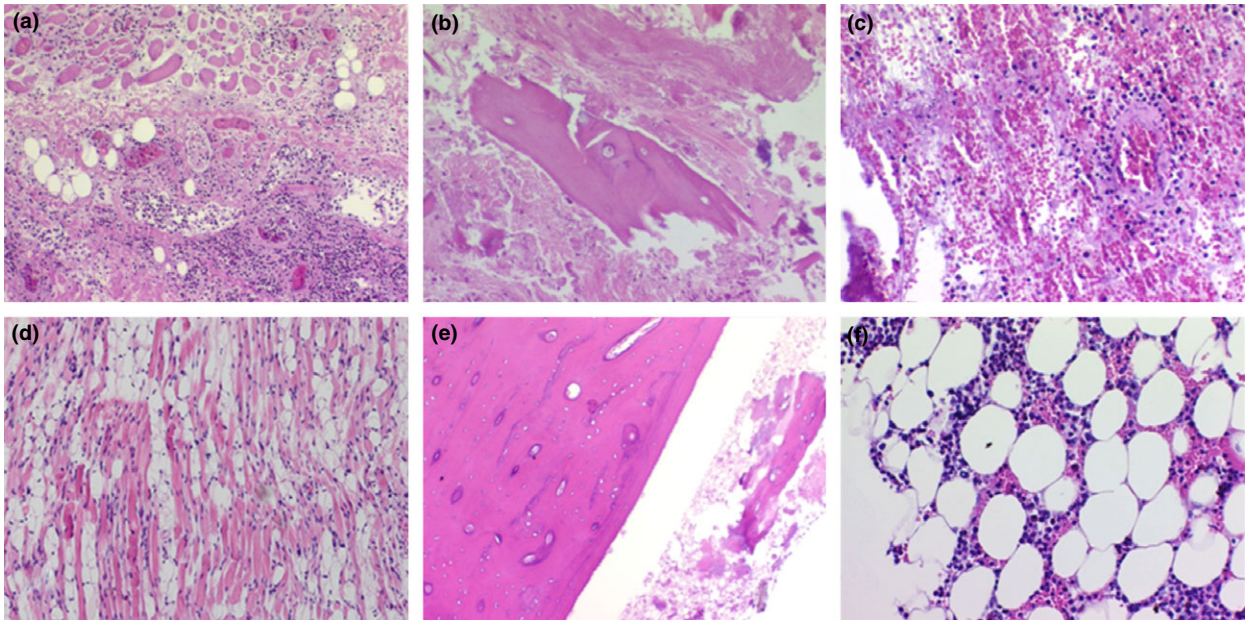

Figure 3 Histologic specimens taken from vascularized composite allografts (VCA) in both chimeric and nonchimeric littermates. (a) Muscle biopsy from donor-matched allograft transplanted into a nonchimeric offspring of sow 827 (Animal 827-10) at day 17. Biopsy shows signs of severe rejection with a prolific lymphocytic infiltrate and vascular thrombosis. (b) Bone biopsy from same VCA demonstrating rejected bone with no viable osteocytes. (c) Bone marrow taken from the same graft again demonstrating signs of severe acute lymphocytic rejection. (d) Muscle biopsy from a donor-matched VCA performed at POD 133 from a chimeric offspring of 827 (827-9). The biopsy shows viable muscle and vessels at this time point. The muscle and vascular supply remained viable at all time points as shown in this specimen at 133 days (H&E, 100×). (e) Biopsy of the bone taken at the same time showing normal bone and viable osteocytes in the bone lacunae. (f) Bone marrow removed from the same transplant demonstrating no signs of rejection and via*ble marrow.*

grafts and rejection of third-party mismatched skin grafts (8 ± 2.0 days). The donor skin grafts demonstrated prolongation (24 ± 7.0 days) but not permanent acceptance (Table 2). All grafts demonstrated some mild lymphocytic infiltration at the day 7 biopsy. All of the animals developed a severe dermal perivascular lymphocytic infiltration with scattered eosinophils and went on to reject their donor skin grafts.

Humoral Assays

Each animal's blood was analyzed both pretransplant, post-VCA transplant, and postskin transplant for the presence of antidonor IgG and IgM antibodies. The chimeric swine did not demonstrate any evidence of antidonor antibodies after the VCA transplant (Fig. 2e). Even after the rejection of the donor skin grafts, they did not demonstrate any evidence of antidonor antibodies. The nontolerant swine did go on to make antidonor IgG after the rejection of the donor-matched VCA (Fig. 2e).

Bone Marrow Chimerism and Function

In two of the four swine transplanted with VCA, it was possible to analyze the recipients' blood for the presence of VCA-derived PAA^+ cells. After transplantation of the VCA, both of the swine were noted to have a small population of VCA-derived donor cells (SLA^{cc}/PAA^+) in their peripheral blood. These cells were analyzed by FACS analysis and noted to be made up of lymphocytes, monocytes, and granulocytes. This population of graft-derived cells remained present for the duration of the experiment and suggested that the donor marrow in the VCA continued to function. Analysis of blood from 827-11 identified a population (2.86%) of graft-derived cells that on postoperative day 100 (Fig. 4a) and on day 198 (Fig. 4b) was 3.61%. FACS analysis of peripheral blood from swine 827-7 at postoperative day 45 showed a population of the cells from the graft (SLA^{cc}/PAA^+) calculated at 4.43% (Fig. 4c) of the total class I^c positive cells, and at day 100, that populations had increased to 9.40% (Fig. 4d).

The donor marrow compartment from the VCAs was evaluated by FACS, and both animals demonstrated a large population of graft marrow cells present in the graft up to the time of sacrifice. This is demonstrated in a representative FACS from swine 827-7 at postoperative day 40 that demonstrates that 53.10% of cells that are both class I^c and PAA positive, the latter of which indicates that these cells could only be coming from the donor graft bone marrow compartment (Fig. 4e).

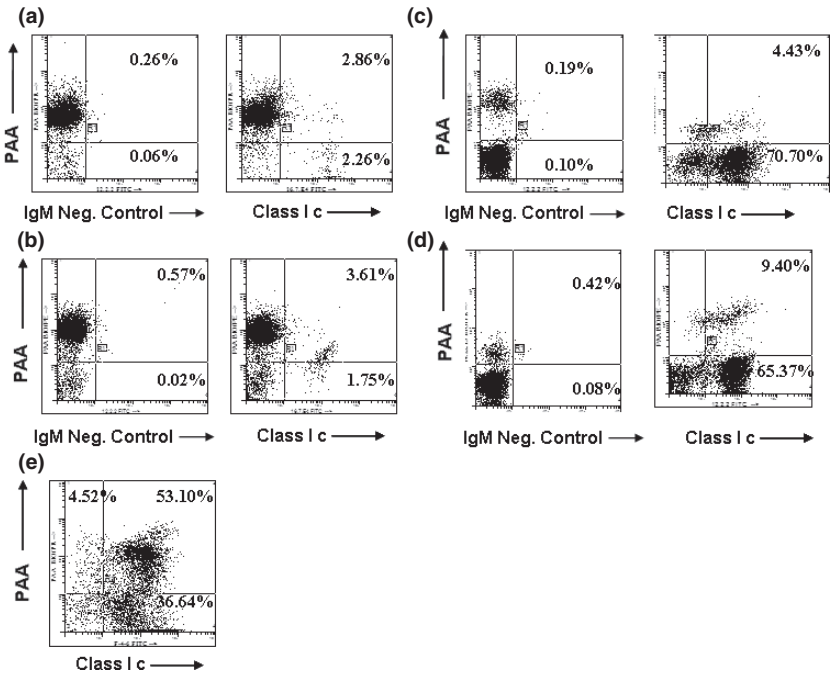

Figure 4 These figures demonstrate the persistence of donor marrow from the vascularized composite allograft in the tolerant swine. (a) FACS analysis of the peripheral blood mononuclear cells (PBMC) taken at pod 100 from 827-11 stained with pig allelic antigen (PAA) versus the IgM negative. FACS of same PBMC stained with PAA and class I^c demonstrating a population of 2.85% of cells that stained positive for both class I^c and PAA. This population of cell is from the donor-matched vascularized composite allografts (VCA). (b) PBMC taken from the same animal at pod 198 demontrates that the population of these cells has increased to 3.61% of the cells. (c) PBMC from 827-7 taken at pod 45 after transplantation of a donor-matched VCA. The first panel demonstrates the negative control. The next panel shows a population of cells from the graft at 4.43%. (d) PBMC take at pod 116 also demonstrates a stable population of cells from the donor graft (9.49%). (e) FACS analysis of the bone marrow from the donor-matched allograft pod 40 from 827-7 demonstrates a large population of cells (53.10%) that are both class I^c and PAA positive.

Discussion

This report describes the successful transplantation of a vascularized composite allograft across a *major histocompatability barrier without long-term immunosuppression* in a robust model for generating mixed hematopoietic chimerism. Transplanting T-cell depleted adult bone marrow intravascularly into the portal vein before birth resulted in stable multilineage macrochimerism. Those animals identified as chimeric at birth demonstrated donor-specific hyporesponsiveness before the transplantation of the donor-matched VCA. After transplantation, these animals went on to demonstrate durable operational tolerance of the VCA. Serial histologic analysis of the transplanted muscle, bone, and bone marrow revealed healthy tissue free of rejection. The flow cytometric analysis of the bone marrow harvested from the VCA identified viable donor BMC within the transplanted bone marrow compartment. In addition, the presence of VCA donor-derived cells was identified in the recipient's peripheral blood. These two observations suggest that the donor VCA continued to function as a donor marrow reservoir after transplantation.

We have previously demonstrated that the injection of partially T-cell depleted adult bone marrow into the portal vein of MHC-disparate swine fetuses leads to donor cell macrochimerism and donor-specific tolerance [27–30]. Our first attempts with IUI in swine resulted in low levels of chimerism, but hematopoietic chimerism persisted in the surviving offspring postnatally without the need for immunosuppression, and there was no evidence of GVHD. The state of donor-specific hyporesponsiveness was confirmed by the use of multiple *in vitro* assays, the prolongation of skin graft survival, and the long-term acceptance of kidney allografts. These experiments provided evidence that the intravascular injection of adult bone marrow into the fetal system can lead to long-term presence of donor cells and that the recipients will accept donor-matched kidney allografts indefinitely.

The objective of this study was to employ this robust model of generating mixed chimerism to test whether VCAs could be accepted by the chimeric offspring to an HSC donor-matched allograft. VCAs are unique in that they allow for the simultaneous transplantation of multiple primarily vascularized tissue types from the donor to the recipient [45]. These allografts are comprised of a combina-

tion of muscle, fascia, cartilage, bone, and bone marrow. The transplant can be seen as a vascularized bone marrow transplant as the graft contains both the HSC and stroma. Theoretically, the bone marrow compartment should function immediately upon transfer in the correct spatial arrangement. This 'bone marrow graft' could provide a continuous source of donor cells, including specialized cells such as bone marrow-derived dendritic cells that have been shown to modulate the host immune response in certain animal models [46].

The sacrifice of the original bone marrow donor after the *in utero* injection of the fetuses necessitated the use of MHC-matched VCA donors to test tolerance of a vascularized allograft. These animals were matched to the major antigens (SLA^{cc}) but are not syngeneic and therefore posses minor antigen differences. Thus, These minor antigens were not present in the donor marrow inoculum and could serve as an antigenic stimulus for chronic rejection of the allograft. We sought to cover these minor antigens by employing a well-described peripheral mechanism of tolerance. We used a 12-day course of cyclosporine that has been previously demonstrated in our laboratory to induce tolerance of the minor antigen differences in a MHC-matched VCA swine model [42]. This technique, however, does not lead to tolerance across major MHC disparities as has been demonstrated in both our VCA and kidney model [28,42,47]. This protocol also failed to prolong the survival of the VCA in the two nonchimeric littermates in this experiment that underwent VCA transplantation (both rejected by 21 days post-transplantation, 9 days after cyclosporine was stopped). The need for coverage of minor antigens remains a controversial subject, but our skin graft data suggests that minor antigens appear to mediate rejection even in situations where tolerance of the major antigens has been achieved.

This experiment again demonstrates the phenomena of split tolerance in which animals demonstrate tolerance of one type of organ while rejecting another from the same donor [48–51]. The eventual rejection of the donor skin graft via skin-specific antigens did not affect antidonor reactivity to the other transplanted tissue including the VCAs nor did it alter the level of donor cell chimerism. This phenomenon has been seen in several experimental models and is thought to be due to the lack of skin-specific antigens expression on the donor marrow cells [6,52]. The observation that there is a low level of cellular infiltrate in the accepted muscle likely represents the migration of T-regulatory cells. We were not able to delineate the phenotype of these infiltrating cells in the muscle in this experiment.

In conclusion, we have demonstrated that the *in utero* injection of adult bone marrow into a swine fetus across a fully MHC-disparate barrier can produce long-term multilineage chimerism and tolerance of a donor-matched vascularized composite allograft. Although this *in utero* injection model may not have direct clinical significance for vascularized allograft reconstruction, the robust and durable chimerism achieved in this model permitted us to transplant VCAs across a full MHC barrier without chronic immunosuppression for the first time in a large animal. In addition, this vascularized marrow transplant continued to function and to produce donor cells. This technique might provide the 'space' necessary to give the donor cells a place to engraft. In addition to providing a continuous source of tolerogenic bone marrow-derived cells, this methodology could, in principle, lead to a new means of treating patients with skeletal abnormalities that can be diagnosed prenatally.

Authorship

DWM: performed surgeries, performed FACS, CML, MLR, analyzed data and wrote paper. MAR: designed study, performed surgeries, analyzed data and edited manuscript. MGS: performed surgeries, performed MLR, CML and edited manuscript. GSG: refined performed the *in utero* injection procedure to reduce mortality. AW: performed surgeries, CML, MLR, FACS and analyzed *in vitro* data. AN: performed limb transplant surgeries and postoperative care. GPN: read and graded histopathology and immunohistochemistry. CAH: analyzed and interpreted FACS, CML, and MLR data. DHS: study design and edited manuscript. WPAL: study design, analyzed data and edited manuscript. PEMB designed study, performed surgeries, edited manuscript.

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