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Vascularized Bone Marrow Cellular Depletion or Discontinuity Abrogates Protection of Vascularized Composite Allografts in Nonhuman Primates

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Background. Vascularized composite allografts (VCA) have demonstrated good clinical outcomes dependent on chronic immunosuppression. Previous work by our group and others supports that cotransplanted vascularized bone marrow (VBM) as a component of VCA offers immunologic protection to prolong graft survival. We aimed to characterize the requirements and potential mechanisms of VBM-mediated protection of VCA by modifying grafts through various strategies. **Methods.** Experimental groups of mismatched cynomolgus macaque recipients received VCA transplants modified by the following approaches: heterotopic separation of the VCA and VBM components; T-cell depletion of either donor or recipient; irradiation of donor VCA; and infusion of donor bone marrow. All groups received standard immunosuppression with tacrolimus and mycophenolate mofetil. **Results.** Experimental modifications to donor, recipient, or graft all demonstrated short-graft survivals (31 d). Chimerism levels without bone marrow infusion were transient and minimal when detected and were not associated with prolonged survival. Donor bone marrow infusion increased levels of chimerism but resulted in alloantibody production and did not improve graft survival. **Conclusions.** VCA graft survival is significantly reduced compared with previously reported VCA with VBM transplants (348 d; $P=0.01$) when the hematopoietic niche is removed, altered, or destroyed via irradiation, depletion, or topographical rearrangement. These experimental manipulations resulted in similar outcomes to VCA grafts without cotransplanted VBM (25 d). These data support the presence of a radiosensitive, T-cell population within the VBM compartment not reconstituted by reinfusion of bone marrow cells.

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INTRODUCTION

Vascularized composite allotransplantation (VCA) has grown as a surgical option for those inadequately addressed by conventional techniques after severe disfigurement, trauma, malignancy, and congenital deformity. Because this reconstructive approach is focused on improving quality of life, concerns on the long-term side effects of chronic immunosuppression have slowed broader clinical application. Currently, chronic lifelong immunosuppression is required to maintain all vascularized composite allografts and often at higher doses as compared to solid organ transplants.[1,2] With this comes acknowledged risks

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of infectious, malignant, diabetic, and renal complications. Renal insufficiency has progressed in some cases to require dialysis and listing for kidney transplantation.[3,4]

Secondary to the life-enhancing rather than life-sustaining nature of vascularized composite allografts (VCA) as a treatment modality, the focus of research efforts has been to reduce the complications of the immunosuppressive therapies. These efforts have focused on inducing graft tolerance, defined as acceptance of donor tissue in the absence of immunosuppressive medication. While immunological tolerance has been achieved in preclinical large animal models of solid organ transplantation, complete VCA tolerance has not been achieved in immunologically mismatched large animal models.[5] Nonhuman primate (NHP) work has supported that prolonged graft survival, and protection against rejection is achieved with standard immunosuppression combined with VCA grafts inclusive of the hematopoietic microenvironment of vascularized bone marrow (VBM).[6,7]

Our previous work utilized an immunologically mismatched NHP model of facial subunit transplantation that contained skin, muscle, and bone with intact marrow. No induction or conditioning therapies were administered, and maintenance immunosuppression was provided by tacrolimus and mycophenolate mofetil. Experiments utilizing VCA and cotransplanted VBM demonstrated significantly prolonged survival up until immunosuppression withdrawal (348 d) as compared to grafts that did not contain VBM graft components (25 d) or tried to reconstitute the removed VBM component with infused bone marrow (76 d; Table 1).[8] These investigations supported a strong a beneficial immunologic role for cotransplanted vascularized bone marrow as a component of VCA but without clearly defined mechanistic pathways.

Tolerogenic models for solid organ allografts have utilized conditioning regimens and bone marrow-based therapies that promote the favorable immunologic state (ie, tolerance) that may have similarities to how VBM may promote prolonged VCA survival without achieving tolerance. Preclinical investigations of methods that interfere and break tolerance in solid organ allografts have been performed to elucidate mechanisms and the specific requirements for these favorable immunologic states.[9] Here, we aimed to build upon our previous work, applying various but specific therapies to both donors and recipients to demonstrate possible interference with the beneficial effect of VBM. Experiments were designed to help define potential requirements of the cell populations within VBM that could confer immunologic protection to VCA grafts or potentially any other transplanted allogeneic tissues or organs.

TABLE 1.

Historical experimental groups 1–3[7,9]

Group	VBM	BMC infusion	Banff I rejection (POD)	Graft survival (POD)	Maximum peripheral chimerism (%)
1	Yes	–	44	267	0.43
1	Yes	–	163	300	2.56
1	Yes	–	261	364	0.29
1	Yes	–	358	462	9.7
2	–	–	7	9	0.39
2	–	–	13	23	1.46
2	–	–	15	42	0.28
3	–	Yes	7	28	0.2
3	–	Yes	13	84	0.60
3	–	Yes	22	115	0.85

Previous reported groups demonstrating the benefit of cotransplanted VBM. Group 1 transplanted both the VCA with VBM, group 2 transplanted VCA without VBM, and group 3 transplanted VCA without VBM and later donor-specific BMC infusion. Banff I rejection and overall graft survival was documented by POD.[7,9]

BMC, bone marrow cells; POD, postoperative day; VBM, vascularized bone marrow.

MATERIALS AND METHODS

Animals

Experiments performed were approved by the University of Maryland School of Medicine's Institutional Animal Care and Use Committee (no 0712001, 0615001) and the Department of Defense Animal Care and Use Committee and conducted in accordance with the Animal Welfare Act and the National Research Council's Guide for the Care and Use of Laboratory Animals. Cynomolgus macaques (*Macaca fascicularis*) were obtained from either Alpha Genesis, Inc. (Yemassee, SC) or Charles River Laboratories (Wilmington, MA) quarantined at the University of Maryland Medical School Teaching Facility. All animals were tuberculin nonreactive and seronegative for herpes B virus, SIV, STLV, LCV, and SRV.

Procedure

Male and female cynomolgus monkeys (*M. fascicularis*), weighing between 2.8 and 8 kg, were utilized in this study. Donor-recipient pairs were established based upon preoperative MLR testing, ABO compatibility, and mismatched by HLA class I antibodies from One Lambda, Inc. (Canoga Park, CA). A heterotopic hemifacial subunit graft was performed as previously described.[5,6]

Groups

Experimental groups (4–8) were compared with previously reported controls (groups 1–3) to reduce animal numbers.[7,8] All animals received immunosuppression with tacrolimus (Astellas, Inc., IL) and mycophenolate mofetil (Accord, Durham, NC). Experimental group 4 investigated heterotopic separation by receiving VCA alone (skin and muscle) to the left abdomen and VBM allograft from the same donor to the right abdomen. Experimental group 5 investigated donor T-cell depletion utilizing 50 mg/kg antithymocyte globulin (ATGAM, Pfizer, D.C.) administered on day 2, 1, and 0 for a goal of <50 CD3+/mm^3. Experimental groups 6 and 7 studied donor graft irradiation utilizing 1.5 Gy bilateral mandible irradiation (Pantak X-RAD HF 320) on day 1, with group 7 also receiving donor bone marrow infusion described below. Experimental group 8 studied recipient T-cell depletion utilizing antithymocyte globulin and similar immunosuppression to other groups with the addition of a 21-day steroid taper from 20 mg/kg.

Bone Marrow Infusion

Donor animals received no treatment before marrow recovery. After euthanasia on day 0, whole bone marrow was isolated from donor animals' lumbar vertebrae under sterile conditions. The bone marrow cells were subsequently isolated and infused at 2.0×10^8 cells/kg through a central venous catheter at 11 mL/h to a total of 30 mL. Heparinized (3 U/mL) bone marrow solution was administered in infusion media (HEPES 25 mmol/L, Albumin 2.5%, RPMI).

Mixed Lymphocyte Reaction

A mixed lymphocyte reaction (MLR) assay was performed to measure proliferation across allogeneic lymphocyte populations. Lymphocytes were isolated using Ficoll-Paque (Amersham Biosciences, NJ) density gradient centrifugation. The cells were washed with culture medium containing RPMI medium (Thermofisher, MA), containing 10% fetal bovine serum, gentamicin, L-glutamine (2 mmol/L), and HEPES buffer (Thermofisher, MA). Donor cells were irradiated with 2000 rads and mixed with an equal number of nonirradiated recipient cells to total 100 000 cells per well in a 96-well plate. Nonirradiated recipient cells mixed with concanavalin A were used as a positive control. Cells were incubated for 72 hours at 37°C, pulsed with 3H thymidine, and incubated for another 18 hours. Incorporation was determined using a liquid scintillation counter (Perkin-Elmer, IL). The stimulation index of each cell mixture was calculated as the ratio of the average counts from 3 wells of allogeneic to autologous responses.

Monitoring

VCA grafts were monitored daily. Physical examination was performed regularly with scheduled and indicated peripheral blood sampling for monitoring of complete blood cell counts, serum chemistry, and tacrolimus levels. Skin biopsies of the VCA graft were performed using a 5-mm punch biopsy. Tissue samples were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin for examination. Rejection was graded based upon the 2007 Banff working classification.[10]

Flow Cytometry

HLA mismatched animals were paired based on their expression or lack of expression of HLA BW6 using anti HLA BW6 (Thermofisher, MA). BW6 positive donors and BW6 negative recipients were selected preoperatively. Chimerism was assessed based on the percentage of the donor HLA BW6 positive cells detected in the recipient peripheral blood sample at regular intervals. Samples were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, OR).

Donor-specific Antibody Detection

IgM and IgG donor-reactive alloantibodies were retrospectively measured by flow cytometry using archived frozen donor peripheral blood lymphocytes and recipient serum samples. Antibody binding was revealed using PE-labeled goat antihuman IgM antibodies (Thermofisher, MA) or biotin-labeled goat antimonkey IgG (Fcγ specific) antibodies (Nordic, Netherlands) followed by PE-labeled streptavidin (BD Biosciences—Pharmingen). FITC-labeled antihuman CD3 (BD Biosciences, CA) was added to gate on T cells. Alloantibody reactivity was defined as an increase of more than 10% in the proportion of IgM- or IgG-positive donor cells relative to donor serum before transplant.

Statistical Analysis

Analyses across and between groups were performed using SPSS software (IBM, Armonk, NY); a probability value of <0.05 was deemed statistically significant. Survival analysis was performed using a Kaplan-Meier log-rank method.

RESULTS

Heterotopic Separation of VCA and VBM Does Not Maintain Survival Advantage (Group 4)

Four of 4 recipients underwent contralateral transplantation of VCA and VBM components. Myocutaneous VCA components were transplanted to the left abdomen and vascularized bone compartment transplanted to the right abdomen and covered by recipient skin. Early graft loss (average 36.8 d ± 18.0) was seen in 4 of 4 recipients. Progressive rejection was seen simultaneously in the VCA skin and bone marrow compartments (animal 1 representative, Figure 1). VCA skin became increasingly erythematous and edematous, starting postoperative day (POD) 5 with subsequent biopsy with mild perivascular infiltrates and progressing to epidermal necrosis. Average time to the first rejection episode was 10.7 ± 4.1 days (excluding animal 2 due to ischemia-related explantation of the myocutaneous flap). Biopsies performed showed early rejection with Banff 1–2 within the first 2 weeks after transplantation. The separated VBM component of the allograft exhibited ongoing overlying edema, with bone marrow at endpoint showing necrosis with acellular marrow space. Peripheral blood chimerism was undetectable in all 4 animals throughout the experimental study period.

Donor Graft Manipulation Results in Early Rejection and Graft Loss

Donor Cellular Depletion (Group 5)

Therapeutic donor T-cell depletion was performed in group 5 (n = 3) by donor-administration of antithymocyte globulin (50 mg/kg) POD 2, 1, and 0 before transplantation. Two of 3 recipients showed very early signs of rejection on POD 3 and 7. Rejection progressed to Banff IV rejection within weeks (day 13 and 28), with no detectable level of chimerism in the peripheral blood (Table 2). Animal 3 developed posttransplant lymphoproliferative disorder (PTLD) associated with lymphocryptovirus (LCV) reactivation, an Epstein-Barr-related herpesvirus, that we have observed previously. Donor-specific alloantibody remained low at experimental endpoint with median IgG and IgM levels of 43.1 ± 3.4 MFI and 488.7 ± 417.0 MFI.

Donor Graft Irradiation (Groups 6, 7)

Group 6 donors (n = 4) underwent 1.5 Gy of focused mandibular irradiation on POD 1. The VCA + VBM was then transplanted as previously described. Four out of 4 recipients experienced Banff 1–4 early VCA skin rejection on 7.5 d ± 1.1, and 3 out of 4 animals progressed to skin necrosis on average 32 d ± 18.5. One animal reached endpoint on POD 56 due to LCV-associated PTLD. Peripheral blood chimerism was undetectable in all 4 recipients (Table 2). The VBM space at time of necropsy was viable and reconstituted with primary recipient in origin CD3+ and CD19+ cells similar among all experimental groups at time of necropsy (Figure 2). Irradiated grafts contained viable cells within the marrow, 83.8% ± 7.74% at time of transplantation, and decreased slightly to 74.0% ± 9.02% at time of necropsy. No donor alloantibody developed in any of the 4 animals.

Group 7 donors (n = 3) also received 1.5 Gy of focused mandibular irradiation on POD 1. In addition, vertebral bone marrow was isolated from donors and infused on POD 1. Peripheral chimerism was detected immediately after donor-specific BM infusion, 0.49%–7.21% (Table 2) on PODs 2–5. This low-level chimerism detected in the periphery disappeared within days and ultimately did not prevent early rejection seen on average

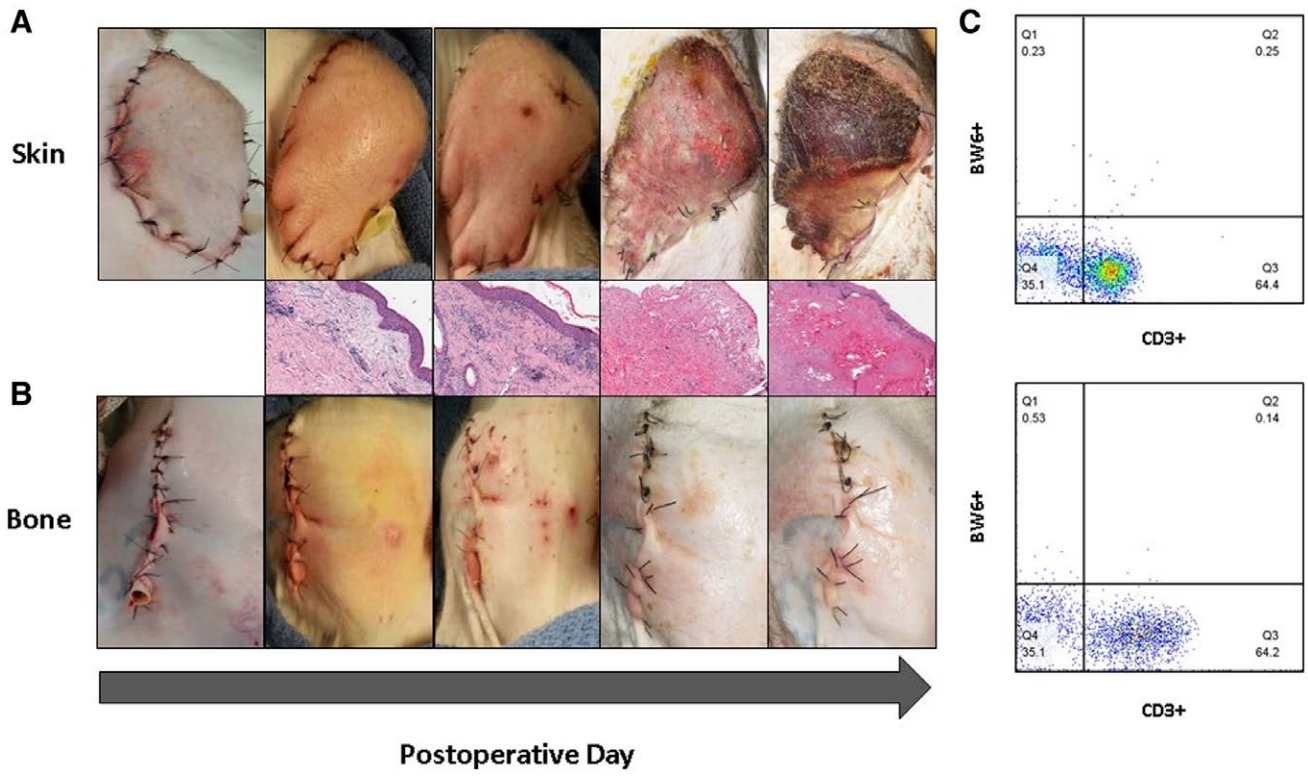

FIGURE 1. Heterotopic separation of graft components does not confer the same survival advantage (group 4). Representative images from contralateral allograft group 4 of (A) progressive evidence of rejection seen in myocutaneous component of allograft. B, Increasing edema and overlying skin changes supporting rejection seen in VBM component of allograft. C, Undetectable levels of peripheral blood chimerism as detected by BW6+CD3+ cell populations. VBM, vascularized bone marrow.

TABLE 2.

Experimental groups 4–8

Group	VBM	Irradiation	BMC infusion	ATGAM	Steroids	Banff I rejection (POD)	Graft survival (POD)	Maximum peripheral chimerism (%)
4	Heterotopic	–	–	–	–	16	16	ND
4	Heterotopic	–	–	–	–	6	22	ND
4	Heterotopic	–	–	–	–	10	52	ND
4	Heterotopic	–	–	–	–	N/A	57	ND
5	Yes	–	–	Donor	–	7	13	ND
5	Yes	–	–	Donor	–	3	28	7.24
5	Yes	–	–	Donor	–	N/A PTLD	64	ND
6	Yes	Donor	–	–	–	6	14	ND
6	Yes	Donor	–	–	–	7	14	1.17
6	Yes	Donor	–	–	–	8	44	ND
6	Yes	Donor	–	–	–	9	56	0.7
7	Yes	Donor	Yes	–	–	6	13	7.21
7	Yes	Donor	Yes	–	–	12	22	3.60
7	Yes	Donor	Yes	–	–	13	27	0.49
8	Yes	–	–	Recipient	Yes	10	20	ND
8	Yes	–	–	Recipient	Yes	29	29	0.21
8	Yes	–	–	Recipient	Yes	13	36	0.65

Group 4 transplanted the myocutaneous portion of the graft to the left abdomen, and the VBM portion to the right abdomen. Group 5 transplanted VCA with VBM after donor T-cell depletion with ATGAM, group 6 transplanted VCA with VBM after 1.5 Gy donor irradiation, group 7 transplanted VCA with VBM after donor irradiation and subsequent donor-specific BMC infusion, and group 8 transplanted VCA with VBM after recipient T-cell depletion with ATGAM administration followed with steroid taper. Banff I rejection and overall graft survival documented by POD.

ATGAM, anti–thymocyte globulin; BMC, bone marrow cells; ND, not detected; POD, postoperative day; PTLD, posttransplant lymphoproliferative disease; VBM, Vascularized bone marrow; VCA, vascularized composite allograft.

POD 10.3 ± 3.1. Early VCA skin biopsies taken demonstrated increased populations of mononuclear cells but minimal inflammation surrounding graft epidermis. Average graft survival was 20.7 ± 5.8 days. At time of necropsy, necrosis was seen throughout the skin and soft tissue, muscle contained significant microthromboses, and the majority of the bone marrow space was necrotic with surrounding dense cortical bone (Figure 3). Alloantibody formation occurred in 3 out of 3 recipients, with

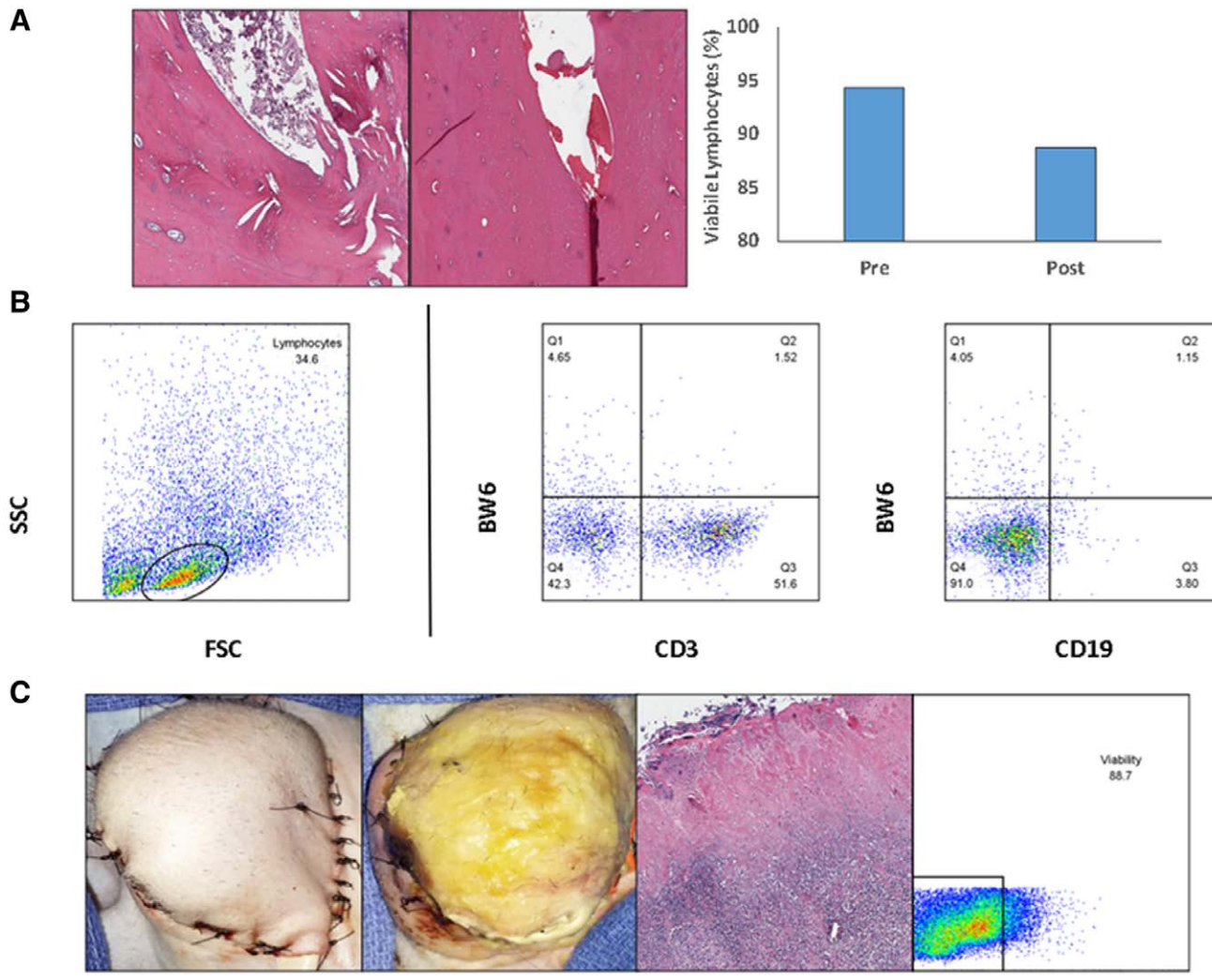

FIGURE 2. Donor irradiation results in graft marrow replaced with recipient cells (group 6). Hematoxylin and eosin staining of allograft bone tissue before (A) transplantation and at time of necropsy with small decrease in lymphocyte viability in percentage. B, Flow cytometry of graft bone marrow composed predominantly of recipient CD3+ and CD19+ cells with minimal residual donor BW6+ cells. C, Allograft appearance at time of transplantation and necropsy, hematoxylin and eosin staining of allograft skin at necropsy, with graft bone marrow cell viability. FSC, forward scatter; SSC, side scatter.

an average increase in IgM alloantibodies 5396 ± 5463 MFI and average increase in IgG alloantibodies 1622 ± 869 MFI.

Recipient Cellular Depletion Interferes With VBM-associated Graft Prolongation

Group 8 (n = 3) received identical VCA with VBM grafts and maintenance immunosuppression to historical group 1. Recipients in group 8 (n = 3) were also administered ATGAM (50 mg/kg) POD 2, 1, and 0 before transplantation and a steroid taper similar to clinical protocols. After ATGAM infusion, recipients' lymphocyte count decreased to 58.3 ± 37.6 CD3+/mm^3. Recipients (3/3) showed perivascular and focal epithelial inflammation between POD 10 and 29, with progression to frank skin necrosis within 23 days. Levels of IgG and IgM donor-specific alloantibodies increased in group 8 recipients an average 2.7- and 18.3-fold, respectively, at time of necropsy.

Immunosuppression Levels

Groups 4–8 were administered a continuous infusion of intravenous tacrolimus for 28 days converted thereafter to daily intramuscular dosing and daily mycophenolate mofetil. Tacrolimus levels were similar between all experimental groups and as compared to the historic cohort (Figure 4). Average tacrolimus levels were maintained above therapeutic targets and were not correlated with experimental endpoints (necropsy).

Prolonged VCA Survival With Cotransplanted VBM Eliminated by Physical or Cellular Manipulation

All experimental groups demonstrated early evidence of Banff I rejection. Rejection was clinically evident and histologically diagnosed within the first 14 days for most experiments. These rejection episodes all occurred while on therapeutic immunosuppression and progressed to more severe rejection grades, graft loss, and experimental endpoint (Figure 5). The effect of interventions on graft survival, as represented in Kaplan-Meier survival plots, supports a similar reduction in graft survival by all experimental manipulations in groups 4–8 (Figure 6).

DISCUSSION

In this study, we established that VBM requires 2 basic criteria to be met to exert its protective effect on VCA graft

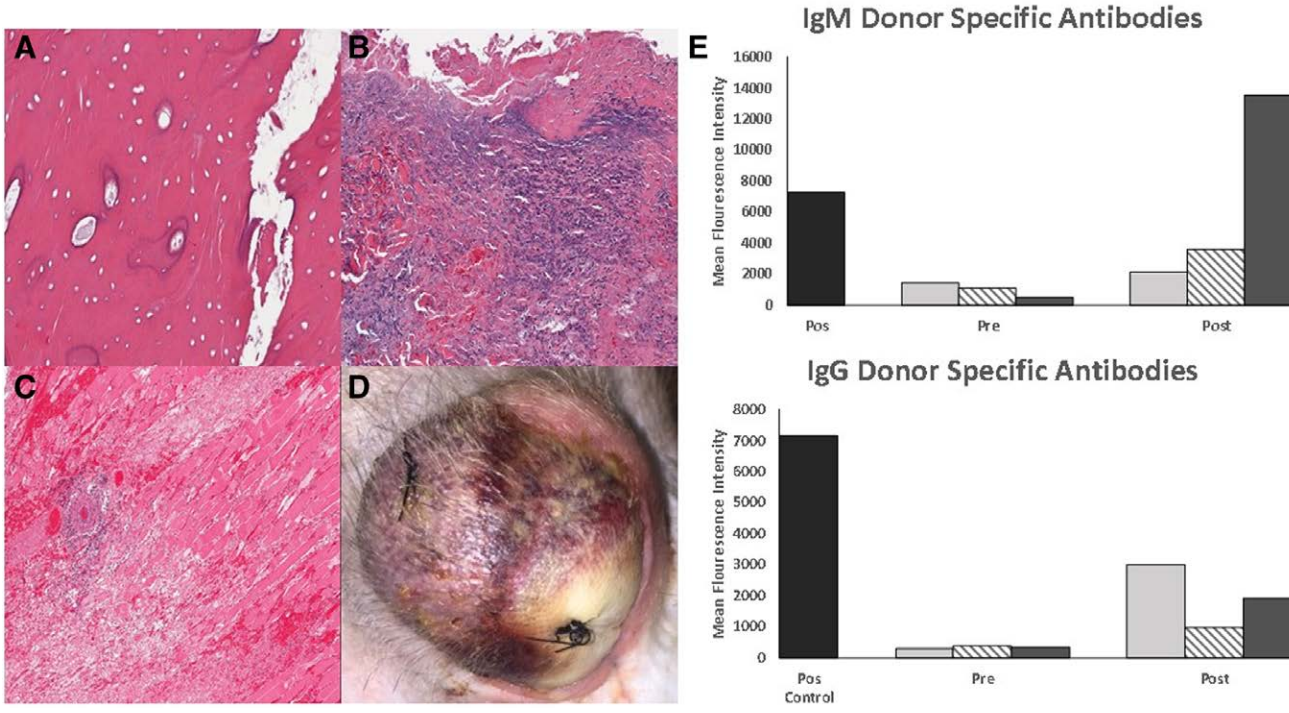

FIGURE 3. Removing and reconstituting bone marrow space promotes humoral response (group 7). Hematoxylin and eosin staining of bone (A), skin (B), muscle (C) at time of necropsy, with correlating clinical appearance of grossly necrotic allograft (D). E, IgM and IgG alloantibodies increased in animal 1–3 at time of necropsy. Ig, immunoglobulin.

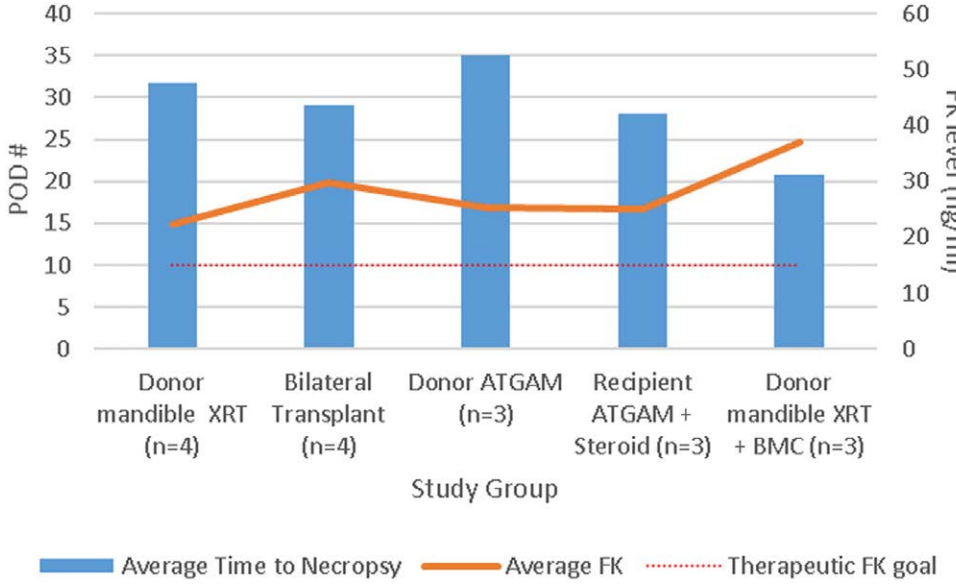

FIGURE 4. Average tacrolimus level (ng/mL) in study groups 4–8 compared with average time of necropsy (POD). All experimental groups 4–8 demonstrated tacrolimus levels above therapeutic targeted goals of at least 15 ng/mL. Rejection and necropsy were not correlated to tacrolimus levels. ATGAM, antithymocyte globulin; BMC, bone marrow cells; CL, contralateral; FK, tacrolimus (FK506); POD, postoperative day; XRT, irradiation.

survival: first, there must exist an intact and unmanipulated hematopoietic niche within the VBM; second, the VBM niche must be contiguous with the VCA and be transplanted at the same location. The survival advantage of incorporating a vascularized bone marrow compartment in composite tissue allotransplantation has been previously described by our laboratory and multiple other investigators in various small and large animal models.

In our model, graft survival appeared indefinite in the setting of standard immunosuppression therapy. Historic control group 1 of VCA with VBM demonstrated graft rejection progressing to loss only when immunosuppression was weaned and withdrawn. Historic control groups 2 and 3 without VBM demonstrated early rejection and graft loss similar to the experimental groups 4–8 reported on in the current study. A significant survival advantage ($P = 0.012$) of

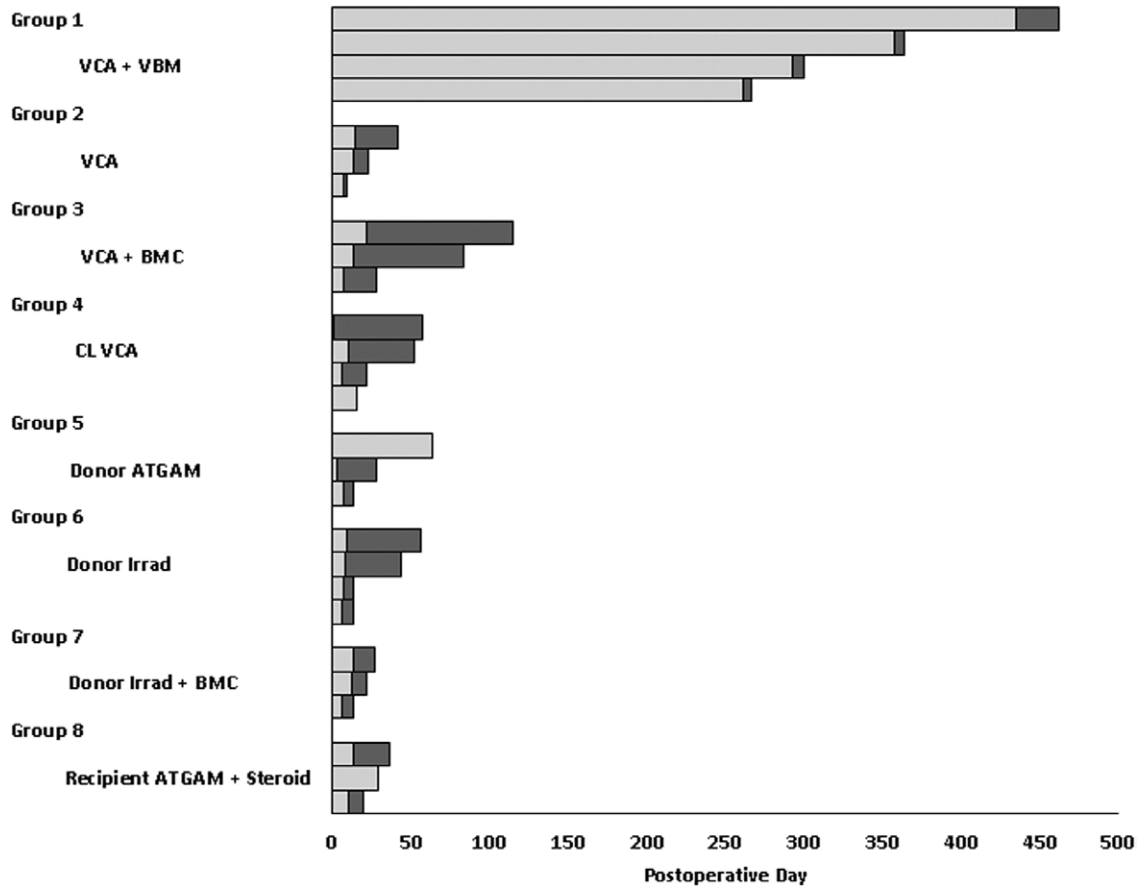

FIGURE 5. Rejection free and overall survival in groups 1–8. Rejection free time defined by Banff I evidence of rejection demonstrated early occurrence in all experimental groups 4–8 and were followed by terminal endpoint. ATGAM, antithymocyte globulin; BMC, bone marrow cells; CL, contralateral; VBM, vascularized bone marrow; VCA, vascularized composite allograft.

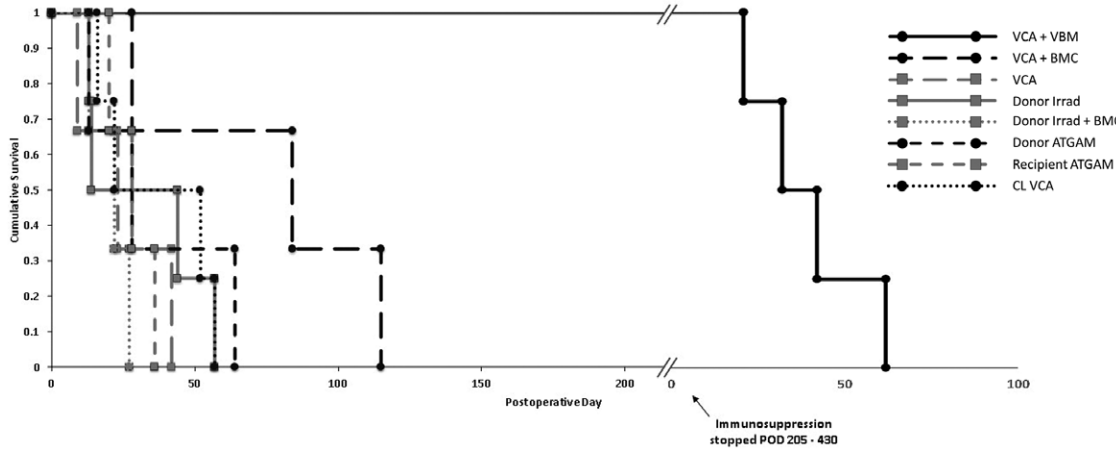

FIGURE 6. Kaplan-Meier curve illustrating graft survival among experimental groups 1–8. Historical control VCA + VBM group with longer allograft survival 348.3 ± 74.4 d ($P = 0.012$) compared with groups 2–8 with various recipient and donor conditioning. All graft and donor manipulations shortened survival times significantly. Reinfusion of bone marrow in a historic cohort (VCA + BMC, Group 3) did not significantly prolong graft survival. ATGAM, antithymocyte globulin; BMC, bone marrow cells; CL, contralateral; POD, postoperative day; VBM, vascularized bone marrow; VCA, vascularized composite allograft.

the historical VCA with VBM group control is supported as compared to any graft manipulation in historic (groups 2 and 3) or current experimental investigations (groups 4–8). Additionally, graft survival persisted in control group 1 for between 21 and 62 days after immunosuppression cessation, and this was not able to be replicated in any experimental group as all rejected their grafts while on continuous therapeutic immunosuppression.

Although strong in their evaluation of the importance of incorporating VBM in VCA, our initial studies were not designed to and did not characterize this hematopoietic niche within the allograft.[7,11,12] In this study, we have begun to

characterize the ways in which VBM conveys its immunologic protection to the VCA.

VCA graft protection appears related to the proximity of the hematopoietic compartment as supported by data from experiments in which the marrow-containing bone was placed contralateral to the myocutaneous flap. These experiments demonstrated an outcome not predicted by our initial hypothesis that VBM may have systemic protective effects. The heterotopic location of the VBM increased VCA rejection and shortened graft survival with identical immunosuppressive therapies. Additionally, the clinical course of the VBM allografts was also altered as the marrow compartments were overwhelmingly repopulated with recipient origin cells. These data were in contrast to prior work that demonstrated not only prolonged survival of the singular component VCA + VBM allograft but also revealed preservation of high proportions of donor origin bone marrow cells.

One possibility may be a requirement for both donor-derived cells in the VBM and proximally cotransplanted myocutaneous tissues that can both present antigen and down-regulate early immune responses. Highly antigenic VCA skin may present self-antigen in an environment favoring regulatory responses. These locally generated and acting donor cells may promote this beneficial down-regulatory response that also impacts donor marrow survival in VBM. Physical separation of VBM from skin could interfere with this potential mechanism and could explain our observation.

Additional experiments examined removing cell populations through means of irradiation and cellular depletion. Because of both the preclinical goals of our NHP model as well as the limitation of specific reagents, we did not target singular cell populations but rather utilized clinically applicable depletional therapies. Antithymocyte globulin is commonly administered as induction and therapy for rejection episodes, and irradiation has been utilized in both NHP models and clinical trials of transplant tolerance. Allograft survival was significantly decreased in our donor irradiation group POD 31.8 ± 18.7, as compared to historical control survival POD 348.3 ± 74.4. The data generated in these experiments support a radiosensitive, T-cell compartment within proximal bone allografts as a key mechanism in promoting VCA survival. Data additionally demonstrated that the effect of this radiosensitive cell population within the vascularized bone marrow cannot be reconstituted by donor bone marrow transfusion. Furthermore, loss of this cotransplanted VBM niche resulted in loss of previously controlled alloantibody production and facilitated marked antibody-mediated responses.

Infusion of donor bone marrow after pretransplantation VCA graft irradiation failed to reconstitute the donor bone marrow niche. This is in contrast to studies by Mathes et al that showed long-term graft survival in the absence of immunosuppression after VCA with donor-specific hematopoietic cell coinfusion. Canine recipients underwent total body irradiation followed by VCA transplantation and bone marrow infusion and supported the importance of recipient rather than donor conditioning.[13] In rodent studies with bone marrow infusion, depletion of specific cell populations could promote chimerism after skin grafting. Our data support that nonspecific depletional conditioning therapies do not promote allogeneic tolerance to VCA (nor prevent early rejection episodes), in contrast to successes reported with conditioning therapies depleting specific cell populations.[14]

Although cell depletion after ATGAM administration in both donor and recipient proved adequate (<50 cells/mm^3), doing so immediately before transplantation proved detrimental for graft acceptance. Skin rejection episodes occurred within days to weeks in all recipients, affecting the early engraftment phase of transplantation. Highly antigenic skin components of VCA are not protected from alloimmune responses despite adequate T-cell depletion and combination therapies of tacrolimus, mycophenolate, and steroid therapies. This correlates to the clinical setting in which nearly universal early skin rejection episodes are seen after using an identical induction protocol. In contrast to our model in which rejection episodes are not treated, in the clinical setting, these acute rejection episodes are successfully reversed with bolus immunosuppression and increased maintenance immunosuppression for a period of time thereafter.

These conclusions are supported by work in a rodent model that has demonstrated that tolerance in a VCA model was dependent on a radiosensitive CXCR4+, Foxp3+, Treg population within the bone marrow. These authors demonstrated that when tolerance was induced by a costimulatory and mammalian target of rapamycin (mTOR)-based therapy, similar interventions of donor irradiation, T- and B-cell depletion, elimination of the bone allograft would prevent the development of tolerance and this effect could not be reestablished with donor bone marrow infusion.[15] This study confirms in a rodent model the observations that we have made in our primate NHP model.

Clinical VCA that can experience early and significant rejection episodes even with the most strenuous induction immunosuppressive protocols proceed to have overall quite good long-term clinical outcomes. In contrast, solid organ allografts experiencing early rejection usually demonstrate worse short and long-term outcomes.[16,17] A potential beneficial effect of the cotransplanted VBM has been supported as 1 component of promoting good VCA outcomes. These studies have supported that VBM graft protection is mediated by the presence of a proximal cellular component in the VBM that is both radiosensitive and susceptible to depletional therapies. Future mechanistic studies should be directed to the specific cell population and pathway by which VBM immunomodulation occurs. These cell populations may offer therapeutic potentials for ongoing allograft protection and therapeutic immunomodulatory therapies for VCA and other solid organ allografts.

The current work has acknowledged limitations characteristic of a preclinical NHP model. This current study utilized historic control cohorts that had been previously reported. The resource intensive nature of these NHP studies represented 1 barrier to reproducing all prior groups. An equally important consideration was the intent to be consistent with the principle of reduction of animal numbers defined by ethical guidelines for animal research.[18] Nonetheless, the possibility exists that the previous results may not have been replicated despite similar MLR responses and mismatching. Another challenge existed in the limited availability of primate-specific reagents to identify the exact cellular populations that are required to achieve the immunologic benefit of cotransplanted VBM. The current study was utilized relatively nonspecific therapies of ATGAM and local irradiation. Ideally, cellular targets within

the bone marrow could be identified for specific depletion and reconstitution. CXCR4 and Foxp3 cell populations would be attractive cell populations to further investigate in our specific NHP model. Rodent studies could be the most appropriate to fully characterize the population of cells that would then be targeted for depletion or augmentation in a future series of large animal studies. The results from our current studies support the presence of a regulatory cell population co-located to the endosteal surfaces of transplanted bone. The presence of a unique endosteal zone where regulatory cells protect allogeneic hematopoietic stem/progenitor cells has been described in a rodent model.[19] The results of our current study support the potential for this immune privileged site within bone marrow to be sensitive to manipulations that eliminate the benefit of cotransplanted vascularized bone for VCA.

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