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RESEARCH PAPER

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Effects of Transient Donor Chimerism on Rejection of MHC-Mismatched Vascularized Composite Allografts in Swine

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Keywords: acute rejection, bone marrow transplantation, mixed chimerism, rejection episodes, tolerance, transient chimerism, vascularized composite allograft

Abbreviations: BMC, bone marrow cell; CsA, Cyclosporine A; HCT, haematopoietic stem cell transplantation; HSC, haematopoietic stem cell; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; SLA, swine leukocyte antigen; TBI, total body irradiation; VCA, vascularized composite allograft.

Background: Despite encouraging outcomes in vascularized composite allograft (VCA) transplantation, the risks of chronic immunosuppression limit widespread applicability. It has been suggested that infusion of donor bone marrow along with the VCA may reduce the level of immunosuppression required to prevent clinical VCA rejection. However, no clear evidence has yet been presented to confirm the role of donor bone marrow in the prevention of rejection. In this study we investigated the immunologic effects of concurrent bone marrow transplantation in a large animal VCA model. **Methods**: MGH miniature swine (n=4) received a non-myeloablative conditioning regimen consisting of low-dose total body irradiation, T-cell depletion, a short course of Cyclosporine A, with or without varying doses of donor bone marrow cells in combination with a complete MHC-mismatched VCA. Animals were monitored daily for signs of rejection or graft versus host disease. Chimerism levels were assessed using flow cytometry and *in vitro* assays were performed to assess for donor-specific responses. **Results**: Transient chimerism was prolonged with increased bone marrow cell doses and total body irradiation. While animals that received BMC infusions did not have significantly prolonged VCA acceptance following cessation of immunosuppression compared to animals that received conditioning without BMCs, they demonstrated better early clinical outcomes and demonstrated donor-specific unresponsiveness during the presence of detectable chimerism. **Conclusions**: Detectable mixed chimerism following bone marrow transplantation and VCA mitigates donor-specific responses and acute rejection episodes, but does not appear to be sufficient for tolerance induction.

Introduction

Vascularized composite allograft (VCA) transplantation refers to the allogeneic transfer of multiple tissue types, including skin, muscle, bone, blood vessels and nerves. Over the past 15 years, the use of VCAs has emerged as a viable reconstructive option for patients with the most severe disfigurements. To date, VCAs of the face, upper extremities, lower extremities, abdominal wall, larynx, uterus and knee have been performed, with follow-up reports demonstrating very encouraging outcomes.[1-4] Face transplant recipients have consistently regained sensation by 8 months and motor function during the first year following the procedure, coinciding with the recovery of important functions such as eating, smelling, smiling, speaking, and swallowing.[4-6] Similarly with hand transplantation, follow-up data has demonstrated restoration of tactile sensibility in 90% of hand transplant recipients, with 84% developing discriminative sensation.[1] Motor recovery at 9 to 15 months following transplantation has resulted in improved ability to eat, drive, grasp objects, shave, use the telephone and write.[1,2]

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This study was presented in part at the 90th American Association of Plastic Surgeons Meeting, Boca Raton, FL, April 9–12, 2011, the 56th Plastic Surgery Research Council, Louisville, KY, April 27–30, 2011 and the 3rd European Plastic Surgery Research Council, Hamburg, Germany, August 2011.

Despite the promising early outcomes in the field of VCA, acute rejection episodes of the skin are common even when under robust conventional triple therapy immunosuppression. The skin is an important immunologic organ- half of all cells in the skin have an immune function, and the high concentration of adhesion molecules within the dermis provides the ideal platform to mount an immune response.[7] Indeed, the immunogenicity of allografted skin was described by Gibson and Medawar 70 years ago,[8] and it is therefore not surprising that different tissues within the VCA have different antigenicity.[9]

Therefore, the establishment of tolerance of fully major histocompatibility complex (MHC)-mismatched VCAs, where recipients can maintain allografts without the need for immunosuppression, remains an important goal to allow more widespread use of VCAs. Unfortunately, while many protocols have achieved transplant tolerance in small animal models, these protocols have generally failed to translate into large animal models or clinical regimens.[10,11]

One approach stands in contrast to this record: the establishment of mixed haematopoietic chimerism through non-myeloablative conditioning and haematopoietic stem cell transplantation (HCT), where lymphoid and haematopoietic cells of both donor and host origin are present in the recipient.[12-18] Mixed chimerism protocols have been used to induce tolerance in clinical trials of kidney transplantation.[12,19] However, while multilineage chimerism was transient with no donor cells detectable after day 21, 7/10 patients remained tolerant of their renal allografts without maintenance immunosuppression. Furthermore, preliminary work investigating the induction of tolerance of VCAs in porcine models using mixed chimerism approaches has suggested that specific tissues within the VCA (e.g. epidermis) may be more resistant to long-term acceptance depending on VCA composition, relative MHC- and minor-antigen matching, source of haematopoietic stem cells, and persistence of chimerism.[20,21]

These outcomes suggest that similar protocols may be applicable to VCA, and strategies to eliminate or reduce the need for immunosuppression have been attempted using infusion of donor HSCs in combination with VCA.[22] While the first recipient of a face transplant received HSCs on days 4 and 11 post-transplant, acute rejection episodes still occurred while on conventional triple-therapy immunosuppression.[23] In contrast, it has recently been reported that hand transplant recipients who received infusions of bone marrow from donors were able to maintain their allografts on tacrolimus monotherapy, with infrequent acute rejection episodes that were easily reversed by either steroid boluses or topical immunosuppression. While these experiences do not demonstrate the ability to induce long-term tolerance of VCAs clinically, they do suggest that inclusion of donor HSCs may provide for a positive immunomodulatory effect on VCA outcomes, providing the basis for these studies.

Methods

Animals

The MGH miniature swine model has fixed MHC (SLA) haplotypes, which has been achieved through a selective breeding program. Recipient animals (n=4, SLA^{dd}) were chosen to be fully mismatched for both haplotypes of class I and class II of MHC from donors (SLA^{cc}). Pig Allelic Antigen (PAA), a non-histocompatibility antigen, was mismatched between donors (PAA-positive) and recipients (PAA-negative) to allow monitoring of origin of cell lineages following BMT. The study was approved by the Institutional Animal Care and Use Committee and conducted in accordance with the NIH guide for the Care and Use of Laboratory Animals.

Conditioning regimen

Recipient animals received a conditioning regimen consisting of low-dose total body irradiation (100cGy on day −2 or 200cGy divided in 2 100 cGy doses on days −3 and −2), T-cell depletion with CD3 immunotoxin (0.05 mg/kg i.v., twice daily from day −4 to day 0) and oral Cyclosporine A (Novartis Pharmaceuticals, East Hanover, NJ) for 45 d starting from day −1, which dose adjusted to maintain levels between 400–800 ng/mL, as previously described.[16,24]

VCA transplantation

On day 0, SLA^{dd} recipient animals received a fully MHC mismatched VCA from the SLA^{cc} donor animals and simultaneous bone marrow transplantation. A gracilis musculocutaneous VCA was raised as previously described[25] from the donor thigh and included skin, subcutaneous tissue, a large portion of the gracilis muscle and a vascular pedicle including the saphenous artery and vein. The VCA was transplanted to the cervical region of the recipient animal and re-anastomosed to the common carotid artery and the internal jugular vein.

Bone marrow harvest and transplantation

Bone marrow was harvested from the long bones and vertebrae of donor animals and collected into 500ml sterile bowls, with all steps performed at room temperature. Bone marrow cells were isolated in harvest medium consisting of Dulbecco's Phosphate-Buffered Saline (Mediatech), 2% Knock-Out Serum Reducer (KSR; Invitrogen), 1× PenStrep (Invitrogen), 20ug/mL Gentamicin (Invitrogen). Large fragments were allowed to settle for 2 minutes prior to collecting released cells. The fragments were twice extracted of additional cells by brief swirling in harvest medium and released cells pooled with the initial concentration. Suspended cells were then filtered through 100u nylon mesh into 500mL conical bottles and centrifuged for 15mins. Cells were then re-suspended in 500ml Dulbecco's Phosphate-Buffered Saline (DPBS), counted and diluted to 500mL with DPBS prior to infusion. After preparation the haematopoietic cells were transplanted over 30 minutes at a dose ranging from 7.8×10^8 to 4×10^9 cells/kg of recipient body weight,

with one animal receiving no cells. The animals were monitored for signs of adverse events following bone marrow infusion.

Post-operative VCA management

VCA recipients were monitored for signs of rejection or technical failure hourly for the first 6 hours and twice daily thereafter. VCAs were assessed for clinical signs of infection, erythema, epidermolysis or skin induration. VCA biopsies were performed at weeks 1, 2, 4 and 8 and at additional time-points if there was clinical suspicion of rejection.

Chimerism monitoring

Chimerism was assessed by flow cytometry twice weekly during the first week following transplantation and once weekly thereafter by monitoring the presence of donor (PAA+) peripheral blood mononuclear cells in the peripheral blood of the recipient (PAA-). The following cell markers were used: CD3 (898H2–6–15; mouse IgGaK), CD4 (74–12–4; mouse IgG2bK), CD8α (76–2–11; mouse IgG2aK), CD172 (74–22–15; mouse IgG1K) and PAA (1038H-10–9; IgMK). Bone marrow and thymic biopsies were performed prior to bone marrow transplantation and at days 50 and 100 and assessed for chimerism by flow cytometry. Chimerism was defined as the temporary (transient chimerism) or long-term (persistent chimerism) coexistence of donor-derived and recipient cells in the host. Tolerance was defined as indefinite persistence of VCA survival assessed by direct observation, histologically and through *in vitro* assays. HSC engraftment was defined as presence of donor-derived bone marrow colony-forming units (CFU's) over 14 weeks following transplantation.

Mixed lymphocyte reaction assays

Mixed lymphocyte reaction (MLR) assays were performed to assess *in vitro* lymphocyte responsiveness to self, donor animals and third-party animals (outbred Yorkshire). Responder (4×10^5 cells) and stimulator (4×10^5 cells, irradiated with 25cGy) peripheral blood mononuclear cells (PBMC) isolated from the blood of animals, were plated together in 96 well plates and incubated for 5 d at 37°C in 5% C02 and 100% humidity. Following this, responder cells were pulsed with H^3 thymidine and incubated further for 5 hours. Proliferation of responder cells was assessed by the uptake of the H^3 thymidine.

Results

Transient chimerism is prolonged with increased bone marrow transplantation doses and total body irradiation

In this study, 4 SLA^{dd} recipients were conditioned using a protocol that includes T cell depletion (using CD3 immunotoxin), total body irradiation at either 100 (n=2) or 200 (n=2) cGy, and a 45 day course of Cyclosporine A (target daily range 400–800ng/mL over days 0–30, tapered to 0 by day 45). Recipients received fully MHC-mismatched bone marrow cells (BMCs) at one of 3 doses: 7.1×10^8, 1.4×10^9, or 4.0×10^9 cells/kg, with one control animal not receiving any cells, and a gracilis myocutaneous VCA from the BMC donor was performed on day 0 of the protocol for all animals (**Table 1**).

Animals that received 100cGy TBI with either 7.1×10^8 or 1.4×10^9 cells/kg (animals 19599 and 19839) had minimal levels of myeloid chimerism (<5 % in both monocyte and granulocyte lineages) detectable within the first 2 weeks and no detectable donor-derived lymphocytes at any time (data not shown). Prolonged and higher levels of chimerism were observed in animal 20198, who received 200cGy TBI and the highest amount of bone marrow cells (4.0×10^9 cells/kg), with donor-derived granulocytes (43.35%) and monocytes (35.30%) peaking on day 30 post-BMT (**Fig. 1**). Similar to the other BMC recipients, only a few donor lymphocytes were detected 4 d following BMT but not at any later time points, suggesting that donor-derived thymopoiesis was not established. In this recipient, chimerism levels decreased quickly and no donor cells were detectable by flow cytometry after day 60, coinciding with cessation of Cyclosporine A on day 45. In the animal that received conditioning without bone marrow transplantation (20011), no donor-derived cells were detected at any time point as expected (data not shown). For all 3 recipients of bone marrow transplantation, PCR analysis of DNA isolated from bone marrow colony forming units, on day 50 and day 100, for donor class I^c was not detected, indicating that long-term engraftment of donor haematopoietic stem cells did not occur (data not shown).

Transient chimerism modulates early clinical VCA progression but does not prolong VCA acceptance following cessation of immunosuppression

In the control animal (20011) that received conditioning without BMCs, the presence of skin changes started on day

Table 1. Summary of experimental findings

Animal No.	Haplotype mismatch	Total Body Irradiation (TBI)	CyA (days)	Mixed Chimerism/ Engraftment	Bone Marrow dose	Skin Rejection crises	Muscle Survival (days)	Skin survival (days)
19599	CC→DD	100 CGy	45	No / No	7.1 × 10^8 cells/kg	Day 8–13	62	49
19839	CC→DD	100 CGy	45	No / No	1.4 × 10^9 cells/kg	Day 5–8	53	46
20198	CC→DD	200 CGy	45	Yes/No	4 × 10^9 cells/kg	No	73	62
20011	CC→DD	200 CGy	45	No / No	No	Day 14–45	15	61

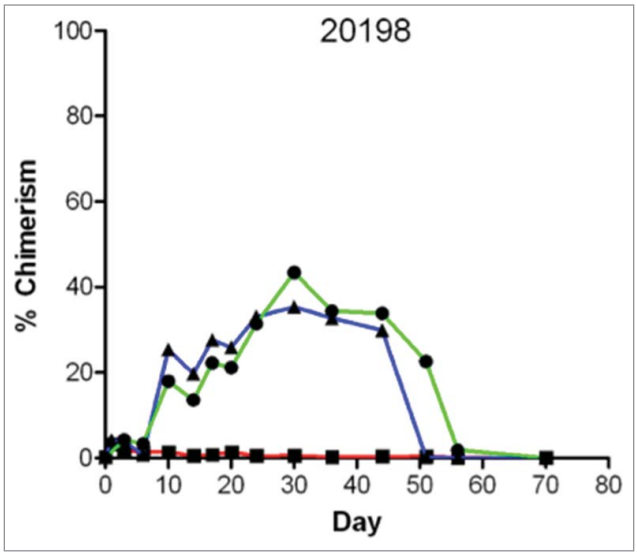

Figure 1. Flow cytometric analysis of peripheral blood chimerism of recipient 20198. PAA^+CD3^+ (lymphocytes), PAA^+CD16^+ (monocytes), and $PAA^+CD172a^+$ (granulocytes) are expressed as a percent of total, respectively.

15 with erythema (**Fig. 2**), and VCA rejection was complete by day 77, with hyperkeratosis and sloughing seen in the overlying skin epidermis. Histological analysis of biopsies confirmed muscle cellular rejection as early as day 15, with perivascular mononuclear cell infiltrate in the dermis and partial degeneration and necrotic muscle (**Fig. 3**).

The two recipients (animals 19599 and 19839) treated with 100cGy total body irradiation and 7.1×10^8 or 1.4×10^9 cells/kg displayed similar outcomes for VCA survival to the control animal (**Table 1**), and both had early acute rejection crises, starting around day 5 but underwent spontaneous resolution (recipient 19839 shown in **Figure 4**, recipient 19599 shown in Supplementary Figure A). As Cyclosporine A was being tapered, VCAs underwent rejection of the skin component, complete by days 46 (animal 19839) or 49 (animal 19599), followed by rejection of the muscle, by days 56 or 62, respectively.

In the animal that received with the highest dose of BMCs (4.0×10^9 cells/kg), during the time period in which donor cells were detectable in the peripheral blood, the VCA displayed no evidence of rejection (**Fig. 5**), and histological examination on day 38 demonstrated viable skin with no evidence of lymphocytic infiltrate (**Fig. 3**). But, while no early skin rejection crises were observed, the VCA was rejected following cessation of Cyclosporine A, with the skin lost by day 62 (**Fig. 5**) and the muscle surviving till day 73.

Bone marrow transplantation induced transient donor-specific unresponsiveness during the presence of detectable chimerism

To assess the effect of donor bone marrow cells on the immune status of VCA recipients, we compared the responses of PBMC from animals 20011 (who received conditioning with 200cGy TBI and no bone marrow cells) and 20198 (who received conditioning with 200cGy TBI and 4.0×10^9 cells/kg) to self, donor and third party antigens. Prior to undergoing the conditioning regimen, both animals demonstrated significant responses to donor and third party antigens (**Fig. 6**). On day 21 post-VCA transplantation, the recipient of donor bone marrow cells was specifically unresponsive to donor while maintaining third party reactivity, whereas at a similar time point (day 14), the control animal still demonstrated responsiveness to donor antigens, illustrating that conditioning without bone marrow transplantation did not confer donor-specific unresponsiveness. Following rejection of the VCA, animal 20198 regained MLR responses to donor antigens (**Fig. 6**), further illustrating that

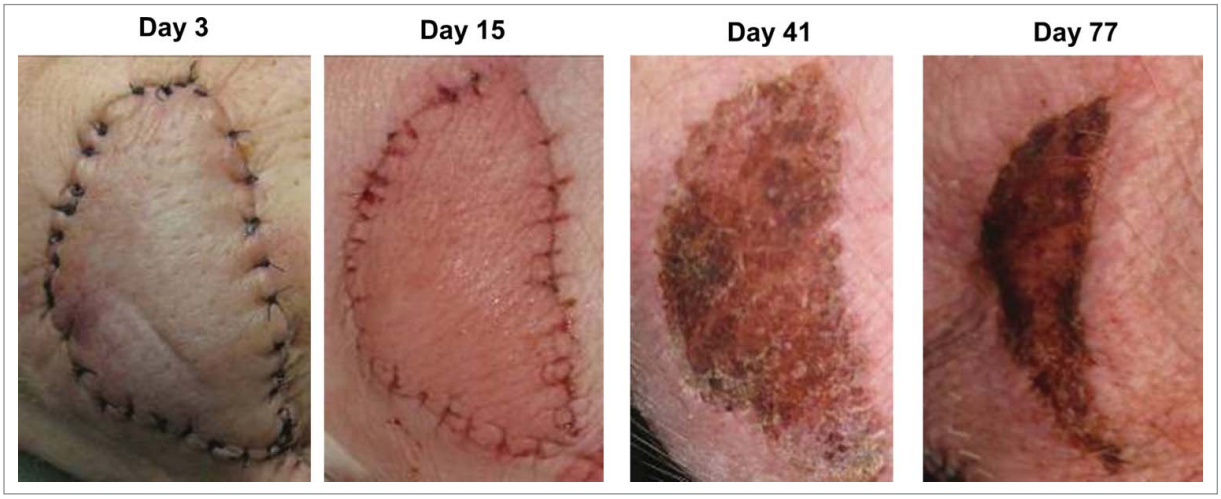

Figure 2. Time course for animal 20011s (higher TBI, no BM cells) VCA rejection. Skin erythema was evident starting on post-transplant day 15. Crusting and rejection of the epidermis occurred by day 40. Full VCA rejection (skin and muscle component) was complete by day 77.

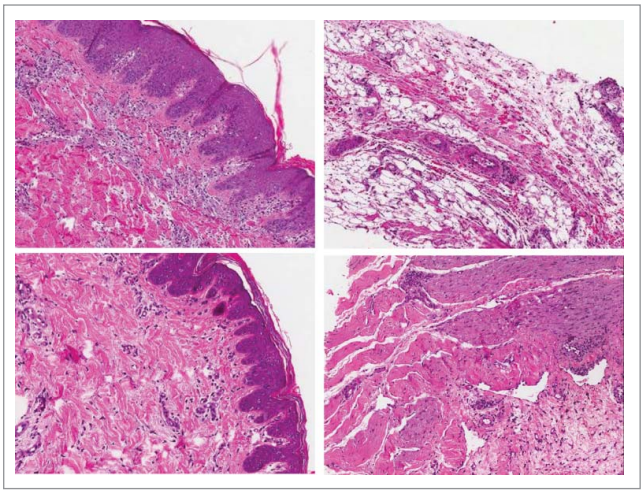

Figure 3. H&E stain of VCA skin from animal 20011 (higher TBI, no bone marrow cells) on day 15 demonstrating focal perivascular mononuclear cell infiltrate in the dermis (top, left) and partial muscle necrosis and neural degeneration (top, right). In contrast VCA skin from animal 20198 (higher TBI, highest BM dose) on day 38 showed minimal mixed lymphohistiocytic inflammation in the skin (bottom, left) and fascia (bottom, right).

the induction of donor-specific non-responsiveness was transient in nature.

Discussion

While VCA transplantation has gained increasing acceptance, with over 100 patients treated with these reconstructive procedures, the inherent MHC-mismatching requires lifetime chronic immunosuppression, thus subjecting the recipient to possible renal failure, infection and malignancy, as well as significant financial burdens. Additionally, even when taken as directed, these drugs often do not prevent patients from developing VCA rejection crises or chronic rejection.[1,4,26-28]

In this study, we investigated the effect of infused BMCs on the outcome of VCA transplantation, comparing animals that received different conditioning regimens and doses of bone marrow. In this study, transient mixed chimerism was observed in the animal receiving the highest dose of BMCs and TBI, and this coincided with lack of early rejection crises and a healthy clinical appearance of the VCA while the animal remained chimeric. Furthermore, early *in vitro* unresponsiveness to the donor was demonstrated in this animal while chimerism persisted, demonstrating the immunomodulatory effect of the transient chimerism.

Notably, chimerism levels fell rapidly following cessation of CyA, and no evidence for engraftment of long-term repopulating stem cells (LTR-SCs) was demonstrated. Signs of flap rejection were observed soon after the loss of chimerism/cessation of CyA in all animals, and the flap was completely rejected soon after chimerism was no longer detectable. Following rejection of all components of the VCA, *in vitro* responses to donor antigens were again observed. These results are in contrast to the animal that received the conditioning without BMT, which displayed a robust *in vitro* anti-donor response throughout the course of treatment. These results suggest that BMCs mitigate donor-specific responses in the early period following infusion and may minimize acute rejection episodes for period of time of detectable mixed chimerism.

While a clinically relevant protocol for induction of permanent VCA transplant tolerance was not achieved in this study, these data may have important implications. The

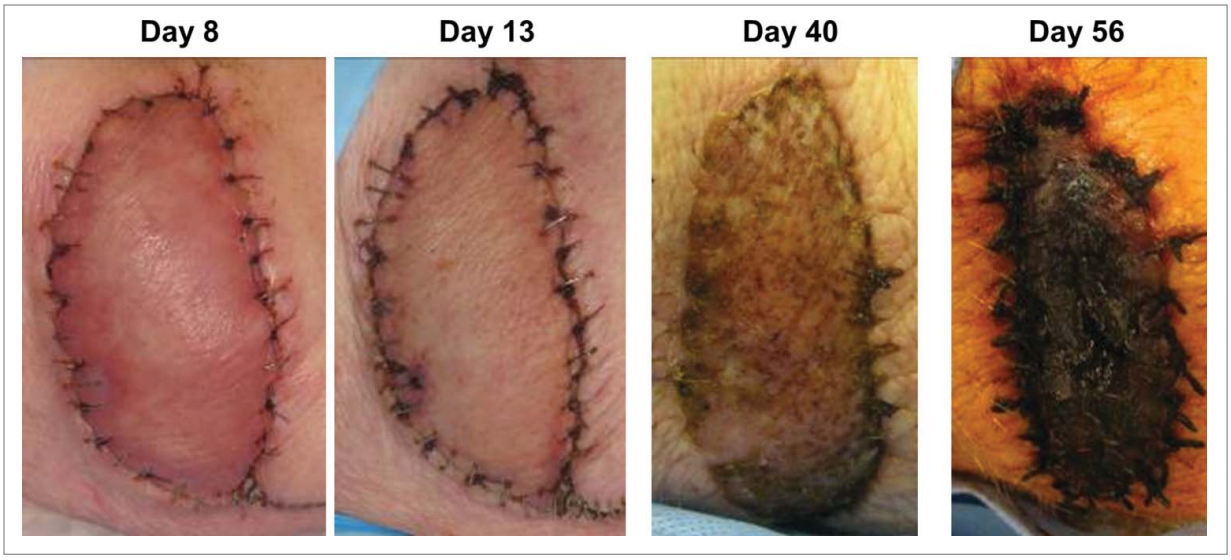

Figure 4. Time course of VCA rejection: animal 19839. An acute rejection crisis of the skin occurred from days 5–8. The VCA appeared healthy until day 40. The overlying VCA skin was rejected by day 46, with the muscle component rejecting by day 56.

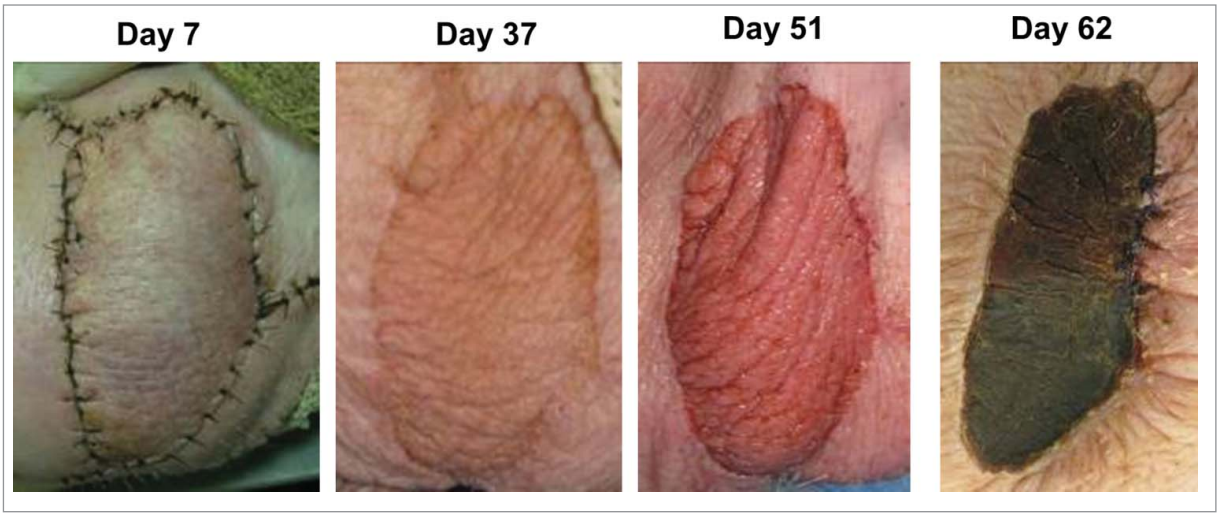

Figure 5. Time course of VCA rejection: animal 20198. Skin erythema and hyperkeratosis started on day 51. Keratosis increased progressively and skin necrosis occurred around day 57. VCA was fully rejected by day 62. Both skin and muscle components of the flap were completely rejected by day 73.

occurrence of acute rejection episodes in clinical recipients of VCA is consistent with our findings, and provide for justifiable rationale for inclusion of donor BMCs as part of the overall VCA protocol given the ability to mitigate early acute rejection episodes. Furthermore, while this study does not address the effect of BMC infusion on the long-term survival of VCA under the cover of immunosuppression, the ability to move hand transplant patients onto maintenance immunosuppression monotherapy when BMCs were administered[22] suggests a positive impact that may have long-term benefits as well.

Currently, the clinical outcome for VCA acceptance when HSCs are included in the regimen have differed than the results observed in the renal transplant protocols,[12,19] suggesting a possible difference in the either the ability to achieve tolerance of a kidney vs. a VCA or the ability of the organ to contribute to the mechanisms of tolerance. It is also possible that in contrast to the kidney recipients, recent VCA recipients did not undergo any conditioning regimen to facilitate the survival or engraftment of the donor HSCs, and thus the duration of the transient chimerism was markedly reduced to the point where donor cells were undetectable in the blood at any time point. However, in all 3 of the animals presented in this study that received both conditioning and BMCs, time of VCA rejection was comparable to the animal that received conditioning alone, suggesting the prolonged transient chimerism observed (presumably through recipient T cell depletion and whole body irradiation) was insufficient in establishing long-term tolerance mechanisms able to prolong VCA survival.

Another possible contributing factor that remains to be explored is the contribution of MHC-matching between donor and recipient, as in the combined kidney/HSC patients, the tolerance was achieved across a single-haplotype from a living-related donor. Finally, tolerance may require more robust approaches for establishing and maintaining tolerance of VCAs, as has recently been described through the induction of stable mixed chimerism and the indefinite acceptance of all VCA components, including skin, across MHC I and II barriers.[29]

In conclusion, this study provides evidence that infusion of donor BMCs and the establishment of transient mixed chimerism may reduce rejection crises in a VCA setting across a full MHC mismatch, but does not induce long-term tolerance of a

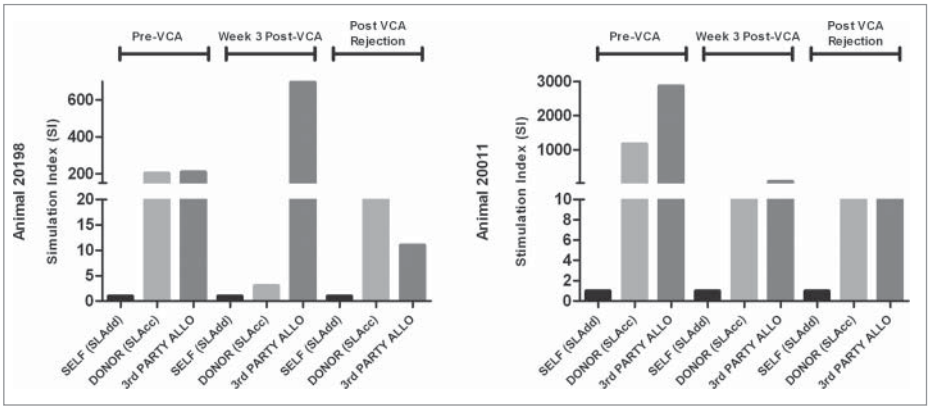

Figure 6. Mixed lymphocyte reaction (MLR) assays. Responsiveness against self, donor and third party was assessed at different time-points following BMT and VCA transplantation. Donor-specific unresponsiveness occurred in animal 20198 while transient mixed chimerism was present (left panel) whereas animal 20011 demonstrated responses to donor antigens at all time points tested (right panel). Data is expressed for each stimulator as stimulation index (SI) with standard deviation.

VCA. This suggests that establishment of long-term, stable chimerism or the induction of more effective, long-term immunomodulatory mechanisms will be necessary to prevent rejection of all components of a VCA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

AALB was responsible for project design, the execution of the overall study, animal care and manuscript preparation; AA and CM were responsible for execution of *in vitro* assays and animal care; KS and MAR was involved in experimental design and provided surgical assistance; RT was involved in *in vitro* assays and provided surgical assistance; DAL provided surgical assistance and assistance with manuscript preparation; CAH, DHS, JMK and CLC were involved in study design, project monitoring and manuscript preparation.

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