Tolerance to Vascularized Composite Allografts in Canine Mixed Hematopoietic Chimeras

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Abstract

Background—Mixed donor-host chimerism, established through hematopoietic cell transplantation (HCT), is a highly reproducible strategy for the induction of tolerance towards solid organs. Here, we ask whether a nonmyeloablative conditioning regimen establishing mixed donor-host chimerism leads to tolerance of highly antigenic vascularized composite allografts.

Methods—Stable mixed chimerism was established in dogs given a sublethal dose (1–2 Gy) total body irradiation before and a short course of immunosuppression after dog leukocyte antigen-identical marrow transplantation. Vascularized composite allografts from marrow donors were performed after a median of 36 (range 4-54) months after HCT.

Results—All marrow recipients maintained mixed donor-host hematopoietic chimerism and accepted composite tissue grafts for periods ranging between 52 and 90 weeks; in turn, marrow donors rejected vascularized composite allografts from their respective marrow recipients within 18–29 days. Biopsies of muscle and skin of vascularized composite allografts from mixed chimeras showed few infiltrating cells compared to extensive infiltrates in biopsies of vascularized

composite allografts from marrow donors. Elevated levels of CD3+ FoxP3+ T-regulatory cells were found in skin and muscle of vascularized composite allografts of mixed chimeras compared to normal tissues. In mixed chimeras, increased numbers of T-regulatory cells were found in draining compared to non-draining lymph nodes of vascularized composite allografts.

Conclusion—These data suggest that nonmyeloablative HCT may form the basis for future clinical applications of solid organ transplantation and that T-regulatory cells may function towards maintenance of the vascularized composite allograft.

Keywords

dog; nonmyeloablative conditioning regimen; mixed hematopoietic chimerism; skin grafting; hematopoietic cell transplantation; CTA transplantation; FoxP3; tolerance

INTRODUCTION

A primary goal in the field of transplantation has been the establishment of immunological tolerance in the patient towards donor-specific tissue antigens. Immunological tolerance would eliminate the need for life-long immunosuppressive therapy required to prevent rejection of the organ graft and eliminate the risks of morbidity, chronic tissue graft rejection, renal toxicity, and malignancy (1,2). Hematopoietic or mixed donor-host chimerism reasserts the immunologic repertoire by encompassing tolerance towards donor antigens and allowing transplantation of solid organs without immunosuppression (3).

Several small animal studies have shown that tolerance towards transplanted solid organs such as kidney (4), heart (5) and pancreas (6) can be achieved as a result of mixed chimerism after hematopoietic cell transplantation (HCT). Using mixed chimerism to induce tolerance to solid organs and vascularized composite allografts in larger animal models has shown signs of success; however, tolerance to the skin has been a major obstacle (7-9). The most common outcome of these experiments, even across minor genetic barriers, has been the establishment of split tolerance where tolerance to the transplanted muscle and bone was established while the skin component was rejected (10,11). While successful transplantation of all components of the VCA have been reported in large animals, they have largely been either cases of a single tolerant animal or significant prolongation observed with the addition of donor mesenchymal stem cells to the regimen (12-14). Presumably, the antigenicity of skin renders it a difficult tissue to transplant under conditions of both immunosuppression (15) and established mixed chimerism (9,16). For any tolerance protocol to be clinically applicable in the emerging field of vascularized composite allotransplantation, tolerance must be established to all components of the allograft including skin (10,11).

We previously reported consistent and sustained hematopoietic cell engraftment in dogs given a nonmyeloablative dose (1-2 Gy) of total body irradiation (TBI) before, and immunosuppression consisting of mycophenolate mofetil (MMF) and cyclosporine (CSP) for 28 and 35 days, respectively, after dog leukocyte antigen (DLA)-identical HCT (17,18). We have also demonstrated that, after establishing mixed chimerism, canine marrow recipients accepted kidney allografts long-term from their marrow donors without the need for immunosuppression (19). However, tolerance was not generally extended to marrow donor skin grafts as rejection occurred in half of the mixed chimeric dogs despite ongoing tolerance to their kidney allografts (20). The intestine, arguably a more antigenic organ to transplant than the kidney, was also successfully transplanted heterotopically into the neck region of four mixed chimeric dogs (21).

A vascularized composite allograft is composed of skin, muscle, fat, nerves, lymphatic and blood vessels. Following nonmyeloablative HCT, vascularized composite allografts allow

for the evaluation of tolerance towards different tissues, which ostensibly express tissue-specific antigens. Furthermore, such grafts represent the best transplantation model for complex tissues such as the hand or any reconstructive surgery involving skin and muscle.

Here, we test the hypothesis that mixed chimerism can induce a state of tolerance towards a vascularized composite allograft in the DLA-identical canine model. We show that the well-tolerated nonmyeloablative conditioning regimen of 1–2 Gy TBI, preceding marrow transplantation and followed by a short course of postgrafting immunosuppression, is sufficient for stable engraftment towards all components of a vascularized composite allograft. Furthermore, engraftment may be influenced by the presence of infiltrating lymphocytes of the T-regulatory phenotype.

RESULTS

Stable Mixed Donor/Host Hematopoietic Chimerism

Table 1 summarizes the conditioning regimen, postgrafting immunosuppression, percentages of donor chimerism in recipient dogs, and engraftment outcome. Donor granulocyte chimerism ranged from 20–98% and lymphocytes chimerism from 40–100%. Between 5.5 months and >4 years after HCT, composite tissue grafts were transplanted from marrow donors to their respective mixed chimeric recipients. Two dogs, G244 and G969, showed an increase in chimerism for granulocytes and lymphocytes from mixed to 98% and 100% donor, respectively. The remaining three recipients showed slight reductions in donor chimerism for both of these cell populations (Table 1 and Supplemental Figure 1).

Vascularized Composite Allotransplantation in Marrow Donors and Stable Mixed Chimeric Recipients

Five mixed chimeric recipients and four marrow donors received vascularized composite allografts from their respective DLA-identical littermates. All nine transplants were initially accepted and were well-healed within 2 weeks. All four non-chimeric donors rejected their composite tissue grafts within 18–29 (median 21) days. Rejection was indicated clinically by erythema and progressive edema, followed by necrosis of skin and muscle (Figure 1A and B). In contrast, the five mixed chimeras accepted the composite tissue grafts from their marrow donors with observation periods ranging from 52-90 (median 52) weeks, at which point composite tissue allografts were considered permanent (Table 1). Acceptance was indicated clinically by normal skin color and hair growth (Figure 1C). The dogs' peripheral blood counts and blood chemistries showed no abnormalities (data not shown). Histologically, representative biopsies of skin and muscle composite tissue grafts obtained from two of the marrow donors at time of rejection revealed lymphocytic infiltrate (Banff Grade IV and Grade 3R, respectively) in both tissues (Figure 2A). Serial biopsies of skin and muscle grafts of the mixed chimeric recipients at 118–167 days after vascularized composite allograft transplantation showed minimal evidence of cellular infiltrate (Banff Grade 0 and Grade 0R, respectively) and no evidence of vascular disease, or epidermal architecture damage (Figure 2B).

FoxP3 and Granzyme B (Grβ) Expression in CD3+ T-cells Isolated from Tissues in Mixed Chimeric and Non-chimeric Recipients after CTA

CD3+ T-cells were isolated from muscle and skin of the vascularized composite allografts and lymph nodes from mixed chimeric recipients and marrow donors. Muscle biopsies obtained from the rejected muscle in marrow donors demonstrated infiltrating T-cells with increased expression of Grβ compared to normal muscle (Figure 3A). CD3+ T-cells obtained from muscle and skin biopsies taken from vascularized composite allografts from mixed chimeric recipients had increased levels of both FoxP3 and Grβ compared to normal

muscle and skin (Figures 3A, 3B). We also examined expression of FoxP3 in the CD3+ cells harvested from lymph nodes from mixed chimeras and found higher expression of FoxP3 in the draining lymph nodes (obtained from regional nodal basin of the composite tissue grafts) than the non-draining lymph nodes or the lymph nodes obtained from untreated control dogs (Figure 3C). Collectively, these data suggest that T-regulatory cells are a component of the cellular infiltrate of indefinitely accepted skin and muscle grafts possibly providing suppression of alloreactive T-cells.

In situ Detection of T-regulatory Cells within Muscle and Skin Biopsies of Composite Tissue Allografts from Long-Term Mixed Chimeric Recipients

Muscle and skin biopsies from untreated control dogs and mixed chimeric recipients that demonstrated long-term acceptance of their vascularized composite allografts were stained for the presence of the intranuclear protein FoxP3. Mixed chimeric recipients maintained a consistent expression of FoxP3 over the course of the transplant (> 1 year), demonstrated both by RT-PCR and immunohistochemistry analyses. Muscle and skin biopsies from normal control dogs were negative for FoxP3 staining (Figure 4A and B). However, significant FoxP3 staining was observed in the muscle and the skin obtained from the vascularized composite allografts of mixed chimeras (Figure 4C and D). Cells staining positive for FoxP3 were localized to limited areas of higher cellularity in both muscle and skin biopsies of mixed chimeric dogs. These results further confirm the observations that T-regulatory cells are present within tissues of chimeric recipients of vascularized composite allografts (22).

Because lymphocytic infiltrates could be observed within some of the biopsies of transplanted skin and muscle in mixed chimeric recipients, we sought to determine whether the percentages of FoxP3+ cells present within those sections were greater than in the vascularized composite allografts taken from the control marrow donors. Analysis of images of FoxP3+ stained cells was done using ImageJ imaging software analysis. As shown in Supplemental Figure 2, the average percent of FoxP3+ cells present in the lymphocytic infiltrate of the skin of the composite tissue allograft on the marrow donor was 0.833 ± 0.508, while that of the mixed chimeric recipient was on average was significantly greater at 5.311 ± 1.926. Similarly, the average percent of FoxP3+ cells in the infiltrate of the muscle of the vascularized composite allograft on the marrow donor was 0.115 ± 0.099, while that of the mixed chimeric recipient was significantly greater at 4.023 ± 2.660.

Mixed Lymphocyte Reaction Confirms that the Immune System is Intact After Nonmyleoablative Bone Marrow Transplant

In order to determine whether mixed hematopoietic chimeras, tolerant to vascularized composite allografts, were immunocompetent, PBMC from these dogs were tested by MLR. PBMC from all mixed chimeric recipients demonstrated robust proliferative responses against PBMC obtained from DLA class II mismatched donors with a median SI of 106, (range = 10 to 126) (data not shown).

DISCUSSION

Vascularized composite allograft transplantation offers an unparalleled opportunity to reconstruct complex defects in patients. To date, approximately 50 hand transplants and 8 partial face transplants have been performed worldwide (23-28). However, it remains an experimental procedure completely dependent on immunosuppressive drug regimens similar to those used in solid organ transplantation. While immunosuppressive drugs have resulted in excellent 1-year graft survival, they have not prevented episodes of acute rejection (29). Additionally, complications from chronic immunosuppression have been widely reported in

both hand and face recipients (30). Immunologic tolerance would allow for long-term survival of a vascularized composite allograft without the need for chronic immunosuppression. Establishment of hematopoietic chimerism following HCT continues to be an attractive strategy for inducing transplantation tolerance (31).

Our study was undertaken to test the hypothesis that nonmyeloablative HCT followed by a short course of postgrafting immunosuppression can establish immune tolerance of host immune cells towards donor hematopoietic cells and vascularized composite allograft from the marrow donors. While small animal models utilizing mixed chimerism to induce tolerance towards this type of transplant have been reported, translation of tolerance to vascularized composite allografts in a large animal model is limited (11,12). Additionally, the use of a canine model in studying tolerance towards vascularized composite allograft has not yet been published. Therefore, we initiated these studies with DLA-identical canines to serve as a platform from which to progress to the DLA-haploidentical and eventually unrelated settings. Because our canine mixed chimeras were DLA-antigen matched but minor antigen mismatched, successful engraftment of vascularized composite allografts was dependent on tolerance to non-MHC antigens shared between hematopoietic cells and the donor composite tissue graft. Non-MHC antigen disparities among dogs can cause potent rejections of skin (32), or kidney allografts (33,34) or cause GVHD (35). Organs that approach the skin in susceptibility to rejection are small bowel (36) and lung (37). There is limited evidence that many of these tissues can be accepted when transplanted into canine mixed hematopoietic chimeras.

Several findings in the present study were noteworthy. First, we observed 100% acceptance of all components of the vascularized composite allografts in the dog, specifically skin and muscle for all five mixed chimeric recipients for periods greater than 1 year; unlike previous large animal models where split tolerance was seen (10,11). In addition, the control transplants that received vascularized composite allografts without the benefit of HCT rejected their grafts (<21 days), confirming the requirement of HCT for inducing tolerance towards the vascularized composite allograft. This strategy of using mixed chimerism to support tolerance towards vascularized skin grafts is supported by studies in the porcine model (12). Second, vascularized composite allografts were performed well after donor marrow engraftment was established. Thus, tolerance against skin-specific antigens was established without the benefit of the tolerance-promoting conditions accompanying HCT. Third, MLR using PBMC from the stable mixed hematopoietic chimeras showed that acceptance of vascularized composite allografts was not due to lack of immunocompetence, duplicating similar studies in our laboratory (17). Fourth, by achieving tolerance against skin, a highly antigenic tissue, these results suggest that this tolerance induction regimen is likely to be successful for other commonly transplanted organs.

A second component of our study was to investigate the putative role of regulatory T-cells in the maintenance of tolerance to donor skin and muscle. Regulatory T-cells have been shown to contribute to the establishment and maintenance of tolerance of the immune system (38). The T-cell subset, CD4+/CD25^{high}/FoxP3$^+$ T-regulatory (T-reg) cells, have even been shown to infiltrate skin of hand transplant recipients maintained on chronic immunosuppression, but the significance of this observation is unknown (39). However, immunohistological analysis of skin biopsies from recipients of allogeneic HCT revealed that an increase in the numbers of T-reg cells correlated with less severe GVHD and was generally associated with positive clinical responses to GVHD treatment (40). Recently, we reported that markers for T-reg cell subsets were increased in CD3$^+$ T-cells isolated from the blood and peripheral tissues after lung transplantation into canine mixed chimeric recipients (41). We have also found that canine peripheral blood CD25+ FoxP3+ cells were highly immunosuppressive in vitro (42). Additionally, lower expression of FoxP3 compared to Grβ

(a cytotoxic protein effecting target cell lysis that is expressed on cytotoxic T-lymphocytes and associated with rejection) has been reported in cells collected from the urine of patients with kidney allografts following episodes of rejection (43). Thus, the long-term presence of T-reg cells after transplantation may indicate a more favorable environment for long-term allograft survival and tolerance induction.

With these studies in mind, we undertook efforts to determine to what extent T-regs (CD3+/CD25 ^{high} /FoxP3+ T-cells) were present in skin and muscle vascularized composite allografts from both marrow donors and mixed chimeric recipients. Elevated levels of FoxP3 expression were found in the transplanted muscle and skin and in the regional draining lymph node of mixed chimeras when compared to normal tissue and to the non-chimeric transplant recipients. In addition, the CD3$^+$ cells derived from biopsies of the muscle and skin of mixed chimeric recipients maintained expression of FoxP3 for a period > 1 year. In contrast Grβ was elevated in the muscle component of the VCA harvested from the non-chimeric control transplants when compared to muscle from the tolerant chimeric VCA recipients. These findings suggest a role for T regulatory cells in the tissue and regional lymph nodes of the chimeric dogs.

In this study we have demonstrated for the first time donor specific tolerance to all components of the vascularized composite allografts in a genetically out-bred DLA-matched canine model conferred through nonmyeloablative allogeneic HCT. We saw no evidence of acute or chronic rejection in skin or muscle, GVHD, or a posttransplant lymphoproliferative disorder within follow-up periods between 52 and 90 weeks. Because of the highly antigenic nature of skin, successful methods of transplantation developed for vascularized composite allografts are likely to translate directly to other less antigenic and more commonly transplanted organs.

MATERIAL AND METHODS

Experimental Animals

Random-bred litters of beagles and mini-mongrel cross-breeds were either raised at the Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, or purchased commercially. The dogs weighed from 7.0 to 13.3 (median 9.6) kg and were 7 to 27 (median 8) months old. They were observed for disease for at least 60 days before study. The Institutional Animal Care and Use Committee of the FHCRC approved the research protocols, and the American Association for Accreditation of Laboratory Animal Care certified the kennels. Five littermate donor/recipient pairs were DLA-identical on the basis of matching for highly polymorphic major histocompatibility complex (MHC) class I and class II micro-satellite markers (44). In addition, specific DLA DRB1 allelic identity was confirmed by direct sequencing (45).

Nonmyeloablative Conditioning and HCT

Conditioning for HCT consisted of a single dose of either 1 or 2 Gy TBI delivered at a rate of 7 cGy/min from a high-energy linear accelerator (Varian Clinac 6, Palo Alto, CA). Two dogs (G244 and G949) received 1 Gy TBI, and three dogs (G767, G500 and G969) received 2 Gy TBI, followed by transplantation of marrow from a respective DLA-identical littermate. Marrow was infused i.v. within 4 hr of TBI at doses of 2.8 to 9.2 (median 4.4) $\times$ 10^8 nucleated cells/kg. The day of HCT infusion was designated as day 0 for establishing mixed chimeric dogs. Postgrafting immunosuppression consisted of MMF (10 mg/kg b.i.d., s.c., from day 0 to day 28) and cyclosporine (15 mg/kg b.i.d. orally from day -1 to 35) for 4 dogs (G767, G244, G969 and G949). One dog, (G500) received sirolimus (0.05 mg/kg s.c. from day -5 to 25) and MMF (10 mg b.i.d., s.c. from day 0 to day 34). All dogs were given

standard postgrafting care (46). The dogs' clinical statuses were assessed twice daily. White blood cell counts with differentials, platelet counts, and hematocrits, were performed daily through day 21 and twice weekly thereafter.

Assessment of Hematopoietic Cell Engraftment

Percent donor chimerism was determined as previously described (47,48). Briefly, a polymerase chain reaction (PCR)-based assay was performed using specific primers for informative microsatellite markers. Primers amplified regions throughout the genome possessing tandem repeats in which the transplant recipient and donor were non-identical. PCR products were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer.

Vascularized Composite Allograft Transplantation

The surgical procedures for composite tissue allotransplantation were done as previously described (49). The skin and muscle were followed via clinical examination for color of the skin and hair growth. Stable engraftment of the graft was determined by punch biopsy of the skin and muscle. Sections of tissue were fixed in 10% formalin, imbedded in paraffin, and cut sections were stained with hematoxylin-and-eosin and Jones' stains. A pathologist (G.S.), blinded to the source of the biopsies, evaluated presence or absence of rejection.

Quantitative Reverse Transcribed-PCR of Intracellular Cytokines and FoxP3 from CD3+ T-cells and FoxP3 Immunohistochemistry

Methods and reagents for cytokine analysis and FoxP3 expression are described under Supplemental Methods.

In vitro Immune Responses

Mixed leukocyte reactions (MLR) were done as described (50). Briefly, peripheral blood mononuclear cells (PBMC) were collected from blood after Ficoll (density 1.074) gradient centrifugation. Cells were washed in phosphate buffered saline PBS and resuspended in 45% Waymouth's Medium (GibCo, Grand Island, NY), 45% Iscove's Modified Dulbecco's Medium (GibCo) with 10% heat-inactivated dog serum, sodium pyruvate, nonessential amino acids and L-glutamine (all except serum from GibCo). Stimulator cells were irradiated with 22 Gy (cesium irradiator). Cells were incubated for 7 days with ^{3}H-thymidine added on day 6.

Statistical Analysis

Comparisons of FoxP3 to Grβ ratios between accepted tissue grafts and rejected tissue grafts, and between tissue grafts and normal controls, were evaluated by Wilcoxon rank-sum tests (2-sided). Significance was defined as a P value less than or equal to 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CSP	cyclosporine
DLA	dog leukocyte antigen
FHCRC	Fred Hutchinson Cancer Research Center
FoxP3	Forkhead Box P3
G3PDH	glycerol-3-phosphate dehydrogenase
Grβ	granzyme B
GVHD	graft versus host disease
HCT	hematopoietic cell transplantation
IL-10	interleukin-10
MLR	mixed leukocyte reactions
MMF	mycophenolate mofetil
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction

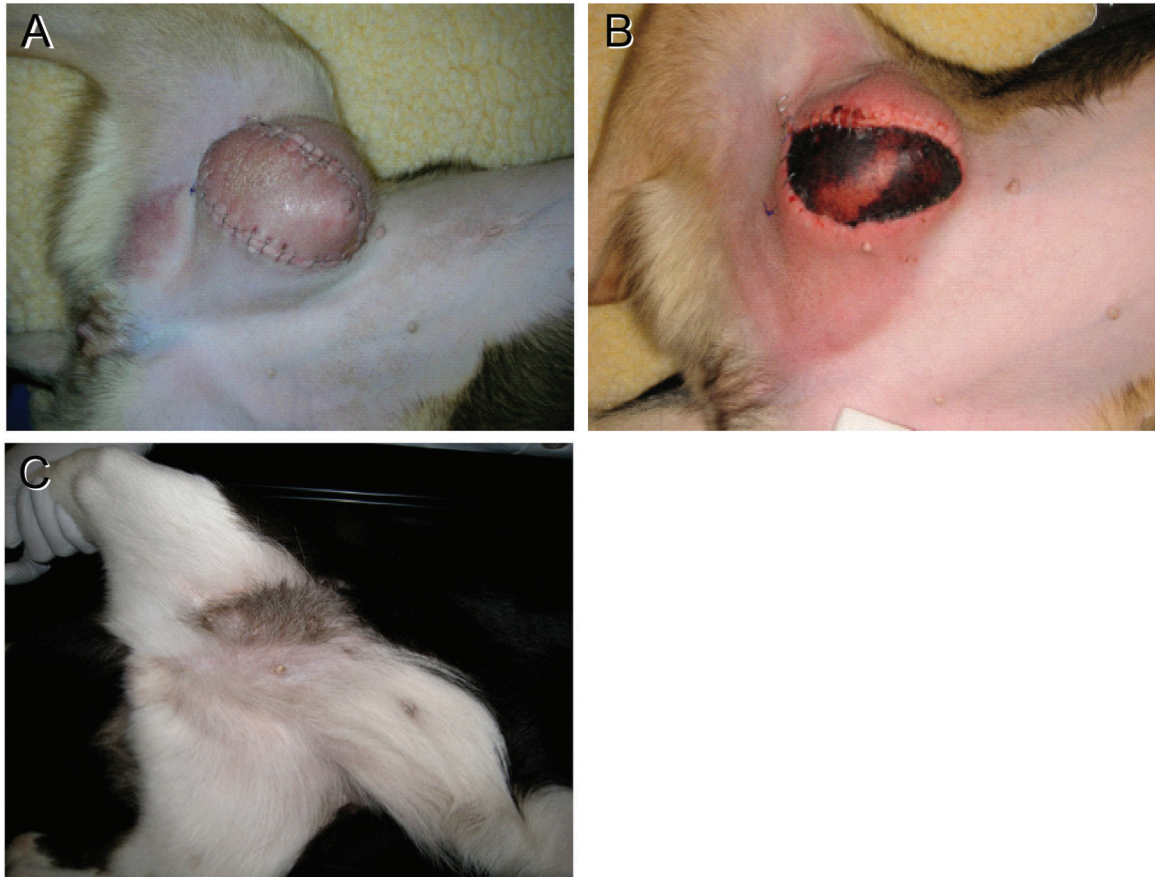| **RT-PCR** | reverse transcribed PCR |
TBI	total body irradiation
TGFβ	transforming growth factor beta
T-reg cells	T-regulatory cells

Figure 1. Rejection and acceptance of vascualrized composite allografts in dogs
Rejection of a vascularized composite allograft in a marrow donor dog (G499), showing early (day 18, **A**) and late (day 21, **B**) signs of rejection, with edema, erythema, and skin necrosis. In contrast, the graft in the mixed chimeric dog (G500) shown here on day 346 with normal hair growth (brown) and no evidence of edema or erythema (**C**).
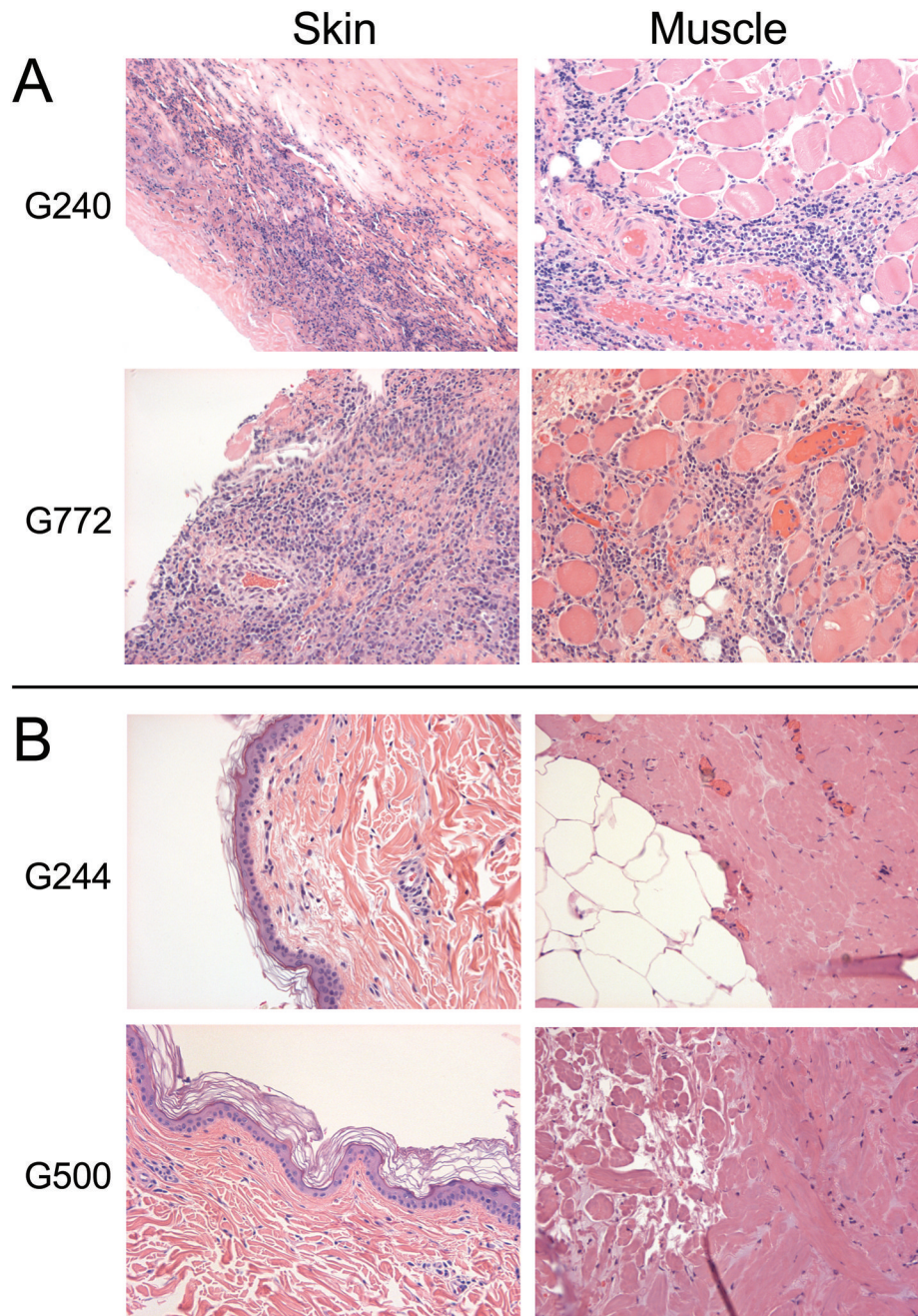
Figure 2. Histology of transplanted skin and muscle tissue from marrow donors and mixed chimeric recipients
Recipient grafts in both marrow donors, G240 and G244 show dense lymphocytic infiltrates in skin and muscle on day 21 and 31 respectively; consistent with rejection (**A**). Marrow donor grafts in both mixed chimeras G244 and G500 showed normal skin and muscle histologies on days 167 and 118, respectively (**B**). Slides were photographed at 200X magnification.
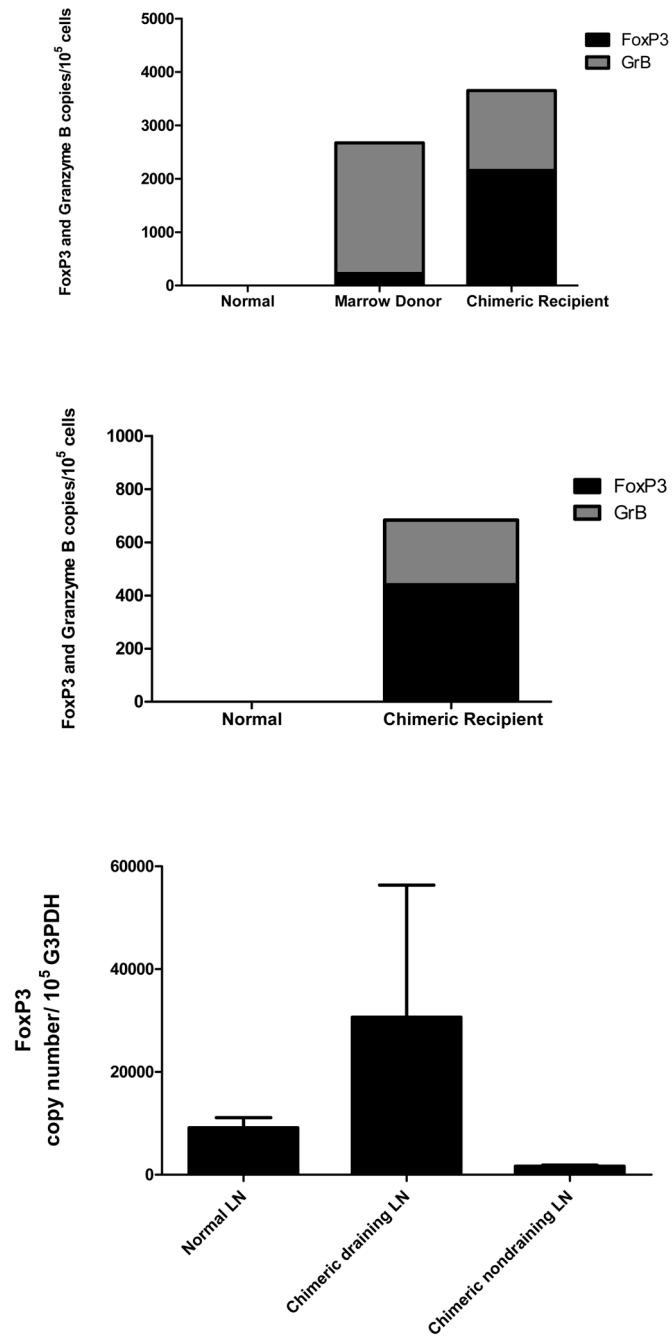
Figure 3. FoxP3 and Granzyme B expression in biopsies of allografted and resident muscle, skin, and draining and non draining lymph nodes from marrow donors and chimeric recipients
(**A**) FoxP3 (black bar) and Granzyme B (grey bar) expression of CD3+ T-cells collected from normal recipient muscle, allograft muscle from marrow donors and mixed chimeric recipients. (**B**) FoxP3 and Granzyme B expression of CD3+ T-cells collected from allografted skin biopsies. (**C**) FoxP3 expression of CD3+ cells isolated from normal dog, and draining lymph nodes or nondraining lymph nodes of the vascularized composite allograft from chimeric recipients (n=3). qRT-PCR methods were used to determine FoxP3 and GrB expression relative to G3PDH.
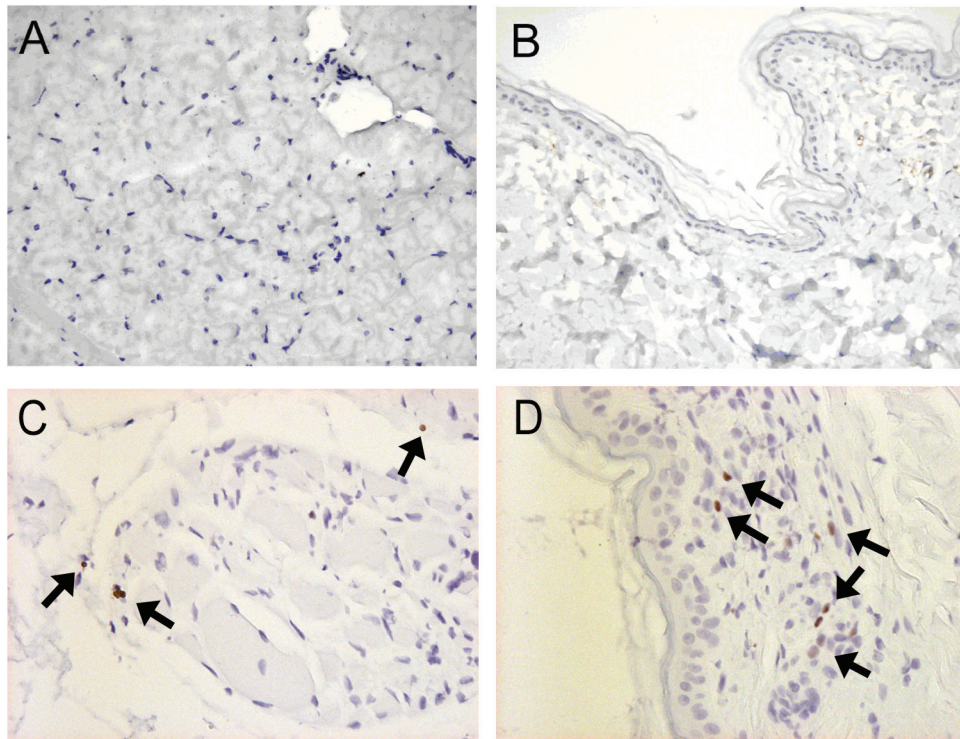
Figure 4. FoxP3+ staining of muscle and skin biopsies from untreated dogs, bone marrow donor dogs and vascularized composite allografts from mixed chimeric recipients
Representative muscle (**A**) and skin (**B**) biopsies from an untreated dog showed no areas of FoxP3+ staining (200x magnification. In contrast, representative muscle (**C**) and skin (**D**) biopsies taken from the composite tissue allograft of mixed chimeric recipients (G767 and G969) reveal increased FoxP3+ staining with limited cellular infiltrate (Arrowheads; 400x magnification).

Table 1

Donor Chimerism after Marrow and Subsequent Composite Tissue Grafts from DLA-Identical Littermates

Recipient #	Date of Marrow Graft	TBI Gy	Donor Marrow Cells × 10^8 / kg	Post-Marrow Grafting Immuno-suppression	Date of Composite Tissue Allograft	% Donor Chimerism[1] (weeks)[2]		Post-Transplant Immuno-suppression	Graft Rejection	Weeks of follow-up[3]
						Before	After			
G767	4/26/07	2	4.3	MMF/CSP	10/15/07	G62.3 (0) L37.5 (0)	G7.4 (35) L25.8 (35)	No	No	>90
G244	5/16/03	1	9.2	MMF/CSP	10/29/07	G62.5 (-1) L49.4 (-1)	G100 (31) L100 (31)	No	No	>77
G500	12/22/04	2	4.8	MMF/Rapa	12/18/07	G50.0 (-3) L66.0 (-3)	G36.1 (26) L42.4 (26)	No	No	>52
G969	12/13/07	2	4.1	MMF/CSP	4/14/08	G94.6 (-4) L49.5 (-4)	G100 (53) L100 (53)	No	No	>52
G949	1/4/08	1	2.8	MMF/CSP	10/27/08	G69.5 (0) L48.4 (0)	G38.3 (52) L45.3 (52)	No	No	>52

[1] Data are presented as percent donor granulocytes (G) and lymphocytes (L).

[2] Chimerism analyzed in weeks before or after composite tissue graft, week = 0.

[3] Weeks of follow-up after composite tissue graft, week = 0.

G767 and G969 received 2 Gy TBI (day = 0) with postgrafting immunosuppression of MMF (10 mg/kg B.I.D. subq., days 0-27) and CSP (15 mg/kg B.I.D. orally , days -1-35) and received HCT on a protocol specifically for this study.

The following dogs received HCT under separate studies and were later transferred to the present study as mixed chimeras. G244 received 1 Gy TBI with postgrafting immunosuppression of MMF (10 mg/kg B.I.D subq., days 0-27) CSP (15 mg/kg B.I.D. orally, days -1-35) and methotrexate (0.4 mg/kg, I.V. days 1, 3, 6, 11).

G500 received 2 Gy TBI with postgrafting immunosuppression of MMF (10 mg/kg B.I.D subq., days 0-34) and sirolimus (0.05 mg/kg, daily, subq, day -5 to 25).

G949 received 1 Gy TBI on day = 0, recombinant canine CTLA4-Ig (4.0 mg/kg I.V. days -7 and -5) donor PBMC (1×10^6 cells/kg on days -7 and -5 injected subq and I.V.) and post grafting immunosuppression consisting of MMF (10 mg/kg B.I.D. subq., days 0-27) and CSP (15 mg/kg B.I.D. orally , days -1-35).