Published in final edited form as:

Transplantation. 2016 December ; 100(12): e120–e127. doi:10.1097/TP.0000000000001496.

Long-Term Tolerance Towards Haploidentical Vascularized Composite Allograft Transplantation in a Canine Model Using Bone Marrow or Mobilized Stem Cells

Jeff Chang, MD^{1,2}, Scott S. Graves, PhD^{1,3}, Tiffany Butts-Miwongtum, BS¹, George E. Sale, MD^{1,4}, Rainer Storb, MD^{1,3}, and David W. Mathes, MD^{1,5,6}

¹Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

²Department of Surgery, University of Washington, Seattle, WA

³Department of Medicine, University of Washington, Seattle, WA

⁴Department of Pathology, University of Washington, Seattle, WA

⁵Department of Surgery, University of Colorado, Aurora, CO

⁶Plastic Surgery Service, VA Eastern Colorado Care System, Denver, CO

Abstract

Background—The development of safe and reliable protocols for the transplantation of the face and hands may be accomplished with animal modeling of transplantation of vascularized composite allografts (VCA). Previously, we demonstrated that tolerance to a VCA could be achieved after canine recipients were simultaneously given marrow from a dog leukocyte antigen (DLA) identical donor. In the present study, we extend those findings across a DLA mismatched barrier.

Methods—Eight Recipient dogs received total body irradiation (4.5 cGy), hematopoietic cell transplantation (HCT), either marrow (n=4) or granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood stem cells (n=4), and a VCA transplant from the HCT donor. Post grafting immunosuppression consisted of mycophenolate mofetil (28 days) and cyclosporine (35 days).

Results—In 4 dogs receiving bone marrow, 1 accepted both its marrow transplant and demonstrated long-term tolerance to the donor VCA (>52 weeks). Three dogs rejected both their marrow transplants and VCA at 5–7 weeks posttransplant. Dogs receiving mobilized stem cells all accepted their stem cell transplant and became tolerant to the VCA. However, 3 dogs developed graft-versus-host disease (GVHD) while 1 dog rejected its stem cell graft by week 15 but exhibited long-term tolerance towards its VCA (>90 weeks).

Address for Correspondence: David W. Mathes, MD, Mail Stop C309, 12631 East 17th Ave., Room 6619, Aurora, CO 80045, david.mathes@ucdenver.edu.

Authors' Contributions: D.W.M. designed the study, performed the surgeries, and co-wrote the manuscript. S.S.G. assisted in the design of the studies and cowrote the manuscript. J.C. assisted in the surgeries and co-wrote the manuscript. T.B.-M. performed chimerism analyses. G.E.S. evaluated the histopathologic samples. R.S. designed and supervised the study and revised the manuscript.

Disclosure: The authors declare no conflicts of interest.

Conclusion—The data suggest that simultaneous transplantation of mobilized stem cells and a VCA is feasible and leads to tolerance towards the VCA in a haploidentical setting. However, there is a higher rate of donor stem cell engraftment compared to marrow HCT and an increase in the incidence of GVHD.

INTRODUCTION

Outcomes from the clinical cases of hand and face transplantation demonstrate improved outcomes over conventional surgical techniques.^{1–7} Despite the benefits, the field of reconstructive transplantation, utilizing vascularized composite allograft (VCA) transplantation, is limited by the need for chronic immunosuppression. VCA transplant recipients have uniformly reported episodes of rejection despite immunosuppression.^{6–9} Increasing the amount of chronic immunosuppression in an attempt to prevent rejection leads to drug toxicity and ultimately can result in opportunistic infections, diabetes, nephrotoxicity, and even malignancy in the recipients.^{10,11} Efforts to limit the use of immunosuppression or to transplant donor marrow without preconditioning to prevent these complications has led to acute rejection and even loss of the allograft.^{12–15}

An alternative approach to immunosuppression is to induce immunological tolerance towards allogeneic tissue transplants through hematopoietic cell transplantation (HCT). Several animal models have been described that successfully utilize this approach. A variety of protocols employing HCT have been used to establish long-term tolerance towards kidney, liver, heart, and lung allografts.^{16,17} In an initial clinical trial, mixed hematopoietic cell chimerism was established to induce tolerance towards renal transplants in recipients with multiple myeloma.¹⁸ However, when this protocol was extended across greater genetic barriers the authors reported immunologic tolerance to the allografts in the setting of transient donor cell chimerism.

The use of HCT to induce tolerance towards VCA transplants has been less established. In murine models, marrow transplantation appears to induce tolerance towards a hindlimb transplant.^{19,20} In a large animal model, transplantation of a hindlimb in conjunction with either marrow or mobilized peripheral blood stem cells in swine induces tolerance to all components of the hindlimb except the overlying skin, a phenomenon termed “split-tolerance”.^{21,22} More recently this group has reported the successful induction of tolerance in haploidentical MGH miniature swine in 4 animals transplanted after the establishment of mixed chimerism and 3 animals transplanted simultaneously with the hematopoietic cell transplantation (HCT).²³ Researchers attempting to induce VCA transplantation tolerance in nonhuman primates were able to demonstrate prolonged survival of the VCA, but ultimately all recipients rejected their allografts.^{24,25}

Previously we have shown that stable mixed chimerism can be reliably induced in a dog leukocyte antigen (DLA)-identical transplant model.²⁶ Furthermore, we have shown that mixed hematopoietic cell chimeras go on to exhibit tolerance towards solid organ transplants such as lung, renal, and small bowel transplants.^{17,22,27} Translating these findings to VCA transplantation, we demonstrated that using marrow also allows for long-term tolerance (> 1

year) towards VCA transplants, regardless of whether HCT is administered months before or given the day of the VCA transplant.^{28,29}

The aim of the present study was to develop a HCT protocol suitable for inducing tolerance towards DLA-mismatched VCA. Here we demonstrate that tolerance in a DLA-mismatched setting can be achieved through a mixed chimeric approach using either marrow or G-CSF mobilized stem cells. Both approaches result in long-term tolerance towards VCA; however, stable donor engraftment and the incidence of GVHD is higher with mobilized stem cells than with marrow HCT.

MATERIALS AND METHODS

Experimental Animals

Random-bred litters of beagle/mini-mongrel cross-breeds were either raised at the Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, or purchased commercially. The dogs weighed from 7.2 to 20.9 kg (median 13.3) and were 18.7 to 82 (median 32) 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. Eight littermate donor/recipient pairs were DLA-haploidentical on the basis of identification of highly polymorphic major histocompatibility complex (MHC) class I and class II micro-satellite markers.³⁰ In addition, specific DLA DRB1 allelic identity was confirmed by direct sequencing.³¹

Nonmyeloablative Conditioning and Hematopoietic Cell Transplantation (HCT)

Our protocol for conditioning and HCT is shown in Figure 1. Conditioning for HCT consisted of 4.5 Gy total body irradiation (TBI) delivered at a rate of 7 cGy/min from a high-energy linear accelerator (Varian Clinac 6, Palo Alto, CA). Eight dogs received TBI followed by HCT consisting of either marrow or G-CSF mobilized stem cells collected earlier from a respective DLA-haploidentical littermate. Marrow or mobilized stem cells were infused intravenously within 4 hours of TBI. Marrow cells were given at doses of 3.2×10^7 to 3.1×10^8 (median 1.3×10^8) nucleated cells/kg. G-CSF mobilized-cells were given at doses of 7.18×10^6 to 2.64×10^9 (median 7.95×10^8) nucleated cells/kg. The day of HCT infusion was designated as day 0 for establishing mixed chimeric dogs. Postgrafting immunosuppression consisted of mycophenolate mofetil (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). All dogs were given standard postgrafting care.³² 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.^{33,34} 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 nonidentical. PCR products were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer.

Vascularized Composite Allograft (VCA) Transplantation

After receiving TBI and HCT, animals were anesthetized and underwent transplantation of a VCA. The surgical procedures for VCA transplantation have been previously described in detail.³⁵ Briefly, the allograft is derived from a myocutaneous rectus abdominus flap based on the deep and superficial inferior epigastric vessels and anastomosed to the recipient femoral vessels. 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, blinded to the source of the biopsies, evaluated presence or absence of rejection.

Skin Graft Placement

After 1 year of stable VCA engraftment, full thickness skin grafts were placed on recipients to determine immunocompetency and donor specific tolerance as previously reported.³⁰ Skin grafts were monitored daily via clinical examination for color of the skin and hair growth. Stable engraftment of the skin graft was determined by punch biopsies. End of study was either rejection of a skin graft or long-term tolerance over 100 days. Fixation and preparation of the skin is as previously mentioned in the previous VCA transplantation section.

Assessment of Donor Hair in VCA and Skin Grafts

To determine whether hair from the donor skin grafts were of donor origin, chimerism was performed on hair follicles extracted from the grafts. Two to 3 hair follicles with their bulbs were isolated and cut into several small pieces, each measuring approximately 3 to 4 millimeters. DNA extraction and PCR from the hair was performed using the Thermo Scientific Phire Animal Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer's directions. All PCR reactions were performed with a control and final products were run on a 2% agarose gel as confirmation before determining percent donor chimerism as described above.

In Vitro Immune Responses

Mixed leukocyte reactions (MLR) were done as described.³⁶ 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 medium (GibCo, Grand Island, NY), 45% Iscove modified Dulbecco 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 ³H-thymidine added on day 6.

RESULTS

Donor Cell Engraftment Outcomes After HCT Using Bone Marrow or G-CSF Mobilized Stem Cells

Table 1 and Figure 2 summarize the outcomes and trends of donor stem cell engraftment following HCT using either donor bone marrow or G-CSF mobilized stem cells. Four dogs were administered donor marrow and another 4 dogs were administered G-CSF mobilized stem cells. Overall, transplantation of donor marrow and mobilized stem cells were well tolerated. H046 became clinically jaundiced on week 13 post-transplant with significant elevation of its liver function tests and was treated for suspicion of liver GVHD. This progressed and at euthanasia, liver biopsies confirmed GVHD. H236 and H495 developed signs of skin GVHD, confirmed with skin biopsies, and were treated with immunosuppression. Because these 3 dogs received immunosuppression for GVHD treatment, we did not include any time points on the day of or after immunosuppression was started for our overall analysis. However, we did follow these particular animals out until euthanasia, which for the skin GVHD recipients was greater than 26 weeks in order to assess if there were any changes to the donor VCA (results not shown).

Other than GVHD, the only other complication was seen in H445, who after a few weeks postmarrow transplant developed recurrent canine herpes virus. This recurrence was likely due to the immunocompromised state secondary to the short course of postgraft immunosuppression. Of the 4 dogs receiving marrow, only H371 demonstrated full engraftment of its donor marrow by week 7 posttransplant whereas H445, H451, and H476 appeared to have initially engrafted, but by week 7, had completed rejection of their donor marrow grafts (Figure 2). Despite full and sustained donor chimerism, H371 did not develop any signs or symptoms of GVHD.

Three of the 4 dogs that received G-CSF mobilized stem cells demonstrated full donor stem cell engraftment. As mentioned earlier, these 3 dogs developed either liver or skin GVHD (Table 1). While we were able to treat the skin GVHD by administering a combination of cyclosporine, MMF, and steroids, we were unsuccessful in treating liver GVHD. Given the failure of treatment and continued rise in liver enzymes, H046 was euthanized. H269 was the only dog to receive mobilized stem cells and reject its hematopoietic graft. As illustrated in Figure 2, this dog, like the others, initially engrafted its mobilized stem cell graft, but by week 15 had 0% donor chimerism.

Outcome of Transplanted Donor VCAs After HCT Using Bone Marrow or G-CSF Mobilized Stem Cells

Table 1 summarizes the outcome of the transplanted VCAs after HCT using either donor bone marrow or mobilized stem cell transplant. Transplanted VCAs were initially accepted in all recipients. However, upon completion of 35 days of post-graft immunosuppression 3 of the 4 dogs that received donor bone marrow rejected their donor VCA. All 3 of these dogs had lost donor cell chimerism by week 7 before the cessation of cyclosporine. In contrast all of the animals that received mobilized stem cells remained tolerant, even H269 that lost detectable donor cells by week 15.

VCA rejection in nontolerant recipients was clinically determined by evidence of erythema, edema and eventual necrosis of the entire VCA (Figure 3). In contrast, tolerant recipients did not exhibit such clinical changes to their transplanted VCA. There did not appear to be evidence of erythema or edema, and the overlying skin appeared to be normal in color with normal hair growth. Hair follicles taken from the VCA were analyzed and determined to be from the respective donor, indicating that the donor VCA was not replaced by recipient tissue.

Histopathological Results of VCA Rejection and GVHD

Skin biopsies taken from nontolerant recipients showed a dense lymphocytic infiltrate with areas of either apoptosis or necrosis, consistent with Banff Grade III and IV as exemplified for dog H445 (Figure 4). The lymphocytic infiltration changes were also seen in deeper tissues such as the muscle. Skin and muscle biopsies sampled from another site on the recipient did not show evidence of dense infiltration, suggesting that rejection was site mediated and directed only to the donor VCA (data not shown). At the time of necropsy, we could not grossly identify obvious vessel thrombosis and, when possible, histology of donor-recipient artery and vein did not show evidence of clot or stenosis. Given the timeline of rejection, it is unlikely that VCA rejection seen was secondary to an acute vascular issue, although chronic rejection involving smaller vessels cannot be ruled out at this time.

Conversely, skin and muscle biopsies taken from tolerant animals show rare to mild perivascular cellular infiltrate with no evidence of apoptosis or necrosis, consistent with Banff Grade 0 and I. When compared side by side, no apparent histological differences were seen between tolerant recipient that received bone marrow (H371) or the recipients that received G-CSF- mobilized stem cell grafts. No histopathological differences were noted after comparing H269, the recipient who rejected a G-CSF mobilized stem cell graft, to the other recipients that attained 100% donor chimerism (H046, H236 and H371).

Donor Specific Tolerance Assayed by Skin Graft Placement and MLRs

To assess whether tolerance observed was donor specific and not secondary to lack of immunocompetency, skin grafts were placed on tolerant recipients. Of the 5 tolerant recipients, 3 recipients were excluded because they had GVHD and were treated promptly with immunosuppression. Of the remaining 2 recipients, H269 and H371, both demonstrated donor-specific tolerance by rejecting third party skin grafts within a week of initial placement and tolerating their respective donor skin grafts greater than 100 days. Histology after 100 days shows minimal infiltrate without evidence of apoptosis or necrosis (Figure 5.).

To confirm that acceptance of the VCA was due to tolerance and not due to in-growth of host skin, we obtained hair from well healed donor skin grafts and determined that it was of donor origin. Additionally we performed MLRs on these 2 recipients demonstrating high proliferative responses to third party donors, but a much lower response to their haploidentical donors (SDC Figure S1.).

DISCUSSION

In our previous study we demonstrated that in a DLA-identical setting, using a nonmyeloablative conditioning regimen consisting of 2 Gy TBI and donor bone marrow transplantation, stable mixed chimeras could be created that went on to accept subsequent VCA transplants from the same donor without additional therapy.²⁸ We extended these findings into a more clinically relevant scenario by simultaneously transplanting both donor bone marrow and VCA.²⁹ This experiment offered proof of principle that tolerance can be established across a minor antigen mismatched DLA-identical barrier in a single-day protocol.

Based on these findings, we modified our protocol to perform transplants across greater genetic mismatch using haploidentical dogs. Here we demonstrate that long-term donor specific tolerance towards haploidentical VCA transplants can be achieved after HCT using either bone marrow or G-CSF mobilized stem cells. The group receiving mobilized stem cells had a higher engraftment rate than those receiving bone marrow but also an increased incidence of GVHD. The reason for the differences in engraftment and rates of GVHD has been previously attributed to both the quantity of cells infused and the difference in the T cell populations in the hematopoietic graft.³⁷ A similar finding was reported in a swine model of HCT and VCA transplantation that used both bone marrow and mobilized stem cells. Studies have demonstrated that use of G-CSF mobilized stem cells results achievement of complete donor chimerism but and long-term survival of the hematopoietic graft due to higher numbers of CD14+ CD34+ cells and CD8+ CD34+ cells.^{37,38} Although we did not delineate specific T cell populations in our hematopoietic grafts before infusion, the difference we observed in engraftment rates between marrow and mobilized stem cells in this study appear to confirm those previous findings.

Four of the 5 recipients expressing long-term tolerance towards their donor VCAs demonstrated full donor cell engraftment. However, 1 tolerant animal (H269) appears to have rejected its donor cell graft while maintaining tolerance to the VCA. This dog lost all evidence of donor cell chimerism by week 15 post-transplantation. This same finding was observed in 1 VCA recipient and all kidney graft recipients in our previous studies in the DLA-identical model.^{29,39} The maintenance of tolerance without detectable donor cell chimerism suggests that donor stem engraftment may not be required for long-term tolerance towards a VCA. Previous studies have postulated different phases of tolerance^{40,41} and our findings support their hypothesis that there might be different mechanisms involved in induction and maintenance of tolerance. The induction of tolerance appears to require the initial engraftment of donor stem cells as evidenced by the high rejection rate of the transplants in the bone marrow group where very poor initial engraftment was noted in 3 of the 4 dogs transplanted. In our matched model we were able to demonstrate a significant elevation of FoxP3+ T cells in the skin and muscle of the transplant.

There have been few reports of tolerance to VCA in large animal models across a class II mismatch. In addition, the majority of these studies required significant preconditioning that would not be possible in a cadaveric VCA transplant. Initial studies in a swine model that used donor marrow or mobilized stem cells with T cell depletion and thymic irradiation and

a VCA transplant yielded “split-tolerance,” that is tolerance only towards certain components of a VCA and rejection directed towards the skin.⁴² Leonard et al recently reported that a protocol infusing mobilized hematopoietic stem cells (collected from peripheral blood by apheresis over 3 days) combined with TBI, antibody mediated T-depletion and postgrafting immunosuppression lead to both multi-lineage mixed chimerism and tolerance to VCA transplanted in 3 haploidentical MGH miniature swine. In contrast to our study where all elements of the transplant (HCT, TBI and VCA) are performed at day 0, in this study T cell depletion was given on days -4 to -1, TBI was performed at day -2, mobilized cells were given at day 0 and day 1 and the VCA transplant was performed on day 3. This protocol also yielded stable mixed chimerism which we did not observe in our model.

In our study we did not observe “split-tolerance” as all components of the VCA were tolerated. We did confirm their results that animals receiving mobilized stem cells had a higher likelihood of fully engrafting and developing GVHD. Other studies in mismatched swine studies using TBI and mesenchymal stem cell transplantation demonstrate prolongation of VCA survival in 2 of 3 pigs up to 120 days; however, further follow-up was not provided and it is unknown whether the VCAs underwent rejection.⁴³ Leonard et al reported the successful induction of tolerance in haploidentical MGH miniature swine in 4 animals transplanted after the establishment of mixed chimerism and 3 animals transplanted simultaneously with the hematopoietic cell transplantation (HCT).²³ However, their protocol require a significant amount of preconditioning and even the in “simultaneous” model the VCA transplant was performed 56 h after first infusion of donor hematopoietic cells.

Two major limitations to our present study is the risk of GVHD accompanying our transplants and the use of G-CSF to mobilize donor stem cells into the periphery. Our central goal is to develop a protocol without the need for preconditioning of the donor so that it can apply to cadaveric transplantation. Our study demonstrates that although long-term tolerance towards a VCA is achieved using mobilized stem cells, the incidence of GVHD is high. The possibility to establish tolerance without the need for long-term engraftment of the donor cells would eliminate this risk. In a protocol designed for human kidney transplantation, those patients only had detectable donor cell chimerism for less than 2 weeks but were rendered tolerant to the allograft.⁴⁴ Our previous work suggested that tolerance is maintained by recipient T-regulatory cells found predominately in the allograft, surrounding lymph nodes and thymus.²⁹

The feasibility of using G-CSF has been reported^{45,46} and currently is being utilized clinically in order to collect donor stem cells in the periphery. In our current model, we administer the G-CSF to the donor a week before collection. However, we recognize that this would not be feasible in the scenario of cadaveric transplantation. In the clinical scenario, we plan to use AMD3100 (Perixafor) instead of G-CSF, as studies have shown similar collection results in terms of quantity and cell populations with the major difference being that AMD3100 mobilizes stem cells into the peripheral blood in a few hours compared to a week when using G-CSF.⁴⁷ Previous work using AMD3100 in canines has demonstrated proof of principle and the drug appears capable of inducing early engraftment even across mismatched barriers.^{48,49} We used G-CSF in our present study because of its

lower cost, ease of administration, and our previous experience in the canine model. Given that our results demonstrate that mobilized stem cells are capable of inducing VCA tolerance, our more recent work has been performed using AMD3100 instead of G-CSF to mimic a more clinically relevant protocol.

In this study, we have demonstrated that tolerance towards VCA transplants across a haploidentical barrier is possible using either bone marrow or G-CSF mobilized stem cells with up to a year follow up. The use of bone marrow does not appear to reliably induce tolerance, whereas the use of mobilized stem cells does but this comes with a higher incidence of GVHD. One of the tolerant recipients rejected its hematopoietic cell graft and lost all evidence of donor cell chimerism but remained tolerant to its VCA, whereas other nontolerant recipients rejected both the hematopoietic cell grafts and VCAs. This disparity likely is due to the time of transient engraftment, with a longer transient engraftment appearing to induce long-term tolerance. This observation highlights the importance of different stages and mechanisms of induction versus maintenance of tolerance and suggests that donor cell engraftment is required only during the initial induction phase but not the later maintenance phase. If so, future strategies could be developed to eliminate donor cell engraftment after a specified period of time required for tolerance, thereby eliminating the risk of GVHD in hand and face transplantation after mixed chimerism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Michele Spector, DVM, Alix Joslyn, and the research technicians in the Comparative Medicine facilities for assistance with animal care; Drs. George Georges, Bruce Swearingen, Zejing Wang, Maura Parker, and Steven Rosinski for weekend dog care; Helen Crawford and Bonnie Larson for manuscript preparation.

Funding: D.W.M. received support from the National Endowment for Plastic Surgery Grant from the Plastic Surgery Foundation. Additional funding was obtained from the National Institutes of Health, Bethesda MD, under award numbers P01CA078902 and P30CA015704 and from the Josef Steiner Krebsstiftung, Bern, Switzerland (R.S.). This study was neither prepared nor funded in any part by a commercial organization, including educational grants. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ABBREVIATIONS

VCA	vascularized composite allograft
G-CSF	granulocyte colony stimulating factor
GVHD	graft versus host disease
FHCRC	Fred Hutchinson Cancer Research Center
DLA	dog leukocyte antigen
MHC	major histocompatibility complex
HCT	hematopoietic cell transplantation

TBI	total body irradiation
MLR	mixed leukocyte reactions
MMF	mycophenolate mofetil
I.V	intravenous
B.I.D	twice daily
S.C	subcutaneous

References

1. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation. 2010; 90(12):1590–1594. [PubMed: 21052038]
2. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation. 2008; 86(4):487–492. [PubMed: 18724213]
3. Ravindra KV, Buell JF, Kaufman CL, et al. Hand transplantation in the United States: experience with 3 patients. Surgery. 2008; 144(4):638–643. [PubMed: 18847649]
4. Schneeberger S, Gorantla VS, Brandacher G, et al. Upper-extremity transplantation using a cell-based protocol to minimize immunosuppression. Ann Surg. 2013; 257(2):345–351. [PubMed: 23001085]
5. Siemionow M, Papay F, Alam D, et al. Near-total human face transplantation for a severely disfigured patient in the USA (Review). Lancet. 2009; 374(9685):203–209. [PubMed: 19608265]
6. Pomahac B, Bueno EM, Sisk GC, Pribaz JJ. Current principles of facial allotransplantation: the Brigham and Women's Hospital experience. Plast Reconstr Surg. 2013; 131(5):1069–1076. [PubMed: 23629089]
7. Lantieri L, Hivelin M, Audard V, et al. Feasibility, reproducibility, risks and benefits of face transplantation: a prospective study of outcomes. Am J Transplant. 2011; 11(2):367–378. [PubMed: 21272240]
8. Dorafshar AH, Bojovic B, Christy MR, et al. Total face, double jaw, and tongue transplantation: an evolutionary concept. Plast Reconstr Surg. 2013; 131(2):241–251. [PubMed: 23076416]
9. Schneeberger S, Kreczy A, Brandacher G, Steurer W, Margreiter R. Steroid- and ATG-resistant rejection after double forearm transplantation responds to Campath-1H. Am J Transplant. 2004; 4(8):1372–1374. [PubMed: 15268743]
10. Lantieri L. Face transplant: a paradigm change in facial reconstruction. J Craniofac Surg. 2012; 23(1):250–253. [PubMed: 22337419]
11. Pomahac B, Gobble RM, Schneeberger S. Facial and hand allotransplantation (Review). Cold Spring Harb Perspect Med. 2014; 4(3)
12. Chang J, Mathes DW. Ethical, financial, and policy considerations in hand transplantation (Review). Hand Clinics. 2011; 27(4):553–560. [PubMed: 22051396]
13. Chang J, Davis CL, Mathes DW. The impact of current immunosuppression strategies in renal transplantation on the field of reconstructive transplantation (Review). J Reconstr Microsurg. 2012; 28(1):19. Jul.
14. Kaufman CL, Ouseph R, Blair B, et al. Graft vasculopathy in clinical hand transplantation. Am J Transplant. 2012; 12(4):1004–1016. [PubMed: 22325051]
15. Kanitakis J, Petruzzo P, Badet L, et al. Chronic rejection in human vascularized composite allotransplantation (hand and face recipients): an update. Transplantation. 2016
16. Kawai T, Cosimi AB, Colvin RB, et al. Mixed allogeneic chimerism and renal allograft tolerance in Cynomolgus monkeys. Transplantation. 1995; 59(2):256–262. [PubMed: 7839449]
17. Nash RA, Yunusov M, Abrams K, et al. Immunomodulatory effects of mixed hematopoietic chimerism: immune tolerance in canine model of lung transplantation. Am J Transplant. 2009; 9(5):1037–1047. [PubMed: 19422333]

18. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008; 358(4):353–361. [PubMed: 18216355]
19. Foster RD, Fan L, Neipp M, et al. Donor-specific tolerance induction in composite tissue allografts.[Erratum appears in *Am J Surg*. 1999 Apr.177(6):529]. *Am J Transplant*. 1998; 176(5): 418–421.
20. Siemionow MZ, Izycki DM, Zielinski M. Donor-specific tolerance in fully major histocompatibility major histocompatibility complex-mismatched limb allograft transplants under an anti-alphabeta T-cell receptor monoclonal antibody and cyclosporine A protocol. *Transplantation*. 2003; 76(12):1662–1668. [PubMed: 14688512]
21. Mathes DW, Randolph MA, Solari MG, et al. Split tolerance to a composite tissue allograft in a swine model. *Transplantation*. 2003; 75(1):25–31. [PubMed: 12544866]
22. Kuhr CS, Yunusov M, Sale G, Loretz C, Storb R. Long-term tolerance to kidney allografts in a preclinical canine model. *Transplantation*. 2007; 84(4):545–547. [PubMed: 17713441]
23. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. *Am J transplant*. 2014; 14(2):343–355. [PubMed: 24405666]
24. Barth RN, Nam AJ, Stanwix MG, et al. Prolonged survival of composite facial allografts in non-human primates associated with posttransplant lymphoproliferative disorder. *Transplantation*. 2009; 88(11):1242–1250. [PubMed: 19996923]
25. Barth RN, Rodriguez ED, Mundinger GS, et al. Vascularized bone marrow-based immunosuppression inhibits rejection of vascularized composite allografts in nonhuman primates. *Am J Transplant*. 2011; 11(7):1407–1416. [PubMed: 21668624]
26. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood*. 1997; 89(8):3048–3054. [PubMed: 9108426]
27. Kuhr CS, Allen MD, Junghanss C, et al. Tolerance to vascularized kidney grafts in canine mixed hematopoietic chimeras. *Transplantation*. 2002; 73(9):1487–1493. [PubMed: 12023629]
28. Mathes DW, Hwang B, Graves SS, et al. Tolerance to vascularized composite allografts in canine mixed hematopoietic chimeras. *Transplantation*. 2011; 92(12):1301–1308. [PubMed: 22082819]
29. Mathes DW, Chang J, Hwang B, et al. Simultaneous transplantation of hematopoietic stem cells and a vascularized composite allograft leads to tolerance. *Transplantation*. 2014; 98(2):131–138. [PubMed: 24918616]
30. Wagner JL, Burnett RC, Storb R. Molecular analysis of the DLA DR region. *Tissue Antigens*. 1996; 48(5):549–553. [PubMed: 8988537]
31. Wagner JL, Hayes-Lattin B, Works JD, Storb R. Molecular analysis and polymorphism of the DLA-DQB genes. *Tissue Antigens*. 1998; 52:242–250. [PubMed: 9802604]
32. Ladiges WC, Storb R, Thomas ED. Canine models of bone marrow transplantation. *Lab Anim Sci*. 1990; 40(1):15. Nov.
33. Yu C, Ostrander E, Bryant E, Burnett R, Storb R. Use of (CA) n polymorphisms to determine the origin of blood cells after allogeneic canine marrow grafting. *Transplantation*. 1994; 58(6):701–706. [PubMed: 7940690]
34. Graves SS, Hogan WJ, Kuhr C, et al. Adoptive immunotherapy against allogeneic kidney grafts in dogs with stable hematopoietic trichimerism. *Biol Blood Marrow Transplant*. 2008; 14:1201–1208. [PubMed: 18940673]
35. Mathes DW, Noland M, Graves S, Schlenker R, Miwongtum T, Storb R. A preclinical canine model for composite tissue transplantation. *J Reconstr Microsurg*. 2010; 26(3):201–207. [PubMed: 20108180]
36. Raff RF, Deeg HJ, Farewell VT, DeRose S, Storb R. The canine major histocompatibility complex. Population study of DLA-D alleles using a panel of homozygous typing cells. *Tissue Antigens*. 1983; 21(5):360–373. [PubMed: 6223409]
37. Bensinger WI, Clift RA, Anasetti C, et al. Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor. *Stem Cells*. 1996; 14:90–105. [PubMed: 8820955]

38. Panse JP, Bastianelli C, Santos EB, et al. Dog leukocyte antigen nonidentical unrelated canine marrow grafts: enhancement of engraftment by both CD4 and CD8 T cells. *Transplantation*. 2003; 76(3):474–480. [PubMed: 12923431]
39. Graves SS, Mathes DW, Georges GE, et al. Long-term tolerance to kidney allografts after induced rejection of donor hematopoietic chimerism in a preclinical canine model. *Transplantation*. 2012; 94(6):562–568. [PubMed: 22929594]
40. Walsh PT, Taylor DK, Turka LA. Tregs and transplantation tolerance (Review). *J Clin Invest*. 2004; 114(10):1398–1403. [PubMed: 15545987]
41. Yamazaki M, Pearson T, Brehm MA, et al. Different mechanisms control peripheral and central tolerance in hematopoietic chimeric mice. *Am J Transplant*. 2007; 7(7):1710–1721. [PubMed: 17564635]
42. Hettiarachy S, Melendy E, Randolph MA, et al. Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine. *Transplantation*. 2004; 77(4):514–521. [PubMed: 15084927]
43. Kuo YR, Chen CC, Shih HS, et al. Prolongation of composite tissue allotransplant survival by treatment with bone marrow mesenchymal stem cells is correlated with T-cell regulation in a swine hind-limb model. *Plast Reconstr Surg*. 2011; 127(2):569–579. [PubMed: 21285761]
44. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008; 358(4):353–361. [PubMed: 18216355]
45. Dreger P, Haferlach T, Eckstein V, et al. G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: safety, kinetics of mobilization, and composition of the graft. *British Journal of Haematology*. 1994; 87(3):609–613. [PubMed: 7527648]
46. Theilgaard-Mönch K, Raaschou-Jensen K, Andersen H, et al. Single leukapheresis products collected from healthy donors after the administration of granulocyte colony-stimulating factor contain ten-fold higher numbers of long-term reconstituting hematopoietic progenitor cells than conventional bone marrow allografts. *Bone Marrow Transplant*. 1999; 23(3):243–249. [PubMed: 10084255]
47. Flomenberg N, Devine SM, DiPersio JF, et al. The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood*. 2005; 106(5):1867–1874. [PubMed: 15890685]
48. Burroughs L, Mielcarek M, Little MT, et al. Durable engraftment of AMD3100-mobilized autologous and allogeneic peripheral blood mononuclear cells in a canine transplantation model. *Blood*. 2005; 106(12):4002–4008. [PubMed: 16105977]
49. Thakar MS, Santos EB, Fricker S, Bridger G, Storb R, Sandmaier BM. Plerixafor-mobilized stem cells alone are capable of inducing early engraftment across the MHC-haploidentical canine barrier (Letter to the Editor). *Blood*. 2010; 115(4):916–917. [PubMed: 20110438]

Figure 1. Protocol for haploidentical tolerance induction using donor bone marrow or G-CSF mobilized stem cells

All recipients receive 4.5 Gy TBI, a HCT consisting of either bone marrow or mobilized stem cells, and a VCA transplant on the same day (designated Day 0). A short course of postgraft immunosuppression of CSP and MMF is administered to prevent GVHD. After 35 days, all immunosuppression is withdrawn and the VCA is monitored clinically and histologically for signs of rejection.

BONE MARROW GROUP

G-CSF GROUP

Figure 2. Donor cell chimerism trends after donor bone marrow or G-CSF mobilized stem cell transplantation

Chimerism was measured in the peripheral blood in both the granulocyte and lymphocyte population. The top graphs depict the trend of donor cell chimerism in all 4 recipients shortly after donor bone marrow and VCA transplantation whereas the bottom graphs depict the trend after transplantation of donor G-CSF mobilized stem cells and VCA.

Figure 3. Clinical findings of VCA rejection and tolerance

Shown here are representative pictures taken from 3 dogs. Rejection in a nontolerant recipient (H476), showing signs of graft erythema and surrounding edema (POD 45: **A**) and ultimately necrosis (POD 72: **B**). In contrast, long-term tolerance of the VCA is seen clinically without evidence of erythema and edema in a recipient receiving donor bone marrow (H371 POD 220: **C**) and in a recipient receiving donor G-CSF mobilized stem cells (H269 POD 357: **D**).

Figure 4. Histology of transplanted skin and muscle of VCAs taken from nontolerant and tolerant recipients

Biopsies taken from a nontolerant recipient (H445, POD 49) shows dense lymphocytic infiltrates in skin and muscle. Biopsies taken from tolerant recipients who developed liver GVHD (H046 POD 91) and skin GVHD (H236 POD 103) show normal skin and muscle. Similarly, biopsies taken from a tolerant recipient not developing GVHD (H371 POD 301) also show normal skin and muscle histology. Biopsies taken from a tolerant recipient who rejected only its HCT (H269, POD 356) again shows normal skin and muscle. All pictures taken at 200× magnification.

Figure 5.

A) Histology of autologous skin graft demonstrating normal skin (H269). **B)** Histology from the donor skin graft demonstrating normal skin (H269). **C)** Histology from a third party skin graft complete rejection of the allograft (H269). **D)** Histologic section from an autologous skin graft stained for foxP3 demonstrating few foxP3 positive cells (H269). **E)** Histologic section from the donor derived skin graft stained for foxP3 showing many foxp3 positive cells suggesting an infiltrate of regulatory T cells (H269).

Table 1

Summary of all 8 canine recipients, transplanted cell doses, donor cell chimerism, outcomes of the transplanted VCAs, and any complications.^a

Recipient	Cell Dose (cells/kg)	Source of Stem Cells	Last Measured % Donor Chimerism (weeks post-op) ^b	VCA Rejection (weeks post-op)	Weeks F/U	Complications
H371	3.64×10^7	Bone Marrow	G: 100% (43) L: 100% (43)	No	52	None
H445	1.43×10^8	Bone Marrow	G: 2.2% (18) L: 2.3% (18)	Yes (6.5)	19	Canine Herpes Virus ^c
H451	3.10×10^8	Bone Marrow	G: 92% (4) L: 42% (4)	Yes (5.5)	20	None ^c
H476	3.2×10^7	Bone Marrow	G: 90% (6) L: 2.5% (6)	Yes (7.1)	10	None ^c
H269	2.64×10^9	G-CSF	G: 0.7% (20) L: 0.8% (20)	No	94 ^d	None
H046	7.18×10^6	G-CSF	G: 100% (13) L: 100% (13)	No	13	Liver GVHD ^c
H236	4.35×10^8	G-CSF	G: 100% (22) L: 100% (22)	No	39	Skin GVHD ^c
H495	1.00×10^8	G-CSF	G: 100% (19) L: 100% (19)	No	14	Skin GVHD

^a As illustrated in this table, 4 recipients received bone marrow and the other 4 received G-CSF mobilized stem cells. All time points are represented in weeks following initial transplantation of HCT and VCA.

^b Data are presented as percent donor granulocytes (G) and lymphocytes (L).

^c Euthanized.

^d H269 lost donor chimerism at 10 weeks for granulocytes and 15 weeks for mononuclear cells