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Simultaneous bone marrow and composite tissue transplantation in rats treated with nonmyeloablative conditioning promotes tolerance¹

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Abstract

Background—Approaches to safely induce tolerance in vascularized composite allotransplantation (VCA) with chimerism through bone marrow transplantation (BMT) are currently being pursued. However, the VCA were historically performed sequentially after donor chimerism was established. Delayed VCA is not clinically applicable due to the time constraints associated with procurement from deceased donors. A more clinically relevant approach to perform both the BMT and VCA simultaneously was evaluated.

Methods—WF (RT1A^u) rats were treated with a short course of immunosuppressive therapy (anti- $\alpha\beta$ -TCR mAb, FK-506, and anti-lymphocyte serum). One day prior to BMT, rats were treated with varying doses of total body irradiation (TBI) followed by transplantation of heterotopic osteomyocutaneous flaps from hind limbs of ACI (RT1A^{abl}) rats.

Results—80% of rats conditioned with 300 cGy TBI and 40% of rats receiving 400 cGy TBI accepted the VCA. Mixed chimerism was detected in peripheral blood at one month post-VCA, but chimerism was lost in all transplant recipients by 4 months. The majority of peripheral donor cells originated from the BMT and not the VCA. Acceptors of VCA were tolerant of a donor skin graft challenge and no anti-donor antibodies were detectable, suggesting a central deletional mechanism for tolerance. Regulatory T cells (T_{reg}) from spleens of acceptors more potently suppressed lymphocyte proliferation than T_{reg} from rejectors in the presence of donor stimulator cells.

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This work is dedicated to the memory of Michael Tanner.

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Conclusions—These studies suggest that simultaneous BMT and VCA may establish indefinite allograft survival in rats through T_{reg}-mediated suppression and thymic deletion of alloreactive T cells.

Keywords

tolerance; vascularized composite allotransplantation; mixed chimerism; non-myeloablative conditioning; T regulatory cell

Introduction

Vascularized composite allotransplantation (VCA) refers to the transplantation of complex primarily vascularized tissues of ectodermal and mesodermal origin (1), including skin, muscle, nerve, bone, tendon, and other tissues. VCA is emerging as a potential treatment for complex tissue defects (1–3). The feasibility of the procedure has been confirmed through over 50 hand transplants, 10 facial reconstructions, and vascularized knee, esophageal, and tracheal allografts around the world to date (3,4). However, a major factor limiting VCA is the requirement for lifelong immunosuppression and the associated toxicities (5). Virtually all expected complications associated with the use of chronic immunosuppression, including renal failure, have occurred now in recipients of VCA (6). The ideal outcome to achieve permanent VCA survival would be induction of donor-specific tolerance, thereby abrogating the need for chronic immunosuppression. Experimental studies have shown that the establishment of donor-specific chimerism in organ transplant recipients can lead to donor-specific tolerance (7–10). The clinical relevance of mixed chimerism has recently been underscored by the results of a prospective clinical pilot trial in which patients became tolerant after receiving kidney and bone marrow transplantation (BMT) from the same donor (11–13).

It is hypothesized that VCA may facilitate the induction and/or maintenance of chimerism since the transplanted part includes a potential niche for bone marrow (BM) stem cells. However, conditioning of recipients to establish chimerism by BMT requires the use of myelotoxic drugs and total body irradiation (TBI) which are associated with significant toxicities. The challenge remains to develop nonmyeloablative conditioning regimens that allow maximal BM engraftment with minimal recipient toxicity. We previously reported that mixed chimerism can be established in an allogeneic rat model by nonmyeloablative conditioning with TBI as low as 300 cGy (9). This model has now been extended to assess tolerance to CTA.

Approaches to induce a tolerogenic state in transplantation often target peripheral and/or central tolerance mechanisms (14–16). Central deletional mechanisms have been demonstrated to be the major mechanism in tolerance induction and maintenance through mixed chimerism. However, tolerance has frequently been associated with suppression of alloreactive T cells by peripheral mechanisms in allograft recipients. Recent studies have shown that CD4⁺/CD25⁺/FoxP3⁺ regulatory T cells (T_{reg}) play an important role in maintaining peripheral tolerance in transplant recipients by controlling alloreactive immune responses through indirect antigen recognition (18–20). T_{reg} also have the potential to influence central tolerance through indirect presentation of donor T_{reg}-derived alloantigens in the thymus and subsequent negative selection of alloreactive T cells, which has been shown to promote limited skin graft acceptance in mice (21). T_{reg} frequencies are higher in BMT compared to peripheral blood (PB) stem cell transplants, which could reduce the overall alloreactivity of the graft and promote a tolerogenic state (22).

We previously used a sequential transplantation model to study VCA in nonmyeloablatively conditioned rats receiving BMT first in order to establish chimerism prior to VCA (9). Although prolongation of the MHC-disparate transplanted tissues were achieved in this study using a short course of immunosuppressive agents and systemic monoclonal antibodies (mAb), a more clinically relevant model would involve performing hematopoietic stem cell transplantation (HSCT) and VCA simultaneously. Our objectives in the present study were to establish a clinically relevant simultaneous BMT and VCA model in rats treated with a nonmyeloablative conditioning regimen, to determine whether tolerance and donor chimerism were maintained over time in graft acceptors, and to examine the role of T_{reg} in maintenance of a tolerogenic state.

Results

Simultaneous BMT with VCA promotes acceptance of VCA

WF rats were conditioned with 100–400 cGy TBI one day prior to BMT and treated with anti- $\alpha\beta$ TCR mAb on day –3, FK-506 on days –1 to +9, and a single dose of anti-rat lymphocyte serum (ALS) on day +9 (Fig. 1). ACI flaps were transplanted into the right gluteal region of WF rats one day prior to BMT. The 5 control rats that received VCA without prior irradiation (0 cGy TBI) rejected their VCA within 25 days. Rejection occurred within 100 days in all animals receiving 100 cGy TBI, and 4 of 5 animals receiving 200 cGy TBI rejected their VCA within 125 days (Fig. 2A). Long-term VCA acceptance (>200 days post-transplant) was observed in 80% of animals receiving 300 cGy TBI and 40% of animals receiving 400 cGy TBI. The reduction in graft survival rate for recipients receiving 400 cGy TBI versus 300 cGy TBI may be the result of a small sample size rather than a direct effect. No clinical manifestations of GVHD were observed in any of the VCA recipients up to 6 months post-transplant.

None of the recipients conditioned with 0 or 100 cGy TBI engrafted with donor cells. 1 of 5 rats treated with 200 cGy TBI had > 1% donor cells in lymphocyte gate of peripheral blood (PB) and accepted their VCA. 5 out of 5 or 4 out of 5 were engrafted in 300 or 400 cGy TBI treatment groups, respectively. As no correlation between the levels of donor chimerism and the graft survival was found according to the TBI doses, recipients were divided into 2 groups as acceptors or rejectors according to the status of VCA acceptance or rejection regardless of the TBI doses received. With simultaneous BMT-VCA, there was no significant difference between the donor chimerism levels of acceptors and rejectors at one month post-transplant with $15.6 \pm 10.4\%$ and $16.3 \pm 8.5\%$, respectively (Fig. 2B), without an obvious reason. However, there was a significant difference between donor chimerism levels at 2 months ($5.6 \pm 0.02\%$ vs. $0.5 \pm 0.003\%$; $P = 0.05$). Donor chimerism was lost in the PB of all chimeras that received simultaneous BMT-VCA by four months post-BMT.

Peripheral donor chimerism originates from the BMT—In this simultaneous VCA/BMT model, the donor cells in the engrafted recipients could originate infused BMC or vascularized bones in VCA. To define the source of circulating donor cells in mixed chimeras, twelve ACI rats were treated with the same immunosuppressive therapy as described above with 300 cGy TBI. WF eGFP rats were used as the source of BM or the VCA so that donor cells could be easily detected by flow cytometry. ACI rats received that eGFP⁺ BMC plus eGFP[–] VCA showed all the donor cells circulating in PB were eGFP⁺ but not eGFP[–] cells at 1, 2, and 3 months post-transplant (Fig. 2C). Furthermore, no eGFP⁺ cells were detected in ACI rats received eGFP[–] BMC plus eGFP⁺ VCA and all the donor cells circulating in PB were eGFP[–]. The difference in donor chimerism at 1, 2, and 3 months between donor BMC from GFP⁺ rats and GFP[–] rats is most likely due to the variation

between experiments. These data suggest that engrafted donor cells come from the BMT but not the VCA.

Secondary antigenic challenge does not break tolerance in VCA acceptors—

There is no method available in rats to directly detect the central deletion of alloreactive T cells, secondary antigenic challenge by skin graft was used to assess the robustness of transplantation tolerance in a chimera that accepted the first VCA graft, since the acceptance of a highly antigenic second graft strongly suggests the existence of central deletional tolerance (25,26). Second-set donor skin grafts were placed at one year post-transplant on four VCA acceptors and six rejectors. Tolerance to skin grafts was maintained in VCA acceptors throughout 120 days follow-up period (Fig. 3A), and no changes in the VCA of acceptors were observed during the secondary challenge. The rejectors rejected the skin grafts promptly within 18 days. Sera from all six VCA rejectors scored positive for anti-donor antibodies in a flow cytometric crossmatch (FCXM) assay, but no sera samples taken from VCA acceptors had circulating anti-donor antibodies (Fig. 3B).

There is no significant difference between the numbers of T_{reg} in tissues of VCA acceptors compared to rejectors— $CD4^+/CD25^+/FoxP3^+$ T_{reg} play an important role in maintaining peripheral tolerance to transplants and may also indirectly influence central tolerance (18,19,21). To determine if the cell numbers of this important tolerogenic mediator were greater in VCA acceptors compared to rejectors, we examined tissues in 3 VCA acceptors and 3 VCA rejectors for $CD4^+/CD25^+/FoxP3^+$ T_{reg} . There was no significant difference between the percentages (Fig. 4A) or absolute numbers (Fig. 4B) of T_{reg} measured in the hematolymphopoietic compartments of acceptors or rejectors. These data suggest that the determining factor in tolerance induction may be T_{reg} immunomodulatory function rather than absolute cell numbers. The majority of T_{reg} were found in the mesenteric lymph node, spleen, and thymus for both acceptors and rejectors, and a lesser percentage of T_{reg} were found in the donor and recipient BM of VCA acceptors (Fig. 4A).

T_{reg} from VCA acceptors more potently suppress lymphocyte proliferation to donor alloantigen than T_{reg} from VCA rejectors—To further study the function of T_{reg} , a MLR suppressor cell assay was used. We incubated irradiated splenic stimulator cells (either donor ACI cells or F344 third-party cells) with naïve WF responder lymph node cells and sorted $CD8^-/CD4^+/CD25^+$ splenic T_{reg} from VCA recipients. Lymphocyte proliferation was measured in samples containing a 1:1 or a 1:0.25 ratio of naïve WF responder cells to T_{reg} (Fig. 4C and D). Splenic T_{reg} from VCA acceptors significantly suppressed lymphocyte proliferation in the presence of donor (ACI) antigen at cell numbers that were equivalent to or even 4-fold less than the number of naïve WF responder cells (Fig. 4C, black bars; $P<0.05$). T_{reg} from VCA rejectors suppressed lymphocyte proliferation in the presence of donor antigen, but this effect was lost when the concentration of T_{reg} was reduced (Fig. 4D). These data suggest that T_{reg} from either acceptor or rejector are functional and maintain their suppressive capabilities. When the effect of suppression reaches the maximal at a certain ratio of responder and T_{reg} , the suppressive effect will remain the same if the number or ratio of T_{reg} increases. That is why almost equal suppression was observed at the 1:1 ratio in both T_{reg} from rejectors and acceptors. The T_{reg} from acceptors still maintained the suppressive effect when fewer T_{reg} were used, but T_{reg} from rejectors did not, suggesting T_{reg} from VCA acceptors have more suppressive capability to donor alloantigen compared with rejector T_{reg} . It is also of interest to note that T_{reg} from both acceptors and rejectors suppressed lymphocyte proliferation much less in the presence of third-party (F344) antigen compared with the donor antigen, suggesting that donor-specific T_{reg} were present in the spleens of rejectors and acceptors.

Discussion

VCA, including hand and face transplantation, significantly improves the quality of life of recipients. However, the toxicities caused by the lifelong use of immunosuppressive drugs have raised ethical concerns for these procedures. A long-term goal in VCA as well as solid organ transplantation would be to induce donor-specific transplantation tolerance to avoid the chronic use of immunosuppressive drugs. Mixed chimerism has been demonstrated to be the most clinically relevant procedure to induce donor-specific tolerance to transplanted tissues and organs (8,11). Mixed chimerism has been shown in rats to induce donor-specific tolerance to the highly antigenic composite tissue allografts (9,24,27,28). These studies demonstrated that chimerism established with a nonmyeloablative conditioning approach could promote VCA tolerance.

However, the VCA were historically performed sequentially with a more than 1 month delay after BMT to establish donor chimerism. The delayed approach for VCA is not applicable in the case of a deceased donor. The clinically relevant VCA model should be that both the BMT and VCA are performed simultaneously. Prabhune et al. reported that tolerance to hind-limb transplants can be established through simultaneous transplantation of hind-limb and BM in recipients ablatively conditioned with high levels of irradiation (27). In our current study, we have established a more clinically relevant transplantation model in rats where both BMT and VCA are performed simultaneously with reduced-intensity nonmyeloablative conditioning. The immunosuppression is halted after nine days post-transplant. Our results show that BMT with nonmyeloablative conditioning can establish long-term tolerance and indefinite survival of simultaneous VCA with measurable levels of donor chimerism at 1–3 months post-transplant. Moreover, the tolerance to grafts is maintained even after PB donor chimerism is lost.

The relationship between stem cell-driven hematolymphopoietic macrochimerism and transplantation tolerance has been well established. Recipients maintained tolerance after losing macrochimerism, which may suggest that transient engraftment exerts an immunomodulatory effect on rejection. Our data are consistent with prior observations in a sequential BMT-VCA rat model. In this study, Rahhal et al. reported that donor macrochimerism was initially achieved with nonmyeloablative conditioning, but the majority of VCA acceptors lost PB chimerism within 6 months (9). In a clinical HLA-mismatched BMT and kidney transplantation report, stable renal function was maintained for years after complete withdrawal of immunosuppressive therapy even though donor chimerism was lost within 2 weeks (11,29). Transient donor chimerism was also found to reduce the rate of acute rejection in human heart recipients (30). The mechanism responsible for this phenomenon may be due to the association between donor microchimerism and graft tolerance. Persistence of donor microchimerism was reported in organ allograft recipients and proposed to facilitate long-term graft survival (31,32).

In the report by Rahhal et al., the donor chimerism was found to be present at significantly higher levels in the transplanted bone compartment compared with other host tissues of VCA acceptors who had lost PB chimerism (9). This observation gives a good explanation to the underlying mechanisms of tolerance. The persistent high level of donor cells in donor BM establishes systemic microchimerism to facilitate tolerance maintenance. This tolerance mechanism may also apply to our current simultaneous VCA/BMT model. Moreover, central deletional tolerance is most likely present in our simultaneous model as reflected by the fact that a second set of donor skin grafts is accepted long-term and does not break tolerance to the VCA. The central tolerance that follows the induction of chimerism by BMT is associated with elimination of cells in the thymus that are reactive to donor antigen.

Donor microchimerism has been reported to be sufficient to induce and maintain tolerance through central deletional mechanisms (35).

We found that the overall number of $CD4^+/CD25^+/FoxP3^+$ T_{reg} did not differ significantly between VCA acceptors and rejectors. However, our MLR assays show that VCA acceptors preferentially suppress lymphocyte proliferation in the presence of ACI antigen, and this suppressive effect was not lost when the concentration of T_{reg} was reduced. The reduced potency of T_{reg} in VCA rejectors points to a loss of peripheral tolerance in these animals. These data suggest that T_{reg} in long-term VCA acceptors exhibit a stronger immunosuppressive capacity and may play an important role in maintenance of tolerance and promoting graft acceptance. The immunomodulative function of T_{reg} seems more critical in tolerance induction and maintenance in comparison with the absolute number of T_{reg} . Interestingly, we also reported that significant local $CD4^+/FoxP3^+$ T_{reg} infiltration was observed in tolerant donor-allograft skin samples but not in skin samples from rejector or naïve rats (36). Taken together, these data may also suggest the importance of T_{reg} in promoting VCA graft acceptance and maintaining tolerance in long-term VCA acceptors.

In summary, our data suggest that both T_{reg} -mediated suppression and central tolerance mechanisms contribute to allograft acceptance in a simultaneous BMT-VCA model. T_{reg} -mediated peripheral tolerance does not appear to be sufficient on its own to induce allograft acceptance. We observed this with our VCA rejectors: T_{reg} from VCA rejectors were able to suppress lymphocyte proliferation in the presence donor antigen, but T_{reg} were unable to prevent VCA or secondary skin graft rejection events. Our data support the hypothesis that maximizing the induction of central tolerance and T_{reg} -mediated suppression, through transient but sufficient levels of donor chimerism, is necessary to achieve a tolerogenic state.

Materials and Methods

Animals

Six- to 10-week old Wistar Furth (WF; RT1A^u) and August Copenhagen Irish (ACI; RT1A^{abl}) male rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). There is fully MHC disparity between WF and ACI rats. WF enhanced green fluorescent protein (eGFP, Wistar-TgN[CAG-GFP]184Ys) male and female rats were purchased (Rat Resource and Research Center, University of Missouri, Columbia, MO) and bred (Institute for Cellular Therapeutics). Animals were housed in a specific pathogen free facility.

Conditioning and bone marrow transplantation

The T cell depleted (TCD) BMC were prepared by magnet negative selection of the TCR^+ cells after incubating with mouse anti-rat $\alpha\beta$ -TCR and $\gamma\delta$ -TCR mAb and combining with sheep anti-mouse IgG-coated magnetic beads (Dynabeads M-450; Dynal, Lake Success, NY). The nonmyeloablative conditioning and BMT were depicted in Fig. 1.

To determine if the origin of donor cells in chimeric rats was from infused donor BMC or the bone of VCA, recipient ACI rats received grafts from WF rats with either enhanced green fluorescence protein positive (eGFP⁺) BMC plus eGFP⁺ VCA or eGFP⁺ BMC plus eGFP⁺ VCA.

Composite tissue allotransplantation

Heterotopic osteomyocutaneous flap transplantation was performed one day prior to BMT. Donor flaps were prepared from male ACI and transplanted into the right gluteal region as previously described (9). Animals were monitored daily for two weeks following

transplantation and then weekly for signs of flap rejection or graft-versus-host disease (GVHD) (9,24). Rejection was defined as loss of the skin paddle.

Evaluation of Donor cell engraftment

The percent donor cells in the periphery of transplant recipients were determined by flow cytometry at months 1–6 post-procedure. PB was stained with anti-RT1A^{abl}-FITC mAb (Becton Dickinson, Mountainview, CA). The number of RT1A^{abl}-FITC positive cells was measured in the lymphoid gate. To determine the origin of donor cells, PB was collected and analyzed by flow cytometry for the number of eGFP-positive cells in the lymphoid gate. Animals whose PB contained 1% donor cells were considered chimeric.

Skin graft placement and FCXM assay

A secondary antigenic challenge was given to VCA acceptors and rejectors by grafting donor tail skin onto a bed prepared in the recipients' mid-scapular region as previously described (9). Sera from skin graft recipients were derived from whole blood samples collected six weeks after the procedure and analyzed for anti-donor antibodies by flow cytometry.

Regulatory T-cell staining panel

To identify CD4⁺/CD25⁺/FoxP3⁺ T_{reg}, we designed a staining panel that included anti-CD4-APC, anti-CD8-PerCP, anti-CD25-PE, anti-αβ-TCR-Pacific Orange, and anti-FoxP3-Pacific Blue (Becton Dickinson).

Mixed lymphocyte reaction (MLR) suppressor cell assay

Briefly, stimulator cells from the spleens of WF (recipient), ACI (donor), or F344 rats (third party) were isolated and incubated overnight. The following day, stimulator cells were irradiated (2,000 cGy) and incubated with naïve WF responder lymph node cells and sorted CD8⁺/CD4⁺/CD25⁺ T_{reg} from VCA recipients. Lymphocyte proliferation was measured in triplicate by (³H)-thymidine uptake.

Statistics

Statistical analysis was performed using the student one-tailed t-test, and *P* values < 0.05 are considered to be significant.

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Abbreviations

ALS	anti-rat lymphocyte serum
ACI	August Copenhagen Irish

BM	bone marrow
BMC	bone marrow cells
BMT	bone marrow transplantation
cGy	centiGray
eGFP	enhanced green fluorescent protein
FCXM	flow cytometric crossmatch
GVHD	graft-versus-host disease
MLR	mixed lymphocyte reaction
mAb	monoclonal antibody
PB	peripheral blood
PBL	peripheral blood leukocytes
T_{reg}	regulatory T cells
TBI	total body irradiation
TCR	T cell receptor
TCD	T cell depletion
VCA	Vascularized composite allotransplantation
WF	Wistar Furth

Reference List

1. Tobin GR, Breidenbach WC III, Ildstad ST, Marvin MM, Buell JF, Ravindra KV. The history of human composite tissue allotransplantation. *Transplant Proc.* 2009; 41:466–471. PMC Journal - NA. [PubMed: 19328905]
2. Swearingen B, Ravindra K, Xu H, Wu S, Breidenbach WC, Ildstad ST. The science of composite tissue allotransplantation. *Transplantation.* 2008; 86:627–635. PMCID: PMC2629383. [PubMed: 18791440]
3. Breidenbach WC, Gonzales NR, Kaufman CL, Klapheke M, Tobin GR, Gorantla VS. Outcomes of the first 2 American hand transplants at 8 and 6 years posttransplant. *J Hand Surg [Am].* 2008; 33:1039–1047.
4. Wu S, Xu H, Ravindra K, Ildstad ST. Composite Tissue Allotransplantation: Past, Present and Future -The History and Expanding Applications of VCA as a New Frontier in Transplantation. *Transplant Proc.* 2009; 41:463–465. PMCID: PMC2858453. [PubMed: 19328904]
5. Ravindra KV, Wu S-L, Bozulic L, Xu H, Breidenbach WC, Ildstad ST. Composite Tissue Transplantation: A Rapidly Advancing Field. *Transplant Proc.* 2008; 40:1237–1248. PMCID: PMC2692668. [PubMed: 18589081]
6. Ravindra KV, Xu H, Ildstad ST. Vascularized composite allotransplantation at a crossroad. *Transplant Proc.* 2011; 43:3501–3503. [PubMed: 22099829]
7. Pan H, Zhao K, Wang L, et al. Mesenchymal Stem Cells Enhance the Induction of Mixed Chimerism and Tolerance to Rat Hind-Limb Allografts after Bone Marrow Transplantation. *J Surg Res.* 2010; 160:315–324. [PubMed: 19524257]
8. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature.* 1984; 307:168–170. [PubMed: 6361574]

9. Rahhal D, Xu H, Huang WC, et al. Dissociation between peripheral blood chimerism and tolerance to hindlimb composite tissue transplants: preferential localization of chimerism in donor bone. *Transplantation*. 2009; 88:773–781. PMID: PMC2780434. [PubMed: 19920776]
10. Yu P, Xiong S, He Q, et al. Induction of allogeneic mixed chimerism by immature dendritic cells and bone marrow transplantation leads to prolonged tolerance to major histocompatibility complex disparate allografts. *Immunology*. 2009; 127:500–511. [PubMed: 19604303]
11. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008; 358:353–361. [PubMed: 18216355]
12. Scandling JD, Busque S, Dejbakhsh-Jones S, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med*. 2008; 358:362–368. [PubMed: 18216356]
13. Leventhal J, Abecassis M, Miller J, et al. Chimerism and Tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. Submitted to *Science Transplantation Medicine*. 2011 2011.
14. Wekerle T, Sayegh MH, Hill J, et al. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *Journal of Experimental Medicine*. 1998; 187:2037–2044. [PubMed: 9625763]
15. Colson YL, Lange J, Fowler KJ, Ildstad ST. Mechanism for co-tolerance in nonlethally conditioned mixed chimeras: negative selection of the V β T-cell receptor repertoire by both host and donor bone marrow-derived cells. *Blood*. 1996; 88:4601–4610. [PubMed: 8977252]
16. Mueller DL. Mechanisms maintaining peripheral tolerance. *Nat Immunol*. 2010; 11:21–27. [PubMed: 20016506]
17. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009; 30:656–665. [PubMed: 19464988]
18. Sanchez-Fueyo A, Domenig CM, Mariat C, Alexopoulos S, Zheng XX, Strom TB. Influence of direct and indirect allorecognition pathways on CD4+CD25+ regulatory T-cell function in transplantation. *Transpl Int*. 2007; 20:534–541. [PubMed: 17362475]
19. Tsang JY, Tanriver Y, Jiang S, et al. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J Clin Invest*. 2008; 118:3619–3628. [PubMed: 18846251]
20. Amarnath S, Costanzo CM, Mariotti J, et al. Regulatory T cells and human myeloid dendritic cells promote tolerance via programmed death ligand-1. *PLoS Biol*. 2010; 8:e1000302.
21. Adeegbe D, Levy RB, Malek TR. Allogeneic T regulatory cell-mediated transplantation tolerance in adoptive therapy depends on dominant peripheral suppression and central tolerance. *Blood*. 2010; 115:1932–1940. [PubMed: 20040758]
22. Blache C, Chauvin JM, Marie-Cardine A, et al. Reduced frequency of regulatory T cells in peripheral blood stem cell compared to bone marrow transplantations. *Biol Blood Marrow Transplant*. 2010; 16:430–434. [PubMed: 19883775]
23. Colson YL, Zadach K, Nalesnik M, Ildstad ST. Mixed allogeneic chimerism in the rat. Donor-specific transplantation tolerance without chronic rejection for primarily vascularized cardiac allografts. *Transplantation*. 1995; 60:971–980. [PubMed: 7491703]
24. Foster RD, Pham S, Li S, Aitouche A. Long-term acceptance of composite tissue allografts through mixed chimerism and CD28 blockade. *Transplantation*. 2003; 76:988–994. [PubMed: 14508367]
25. Billingham RE, Brent L, BROWN JB, Medawar PB. Time of onset and duration of transplantation immunity. *Transplant Bull*. 1959; 6:410–414. [PubMed: 13800708]
26. Graff RJ, Silvers WK, Billingham RE, Hildemann WH, Snell GD. The cumulative effect of histocompatibility antigens. *Transplantation*. 1966; 4:605–617. [PubMed: 5339407]
27. Prabhune KA, Gorantla VS, Perez-Abadia G, et al. Composite tissue allotransplantation in chimeric hosts part II. A clinically relevant protocol to induce tolerance in a rat model. *Transplantation*. 2003; 76:1548–1555. [PubMed: 14702522]
28. Gorantla VS, Prabhune KA, Perez-Abadia G, et al. Composite tissue allotransplantation in chimeric hosts: part I. prevention of graft-versus-host disease. *Transplantation*. 2003; 75:922–932. [PubMed: 12698075]

29. Farris AB, Taheri D, Kawai T, et al. Acute renal endothelial injury during marrow recovery in a cohort of combined kidney and bone marrow allografts. *Am J Transplant*. 2011; 11:1464–1477. [PubMed: 21668634]
30. Pham SM, Rao AS, Zeevi A, et al. A clinical trial combining donor bone marrow infusion and heart transplantation: intermediate-term results. *J Thorac Cardiovasc Surg*. 2000; 119:673–681. [PubMed: 10733755]
31. Starzl TE, Demetris AJ, Murase N, Ildstad ST, Ricordi C, Trucco M. Cell migration, chimerism and graft acceptance. *Lancet*. 1992; 339:1579–1582. [PubMed: 1351558]
32. Starzl TE, Demetris AJ, Trucco M, et al. Cell migration and chimerism after whole organ transplantation: The basis of graft acceptance. *Hepatology*. 1993; 17:1127–1152. [PubMed: 8514264]
33. McSherry C, Hertz MI, Jackson AM, et al. Allogeneic microchimerism and donor antigen-specific hyporeactivity in lung transplant recipients. *Clin Transplant*. 1995; 9:442–449. [PubMed: 8645886]
34. Corry RJ, Chakrabarti PK, Shapiro R, et al. Simultaneous administration of adjuvant donor bone marrow in pancreas transplant recipients. *Ann Surg*. 1999; 230:372–379. [PubMed: 10493484]
35. Bonilla WV, Geuking MB, Aichele P, Ludewig B, Hengartner H, Zinkernagel RM. Microchimerism maintains deletion of the donor cell-specific CD8+ T cell repertoire. *J Clin Invest*. 2006; 116:156–162. [PubMed: 16395404]
36. Bozulic LD, Wen Y, Xu H, Ildstad ST. Evidence That FoxP3+ Regulatory T Cells May Play a Role in Promoting Long-Term Acceptance of Composite Tissue Allotransplants. *Transplantation*. 2011; 91:908–915. [PubMed: 21304439]

VCA-BMT Simultaneous Protocol

Figure 1. A clinically relevant simultaneous BMT-VCA model

Male WF rats were treated with a short course of immunosuppressive therapy that included the following: one dose of anti- $\alpha\beta$ -TCR mAb delivered three days prior to BMT (day -3), eleven doses of FK-506 delivered daily beginning one day prior to BMT, and one dose of anti-lymphocyte serum (ALS) delivered 9 days after BMT. WF rats received TBI at doses ranging from 100–400 centigray (cGy) one day prior to BMT. Heterotopic osteomyocutaneous flaps were taken from the hind limbs of male ACI rats and transplanted into the right gluteal region of WF rats one day prior to BMT. 100×10^6 TCD ACI BMC were delivered on day 0. Monthly peripheral blood leukocyte (PBL) typing was performed up to six months after BMT and VCA. A secondary antigenic challenge and subsequent flow crossmatch assay were performed one year post-VCA/BMT. Identification of T_{reg} cells by flow cytometry was also performed at the termination of the experiment.

Figure 2. Simultaneous BMT with nonmyeloablative conditioning approaches promotes long-term graft survival in VCA acceptors

Five control WF rats received VCA without prior irradiation (0 cGy TBI). Twenty WF rats were treated with a nonmyeloablative conditioning regimen and with TBI doses from 100 – 400 cGy (5 rats per treatment group). All other treatments were depicted in Fig. 1. Control and treated WF rats received simultaneous BM and VCA grafts from ACI rats. (A) Cumulative graft survival over time is shown for animals treated under the simultaneous protocol. Acceptance was defined as integration of the skin flap into surrounding skin with growth of black donor hair. Graft survival was followed up to 200 days. (B) Peripheral blood chimerism kinetics of VCA recipients (ACI to WF; N = 6 for acceptors, and N = 14 for rejectors) over a 6 month follow-up period are shown. PBL were stained for the donor MHC class I marker RT1A^{abl} and processed by flow cytometry. The percentage of donor cells in the lymphoid gate was measured, and the average percent level of donor chimerism is shown. Engraftment was defined as >1% donor cells in the lymphoid gate (dotted line indicates 1.00%). Error bars indicate standard error of the mean. An “*” indicates significant differences ($P < 0.05$) between percent chimerism levels of acceptors compared to rejectors as measured by the student’s one-tailed t test. (C) To determine the origin of donor cells in chimeras from infused donor BMC or the bone of VCA, twelve ACI rats were treated with the same immunosuppressive therapy as described in Fig. 1 and received 300 cGy TBI. Half

of these recipient received grafts from WF rats either eGFP⁺ BMC plus eGFP⁻ VCA or eGFP⁻ BMC plus eGFP⁺ VCA. A total of 100×10^6 TCD WF (eGFP⁺ or eGFP⁻) BMC were delivered at Day 0. PB typing was performed at 1, 2, and 3 months post-procedure in VCA acceptors, and percent eGFP⁺ or eGFP⁻ donor cells in the lymphoid gate were measured. Error bars indicate standard error of the mean.

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Figure 3. Secondary antigenic challenge does not break tolerance in VCA acceptors

Four VCA acceptors and six VCA rejectors treated according to the protocol in Figure 1 were given skin grafts at one year post-VCA, and sera from these animals were collected 1 month after skin transplantation. Skin graft survival was monitored up to 120 days (A). Rejection was defined as complete when no residual viable graft could be detected. Anti-donor specific antibodies in sera were examined for anti-donor antibodies by FCXM assay (B).

Figure 4. T_{reg} from VCA acceptors more potently suppress lymphocyte proliferation than T_{reg} from VCA rejectors in the presence of donor stimulator cells
 (A and B) Tissues from 3 VCA acceptors and 3 VCA rejectors were collected six months after secondary antigenic challenge and stained for T regulatory cells (CD4⁺/CD25⁺/FoxP3⁺). Samples were analyzed by flow cytometry. The mean cell percentage (A) and the mean absolute cell number (B) are shown. Error bars indicate standard error of the mean. (C and D) Responder cells were prepared from naïve WF recipient rats and T_{reg} were isolated from VCA acceptors (Part C) and VCA rejectors (Part D) at six months after secondary antigenic challenge. Experiments were performed in triplicate. Stimulator cells from the spleens of ACI rats (VCA donor) or F344 rats (third party) were isolated and incubated with

responder cells and T_{reg} , and lymphocyte proliferation was measured by (H^3)-thymidine uptake. Ratio of responder cells from naïve WF rats to T_{reg} is shown as 1:1 and 1:0.25. Error bars are indicative of the standard error of the mean between the samples tested. Values are shown as average $\pm$ standard error of triplicate cultures. Statistical analysis was performed using the student one-tailed t-test, and an “*” indicates a significant difference in lymphocyte proliferation in the presence of T_{reg} compared to responders without T_{reg} ($P < 0.05$). The allogeneic response (Allo; ACI stimulator cells incubated with WF or F344 responder cells but no T_{reg}) and the autogeneic response (Auto; WF stimulator cells incubated with WF responder cells but no T_{reg}) are also shown as controls.

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