Clinical and Developmental Immunology

# Immunology of Vascularized Composite Allografts

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*Editorial*

# Immunology of Vascularized Composite Allografts

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Over the past decade, vascularized composite allotransplantation (VCA), such as hand and face transplantation, has become a clinical reality and a viable treatment option for those patients suffering from complex tissue injuries or defects not amenable to conventional reconstruction. Despite the fact that early and intermediate functional outcomes are highly encouraging rejection and the need for chronic immunosuppressive treatment continue to be the bane of VCA, preventing its broader clinical application.

A thorough understanding of the mechanisms underlying alloimmune responses in VCA is key to establish novel protocols for immunomodulation and tolerance induction after this type of transplant, without the need for long-term immunosuppression. Such advances would diminish the risks and favor the benefits for these non-life-saving but life-changing transplants.

This special issue is devoted to the immunology of vascularized composite allografts. The individual authors are leaders in the field, with extensive knowledge and expertise in this novel and emerging field of transplantation. The goal of this issue is to cover various specific aspects, as well as some of the immunological challenges related to VCA. The main focus is on tolerance strategies and how those apply to VCA.

Greater scrutiny is applied to immunosuppression-induced complications in VCA recipients compared to solid organ transplant patients since VCA is deemed life-enhancing rather than life-saving interventions. K. V. Ravindra et al. discuss the pressure to develop tolerance-inducing strategies in VCA.

D. A. Leonard et al. provide a comprehensive review of the use of mixed chimerism approaches for tolerance induction in the field of VCA with a particular emphasis on translational large animal (MGH miniature swine) protocols. The authors also provide an interesting overview of novel cellular therapies, such as regulatory T cells, regulatory dendritic cells, or mesenchymal stem cells as potential adjuvants to mixed hematopoietic chimerism in the development of tolerance induction protocols for clinical VCA.

M. Siemionow and A. Klimczak discuss the Cleveland Clinic research experience with chimerism-based experimental small animal models for tolerance induction and highlight in particular the complexity of immunomodulatory protocols in VCA as well as their relevance and applicability for clinical practice.

W.-C. Huang et al. review approaches to improve the safety of tolerance inducing regimes currently applied to VCA and discuss immune monitoring and tolerance assays that are critically required prior to translating such protocols in the clinic.

Y.-R. Kuo et al. summarize the current understanding of immunomodulation achieved by mesenchymal stem cell (MSC) therapy and provide a possible outline for its future clinical application in VCA.

R. Starzl et al. provide a historically oriented discussion of cross-disciplinary approaches to develop novel points of view for some of the most challenging immunological problems in VCA, particularly the early diagnosis and assessment of rejection. Some of these approaches and methods lie at the intersection of medicine, immunology, mathematics, and computer science. By leveraging the strengths and capabilities of each discipline to solve problems that have been resistant to analysis in another, more rapid progress can be made in

delivering novel and clinically relevant findings, diagnostics, or therapeutic agents.

T. Hautz and colleagues review the key players and molecular events of skin inflammation and discuss new therapies originally developed in solid organ transplantation with particular emphasis on skin allograft rejection and how such therapies could relate to VCA. This is of interest since the mechanisms and dynamics of acute and chronic skin allograft rejection are incompletely understood and remain the subject of numerous ongoing trials aimed at better understanding of the underlying pathophysiology and at novel and targeted drug development.

J. T. Schnider et al. provide an overview of graft-targeted and delivered site-specific immunosuppression, which is uniquely well suited for VCA due to their direct accessibility to such interventions. The authors highlight the fact that site-specific therapeutic effects and efficacy of systemically active agents may enable optimal dosing, frequency, and duration of overall immunosuppression in VCA, with minimization or elimination of long-term drug-related toxicity.

C. G. Wallace et al. show that intrajejunal treatment with donor splenocytes could render recipients immunologically hyporesponsive in a donor-specific manner *in vitro*. When the regimen was tested in a rodent setting *in vivo*, VCA rejection was delayed but did not result in immunosuppressive drug-free tolerance. These encouraging data warrant further investigation to assess the exact role and mechanisms of intrajejunal treatment as a low-risk adjunct to prevent VCA rejection.

Q. Liu et al. provide interesting evidence that a short-course of combined antilymphocyte serum and Cyclosporine A treatment enables indefinite VCA survival, which induces secondary donor-specific skin and heart allograft tolerance despite the loss of peripheral chimerism. These findings open up new perspectives for the role of VCA in the induction and maintenance of tolerance to solid organ transplants.

We hope that this series of articles will stimulate continuing efforts to better understand the unique immunological features of VCA compared to solid organ transplants, and the development of novel strategies to induce tolerance.

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*Review Article*

# Chimerism-Based Experimental Models for Tolerance Induction in Vascularized Composite Allografts: Cleveland Clinic Research Experience

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The preclinical experimental models of vascularized composite allografts (VCAs) have been rapidly developed for the assessment of immunomodulatory protocols for clinical application. Recently, researchers have focused on immunomodulatory protocols which overcome the immunologic barrier between the allogeneic donor and recipient and may lead to tolerance induction. In order to test the feasibility of chimerism induction, experimental VCAs have been performed in different models including rodents, large animals, and nonhuman primates. These models differ in the complexity of transplanted tissue and in their responses to immunomodulatory protocols. In most applications, VCA contains multiple-tissue components; however, each individual component of CTA possesses unique immunologic characteristics that ultimately contribute to the chimerism induction and successful outcome of the VCA. Heterogenic character and complexity of tissue components in different VCA models determine the quality and robustness of donor-specific chimerism. As introduced in experimental studies, variable immunomodulatory options have been studied to achieve tolerance to VCA in rodents and large animal models allowing for widespread application in clinic. In this paper, based on our own experience, we have analyzed the current knowledge of tolerance-inducing strategies via chimerism induction in VCA experimental models in the context of immunomodulatory protocols and VCA complexity and their relevance and applicability to clinical practice.

## 1. Introduction

Experimental models of vascularized composite allografts (VCAs) successfully preceded clinical application of VCA, especially hand and face transplants, which have become a breakthrough in the fields of reconstruction for patients suffering from massive complex tissue injury. Although allotransplantation, as a reconstructive option, has become widely accepted as an experimental procedure in clinic, it still raises a lot of attention due to lifelong immunosuppression. To date, 59 hands in 41 patients and 24 partial or full face transplants, which are considered the most challenging VCAs, have been successfully performed in clinic (IRHCTT; http://www.handregistry.com/) [1, 2].

Experimental models of VCAs were created not only to assess the surgical feasibility and functional recovery after allotransplantation, but also to test tolerance-inducing strategies based on immunomodulatory protocols which will have potential application in clinic [3, 4]. Extensive research on tolerance induction performed during the last two decades has proven that development of donor-specific chimerism may accompany induction of tolerance in VCA; however, the role of chimerism in tolerance induction is still debatable [5–7].

Tissue resident cells, which are present within the transplanted tissue, may play an immunomodulatory role when the proper immunosuppressive regimen is applied. Immunocompetent cells present within the transplanted tissue are known as passenger leukocytes and, after vessel anastomosis between the transplanted VCA and recipient vessels, they may migrate into different compartments of the recipient and contribute to chimerism induction. The role of passenger

leukocytes was confirmed by Starzl in his pioneered studies on the role of chimerism in solid organ acceptance [8].

The heterogenic character of tissue components in different VCA models determines the quality and robustness of donor-specific chimerism. A rodent MHC-mismatched model offers the advantage of identification of donor versus recipient cells, using monoclonal antibodies specific for MHC strains of rodents.

Our own observations indicate that a universal tolerogenic protocol for VCA still does not exist, and the success of VCA acceptance depends on the immunologic character of transplanted tissues, their complexity, and the genetic barrier between donor and recipient.

In this paper we analyze our experience and the current knowledge on tolerance via chimerism induction strategies in experimental VCA models. Immunomodulatory protocols used in experimental models include (i) monotherapy protocols using calcineurin inhibitors such as cyclosporine A (CsA) or tacrolimus, (ii) T-cell depletion protocol, and (iii) protocols augmented with donor bone marrow cells (BMCs). These protocols will be analyzed in the context of chimerism induction and VCA complexity.

## 2. Monotherapy Protocol with Calcineurin Inhibitors for Chimerism Induction in VCA

Monotherapy protocol with CsA has been applied in many experimental VCA models including models with a single component of allograft (skin) and in more complex models such as limb and face allografts [9–23].

*2.1. Vascularized Skin Allograft: The Model of a Single Tissue Component.* Skin represents an important component of VCA and may be transplanted as a single component to cover large skin defects or as an integral part of composite tissue allograft including hand and face transplants.

Many immunocompetent cells, including Langerhans cells (LCs) and dermal dendritic cells (DDC), are present in the skin, both with an antigen-presenting function, as well as dermal T lymphocytes. The highly immunogenic character of skin represents a significant challenge for skin acceptance and an experimental skin model is the most frequently used model for tolerance induction studies [24].

In our experimental design of VCA, we have performed a study to determine if there is correlation between the vascularization and development of donor-specific chimerism in different sizes of vascularized skin allografts (VSAs) and nonvascularized skin allografts (NVSAs) in the rat model, under low-maintenance dose of CsA monotherapy (2 mg/kg/day) [9]. In this study, we have documented that vascularization and size of the skin allograft contribute to both skin allograft survival and donor chimerism induction. We observed the presence of donor chimerism in both vascularized and nonvascularized skin grafts; however, the dynamics and level of chimerism differed between transplanted groups. We have confirmed that larger graft size correlates positively with chimerism level, only in the VSA recipients, and initially, at one week posttransplant, chimerism was assessed at 12.2% in large skin allograft recipients ($6 \times 6$ cm) versus 8.0% in the group receiving smaller ($2 \times 2$ cm) skin allografts ($P < 0.05$) [9].

In contrast, in NVSA, recipient's larger skin diameter correlated inversely with blood chimerism level and at day 7 following-transplant; the mean value of total donor chimerism was assessed at 2.53% in the group receiving large (6×6 cm) skin grafts versus 3.92% in the group receiving small ($2 \times 2$ cm) skin allografts ($P < 0.05$) [9].

In both types of skin transplants, VSA and NVSA, chimerism declined during the follow-up period, and two months after transplantation, it revealed levels of 1.1% to 1.6% in the VSA group and was found to be below 0.5% in the NVSA group. The level of chimerism correlated with allograft survival and skin vascularization. The differences in chimerism level in VSA when compared with NVSA are dependent upon the progress of allograft vascularization. After transplantation of VSA, blood supply returns to the allograft within 1-2 hours after donor-recipient vessel anastomosis and this minimizes ischemic as well as reperfusion-related damage. Moreover, graft-resident cells rapidly migrate into the recipient's blood circulation, which contributes to chimerism induction. In contrast, in NVSA transplants, graft revascularization takes at least a few days, and this extends relative ischemia time, with its known complications. During this early period, there is sprouting of new vessels from the recipient bed and neighboring recipient skin which are reaching the graft; thus, there is no direct connection between donor-origin cells from the graft and the recipient's immune system, via blood circulation, as is the case in VSA models. The smaller size of skin graft is more susceptible to revascularization and this may explain higher chimerism-level small-size allografts when compared to the larger-size NVSA.

Our observation confirmed the dynamics of the skin allograft vascularization in non-VSA and VSA models, as well as graft size, to have a significant effect on the development of donor chimerism.

Total abdominal wall (TAW) transplant in a rat model has been developed in our laboratory to monitor immunologic responses in the largest VSA transplant ($8 \times 12$ cm$^2$) [10]. This is the first model of large vascularized skin allograft transplant in a small animal, simulating a clinical abdominal wall transplantation with consistent anatomy, straightforward surgical technique, and reliable blood supply, which are essential for the success of experimental transplantation studies. The transplantation procedure was performed under a maintenance dose of CsA monotherapy started from 16 mg/kg/day and maintained at 2 mg/kg/day after 4 week posttransplant.

Chimerism levels were monitored and at day 7 posttransplant, the mean value of total chimerism was assessed at $6.7 \pm 1.32\%$ or the presence of donor-origin cells; however, over time, chimerism declined and at day 100 posttransplant revealed $1.3 \pm 0.38\%$.

These studies on skin allograft transplants have proven that skin is an abundant source of donor-origin cells which are able to migrate and engraft to the recipient compartments, leading to chimerism induction and maintenance when supported by adequate immunosuppressive therapy.

*2.2. Complexity of the VCA: The Multitissue Models.* Complexity of the VCA introduces surgical and immunological challenges and requires adjustment of immunosuppressive protocols. In most clinical applications, such as hand and face transplants, VCA contains multitissue components including skin, subcutaneous tissue, muscle, bone with bone marrow, lymph nodes, nerve, tendon, and mucosa. The most commonly used experimental model of VCA is the orthotopic and heterotopic limb allograft transplant.

*2.2.1. The Limb Allograft Model.* The limb represents a specific model of the VCA since vascularized bone, with bone marrow cells, constitutes a structural component of the VCA in addition to muscles, skin, nerves, and tendons. We have shown that a limb allograft contains approximately $50 \times 10^6$ of the bone marrow cells which may play a significant role in chimerism induction [11].

Experience with successful experimental limb transplantation across MHC-mismatched rat strains was reported by Kim et al. [12], where successful limb allograft survival was accomplished under a maintenance dose (10 mg/kg/day) of CsA monotherapy. Kim reported that continued CsA delivery is mandatory for limb allograft survival, since animals rejected transplanted limbs within 1 week following CsA cessation. However, Black et al. reported indefinite limb allograft survival under a moderate daily dose of CsA (8 mg/kg/day), given for 20 days posttransplant, followed by a maintenance dose of CsA given twice a week [13]. These studies proved that maintenance CsA therapy is essential for limb allograft survival.

Our experience with limb allograft model under continued CsA monotherapy resulted in long allograft survival [14]. In this study, semiallogenic rat hind-limb transplantations were performed under low-dose CsA protocol (4 mg/kg/day) combined with topical steroids, fluocinolone acetonide (6 mg/cm$^2$/day), both started at the day of surgery and maintained during the entire follow-up period. Synergistic therapeutic effect of the low dose of CsA and topical application of steroids allowed for extended limb allograft survival, up to 51 days.

The first studies reported by Kim, Black, and Inceoglu documented the technical feasibility and beneficial effect of CsA in limb VCA survival, but chimerism was not assessed in these studies.

Hewitt et al. reported hind-limb transplants between Lewis and Lewis × Brown-Norway (LBN) rats, in immunologically unmodified limb allograft recipients [15]. The authors documented that development of a high level of hematopoietic donor-specific chimerism of 60.2 ± 14.5% was associated with development of GvHD, whereas the presence of a stable, low level of mixed T-cell chimerism, below 18.3 ± 3.9%, was associated with tolerance induction in most of the limb allograft recipients ($P < 0.002$).

Several studies on limb allograft under the CsA protocol were also performed in a large animal model. Bourget et al. tested the effect of a 12-day course of CsA monotherapy (13 mg/kg/day) in MHC-matched, minor antigen-mismatched miniature swine model [16]. The authors reported long-term survival of the musculoskeletal component of limb allograft recipients under CsA monotherapy, and this was associated with the presence of transient chimerism which was detectable until day 19 posttransplant. Authors concluded that transient hematopoietic chimerism is sufficient for tolerance induction in the large-animal model of VCA [16].

*2.2.2. Face Allograft Model.* Face allograft is an example of the most complex VCA models and may be transplanted with or without a vascularized bone component.

(1) *Face Allograft without Bone Component*. The first full face/scalp allograft model was introduced in a rat, in the year 2000, by Siemionow et al., in the Microsurgery Laboratory of Cleveland Clinic. Since that time, Siemionow's team has developed different experimental models of rat face transplantation that differ in their content of transplanted tissue and immunosuppressive protocols. In 2003, first reports that documented successful face/scalp allograft survival between LBN donors and Lewis recipients under CsA monotherapy (16 mg/kg/day), tapered within four weeks to low maintenance dose of 2 mg/kg/day, were introduced [17]. Following full face transplantation, we developed a hemiface transplant model to test the feasibility of tolerance induction and immunological response to different protocols [18]. The immunosuppressive protocol of CsA maintenance monotherapy (2 mg/kg/day) was tested in semiallogenic (LBN to Lewis) and fully MHC-mismatched (ACI to Lewis) models, corresponding to a more stringent and clinically relevant scenario [19]. Long-term survival in both models was associated with the presence of donor-specific chimerism in both T-cell and B-cell lineages, assessed both in the peripheral blood and bone marrow compartments, and was associated with engraftment of donor-origin cells to lymphoid organs of recipients. In semiallogenic hemiface model, T-cell and B-cell chimerisms were assessed at 10.14% for the CD4 and at 6.38% for CD8 T-cell population and at 10.02% for B-cell lineage represented by CD45RA antigen. In complete MHC-mismatched (ACI to Lewis) face transplant model, a high level of donor chimerism was detected (17.54% for CD4 and 9.28% for CD8) in T-cell population; however, low chimerism (below 1%) was assessed for B lymphocytes. Moreover, we have confirmed the engraftment of cells of allograft origin into spleen and lymph nodes, but not to the thymus, of the face transplant recipients [19].

Development of chimerism in a face allograft model may be explained by the rich representation of dermal T lymphocytes within skin component, as well as lymph nodes which are an abundant source of donor T and B cells.

(2) *Face Allograft Model with Bone Component*. The clinical need to cover extensive craniomaxillofacial defects, including bony and soft tissue components, encouraged us to develop rat model of composite hemiface/calvaria, maxilla, and hemiface/mandible/tongue transplantation models [20–23]. These surgically challenging models were maintained under low nontoxic dose of CsA (2 mg/kg/day) monotherapy and immunologically assessed for the presence of chimerism

at different time points starting from day 7 posttransplant with the end-point at the sacrifice day.

In hemiface/calvaria model, viable bone marrow cells were detected within vascularized bone component, and peripheral blood chimerism was supported predominantly by B-lymphocyte population.

In a heterotopic rat maxilla model, which contains only bone and mucosal tissue (without skin), donor chimerism was detectable in long-term survivals (over 100 days posttransplant) and was represented by CD4 (12.5%) and CD8 (5.3%) T lymphocytes and by 4.7% of B lymphocytes [21].

The purpose of developing an orthotopic composite hemiface/mandible/tongue model was to extend application of our standard face transplantation model in the rat by incorporation of the vascularized mandible, masseter, and tongue; to test its feasibility across the MHC barrier; and to assess the immunomodulatory effect of different tissue components of hemiface/mandible/tongue allograft and their contribution to the development and maintenance of multilineage chimerism [23].

Under CsA monotherapy, chimerism was initially characterized by a high level of donor-origin T cells assessed at 12.3% for CD4 and at 11.3% for CD8 T-lymphocyte subpopulations, whereas B-cell chimerism was lower (2.8%), assessed for CD45RA B-cell-specific antigen. Chimerism kinetics switched over time and T-cell chimerism declined, whereas B-cell chimerism at day 300 posttransplant was maintained and was assessed at 4.4%. Donor-origin cells were also detected in the bone marrow compartment of hemiface/mandible/tongue recipients, at 2.33%, and 1.21% of total chimerism was represented by immature $RT1^n$/CD90+ cell phenotype [23].

In maxilla and hemiface/mandible/tongue models, the oral mucosa contains submaxillary and submandibular lymph nodes and salivary glands. Salivary glands contain a diverse population of lymphocytes represented by T cells, B cells, and natural killer cells. These cells are distinct from cells present in peripheral lymphoid organs and are known to be responsible for regulation and mediation of humoral and cellular immune responses in the mucosal immune network [25].

These findings indicate that bone marrow, lymphoid, and glandular components of the hemiface/calvaria, maxilla, and composite hemiface/mandible/tongue allograft have a positive immunomodulatory effect supporting development of donor chimerism and long-term allograft survival [26]. Maintaining a balance between chimerism induction and maintenance is crucial for long-term survival of facial VCA in a rat model.

Recently, therapy with tacrolimus and mycophenolate mofetil (MMF) was introduced in large-animal model of heterotopical facial VCA in nonhuman primates [26]. The heterotopically transplanted facial segment contained vascularized bone marrow (VBM) contained within donor mandible. Facial allograft recipients were maintained on tacrolimus (blood level 15–25 ng/mL) and MMF (50 mg/kg/day). Recipients of the facial allograft with VBM component demonstrated prolonged allograft survival when under maintenance immunosuppression; however, discontinuation of immunosuppression resulted in facial allograft rejection. Facial VCAs without bone component were rejected within 7–15 days despite continuous immunosuppression. Macrochimerism was detectable in both groups in blood and peripheral lymphoid tissues, spleen, and lymph nodes. These observations support the immunomodulatory role of hematopoietic cells present within VCA that facilitate stable graft acceptance with a modest requirement for immunosuppression [26].

## 3. The Role of Combined T-Cell Depletion and Immunosuppression in Chimerism Induction

Elimination of memory T lymphocytes or inhibition of T-cell activation represents a critical mechanism in the induction of transplantation tolerance [27]. Currently, immunodepletive protocols are widely used as part of an immunosuppressive regimen, both in clinic and in experimental models. Nonselective depletion of T cells is accomplished by either polyclonal anti-lymphocyte serum (ALS), anti-thymocyte globulin (ATG), or monoclonal antibodies such as anti-CD3 (muromonab-CD3) and anti-CD52 (Campath-1H) antibody. In contrast, selective depletion of specific populations of T lymphocytes eliminates only alloreactive T cells [28]. When recipients are submitted to depletive protocols, they are protected against graft-versus-host disease (GvHD), since immunodepletive agents eliminate graft-derived alloreactive T cells.

In our experiments with chimerism induction under immunodepletive protocols, we have used ALS and anti-$\alpha\beta$-TCR monoclonal antibodies (anti-$\alpha\beta$-TCR mAb) to achieve tolerance.

The polyclonal nature of ALS results in diverse immunosuppressive effects. ALS successfully eliminates all subpopulations of T lymphocytes (Figure 1) in peripheral blood and tissues via cytotoxicity and/or opsonization [29]. Moreover, ALS mediates leukocyte/endothelial level interactions by modulation of adhesion molecules or chemokine receptor expression. The immunomodulatory activity of ALS is also accomplished by interference with dendritic cell function; ALS acts as costimulatory blocker inhibiting maturation of dendritic cells and reduces the stimulatory capacity of dendritic cells for T-cell proliferation. In addition, ALS substantially depletes blood monocytes and NK cells, and this diminishes their innate immunity, contributing to prevention of allograft rejection, in addition to T-cell depletion. This action may, however, lead to development of opportunistic infections.

In contrast, by selective depletion with anti-$\alpha\beta$-TCR mAb, only alloreactive T cells are targeted by specific inhibition of $\alpha\beta$-TCR, but other cells such as $\gamma\delta$ T cells, natural killer (NK) cells, monocytes, and other leukocytes are preserved [30] (Figure 1). The $\alpha\beta$-TCR is expressed on the vast majority of immature and mature rat T lymphocytes and is responsible for the first signal of T-cell activation. By inhibiting the first signal of T-cell activation with anti-$\alpha\beta$-TCR mAb, alloreactive T cells, which are the main players of

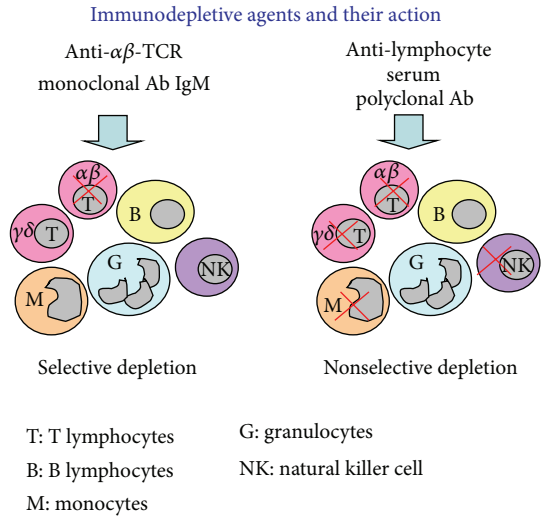


Figure 1: Selective immunodepletion under anti-$\alpha\beta$-TCR monoclonal antibody and nonselective depletion of leukocytes under anti-lymphocyte serum.

acute rejection, are selectively eliminated leading to peripheral anergy. The anti-$\alpha\beta$-TCR mAb acts as a depleting agent on target cells; however, functional inhibition has also been reported [31]. Immunocytochemistry has confirmed reduced TCR intensity staining after anti-$\alpha\beta$-TCR mAb therapy, both in our studies and the reports of other investigators [32, 33]. Moreover, this antibody is not mitogenic and initiates low first dose of cytokine release as compared to some other anti-T-cell monoclonal antibodies [34]. In addition, anti-$\alpha\beta$-TCR therapy downregulates endothelial activation and expression of many proinflammatory cytokines (e.g., IL-2 and IFN-$\gamma$) which are associated with allorecognition and development of rejection, as confirmed in the rat model of cardiac allografts [31, 35].

Immunodepletive agents are not that effective in tolerance induction when administered alone, but when induction therapy with immunodepletive agents is supported with short-term immunosuppression, irradiation, or costimulatory blockade, this type of protocol represents a powerful tool for chimerism development and tolerance induction.

*3.1. Immunodepletive Protocols in the Limb Allograft Model.* We have investigated tolerance induction in a limb allograft model, using a 21-day combined protocol of ALS and CsA therapy. Transplantations were performed in semiallogenic rat model between LBN ($RT1^{l+n}$) donors and Lewis ($RT1^{l}$) recipients. The combined immunodepletive protocol of ALS and CsA significantly prolonged limb allograft survival (over 420 days) compared to monotherapy with ALS or CsA alone (6 and 23 days, resp.), and tolerance was confirmed *ex vivo* by MLR assay showing hyporesponsiveness to the donor antigens and *in vivo* by acceptance of donor skin grafts. In addition, at 100 days posttransplant, immunocompetence of the recipients was confirmed by rejection of the third-party skin allograft. Tolerant animals demonstrated a donor-specific hematopoietic chimerism in the peripheral blood ranging from 35% to 42%, whereas in nontolerant animals chimerism was not detected [36].

After achieving success in tolerance induction in a semiallogenic limb transplant model, we applied the immunodepletive protocol of ALS and CsA to a more immunogenetically challenging model in fully MHC-mismatched animals ($BN(RT1^{n})$ donors and Lewis ($RT1^{l}$) recipients. Under the ALS/CsA protocol, limb allograft survival was extended by up to 56 days; however, tolerance was not achieved [37]. Only transient, donor-derived chimerism ($17 \pm 1.1\%$ at day 35) was detected and dropped down to 0 at the time of rejection. This study confirmed that transplantation across a strong MHC barrier mandates adjustments in immunosuppressive protocols.

The success of tolerance induction in a limb allograft model under combined ALS and CsA therapy encouraged us to develop a new protocol of selective inhibition of potentially alloreactive $\alpha\beta$-TCR T cells, in combination with a short course of CsA therapy (Figure 2). Initial studies tested the dose and duration of anti-$\alpha\beta$-TCR mAb CsA therapy and resulted in establishment of dose of anti-$\alpha\beta$-TCR monoclonal antibody, at 50 $\mu$g/day, in combination with tapered dose of CsA, from 16 mg/kg/day to 2 mg/kg/day, over 35-days posttransplant under this protocol [38]. Limb allograft survival (over 720 days) was associated with the presence of donor-specific chimerism in CD4 (6.7%) and CD8 (1.2%) T-cell subpopulation. Tolerance to alloantigens was confirmed *in vivo* by acceptance of the donor skin graft, and the immune competence of recipients was confirmed by rejection of third-party grafts. In contrast, a 35-day protocol of CsA monotherapy resulted in limb allograft rejection within two weeks after cessation of immunosuppression.

To further test the efficacy of short-term anti-$\alpha\beta$-TCR/CsA protocol, we investigated the effect of 21-, 7-, and 5-day protocols for chimerism development, allograft survival, and tolerance induction [39]. Indefinite limb allograft survival and functional recovery were associated with the presence of a stable level of donor-specific chimerism ranging from 10 to 12% in CD4 and 6 to 9% in CD8 T-cell subpopulation. Tolerance to donor antigens was confirmed *in vivo* by skin grafting and immunocompetence was confirmed by MLR assay. In this study, a combined anti-$\alpha\beta$-TCR/CsA protocol resulted in over 95% depletion of $\alpha\beta$-TCR-positive cells at, as early as, posttransplant day 7, and T-cell repopulation was present at 35 days after treatment cessation. The timing of deletional effect under 5-day protocol correlates with the maturation process of newly developed T cells (both from the donor and the recipient) in thymus, which takes approximately 28 days, and thus the short period of immunodepletion is sufficient to create a chronological window of unresponsiveness to the new repertoire of T lymphocytes [39]. We have confirmed that 5-, 7-, and 21-day immunodepletive protocols with anti-$\alpha\beta$-TCR/CsA resulted in long-term limb allograft survival, and we have chosen 7-day therapy as a standard immunodepletive protocol for tolerance induction in VCA. The rationale to choose 7-day protocol of $\alpha\beta$-TCR/CsA is the opportunity to use this protocol at the day of transplantation without recipient preconditioning and this

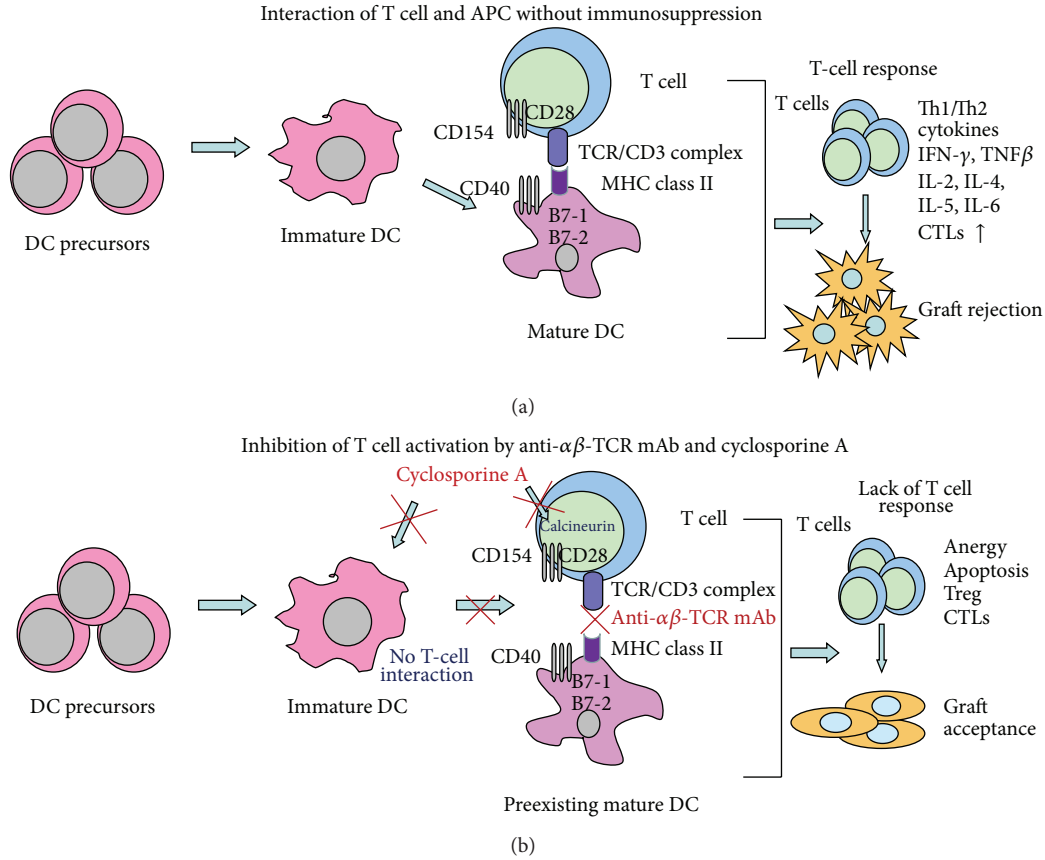


Figure 2: (a) Interaction of memory T cells and antigen pretention cells (APCs) without immunosuppression induced T-cell response and allograft rejection. (b) Selective targeting of $\alpha\beta$-TCR of TCR/CD3 complex inhibits the first signal of T-cell activation. Inhibition of immune response is enhanced by CsA, which inhibits IL-2 production by T cells and reduces expression of costimulatory molecules of dendritic cells. Lack of immune response by T cells facilitates allograft acceptance.

has the advantages of direct clinical application since in clinical VCA a preconditioning protocol rather will never be accepted.

Central (intrathymic) clonal deletion provides a robust form of tolerance in all chimerism-related approaches, even to the most immunogenic tissue, such as skin. Clonal deletion is usually considered superior to regulatory or anergic mechanisms since clonal deletion physically eliminates T cells with certain specificity [7]. To assess the role of thymus in tolerance induction in VCA, a series of experiments were designed using 7-day combined immunosuppressive protocol of CsA with T-cell depletion using anti-$\alpha\beta$-TCR/CsA in rat limb allograft model [33]. Allotransplants were performed between semiallogenic LBN donors and euthymic and thymectomized Lewis rat recipients without maintenance therapy. Treatment with $\alpha\beta$-TCR/CsA resulted in indefinite limb allograft survival (median survival time = 370 days) in euthymic recipients; however, a combined protocol of anti-$\alpha\beta$-TCR/CsA applied to thymectomized Lewis recipients should cover 51 days, the median survival time (MST) of limb allografts. In contrast, in control monotherapy groups with $\alpha\beta$-TCR or CsA in euthymic Lewis recipients, the MST of limb allografts was 13 and 22 days, respectively.

Stable T-cell chimerism of donor origin was achieved at 17.3% for CD4 and at 13.9% for CD8, in euthymic rats, whereas only transient chimerism, 7%–9% for CD4 and 2%–4% for CD8 T cells, was detected in the thymectomized rats. Immunoperoxidase staining confirmed engraftment of donor-origin cells into lymphoid organs (spleen, lymph nodes, and thymus) of the recipients in the euthymic rats under anti-$\alpha\beta$-TCR/CsA protocol. The morphology of many of the engrafted cells resembled that of dendritic cells. In contrast, in thymectomized limb allograft recipients, donor-origin cells were detected in the spleen and lymph nodes at the time of anti-$\alpha\beta$-TCR/CsA immunosuppressive protocol cessation but were absent in the lymph nodes, and only scattered cells were found in the spleen, at the time of allograft rejection.

This study confirmed that mixed chimerism ensures intrathymic T-cell deletion of donor-reactive cells, as long as chimerism persists. This is mediated mainly by bone-marrow-derived dendritic cells of both donor and recipient origins. In this limb allograft model, the constant delivery of bone marrow cells of donor origin was permitted from the transplanted limb containing both the femoral and tibial bones containing hematopoietic cells. Mixed chimerism

provides cells with an antigen-presenting function of both donor and recipient acting in the periphery and preserving recipient's immunocompetence to the third party antigens. In our experimental limb allograft model, MLR assay and skin grafting confirmed donor-specific tolerance in euthymic limb allograft recipients. Based on these observations, the authors suggest that the nonmyeloablative 7-day protocol of selective targeting of $\alpha\beta$-TCR-positive cells, in combination with CsA therapy, may facilitate engraftment of donor cells into the thymus, leading to negative selection of newly developing alloreactive host T cells. Both a central and peripheral mechanism may be involved in chimerism maintenance and tolerance to limb allograft.

A successful protocol of combined anti-$\alpha\beta$-TCR/CsA with selective depletion of potentially alloreactive T cells was also applied in a fully MHC-mismatched rat limb allograft model, making this short-term, nonmyeloablative VCA conditioning, clinically applicable. Tolerance to the limb allograft was associated with stable, multilineage, donor-specific chimerism in the T-cell population: CD4 (7.6%) and CD8 (1.3%), and chimerism maintenance was supported by the B-cell lineage (16.5% of $RT1^n$/CD45RA) [40].

In all limb allograft models, a vascularized bone component containing bone marrow cells of donor origin contributed to long-term femur allograft survival. Following revascularization, bone marrow cells migrated from the VCA donor and engrafted and repopulated in different tissues of the limb recipients, including the recipient's bone marrow compartment and, in this way, contributed to chimerism maintenance.

Our experience with VCA models has confirmed that reliable and stable chimerism, particularly in T-cell population, is a critical component for successful tolerance induction in VCA models without bone component, whereas more robust chimerism, in B-cell lineage, contributes to long-term survival when VCA contains vascularized bone compartment with donor hematopoietic cells.

*3.2. Immunodepletive Protocol for Single Components of VCA.* To test the effect of nonmyeloablative selective depletion of alloreactive T cells and to evaluate the contribution of skin and bone with bone marrow cells (single components of limb and face VCA) to chimerism induction, we have developed models of vascularized skin allograft from the groin region (groin flaps) [41] and unilateral and bilateral vascularized femoral bone transplantation [42–44].

*3.2.1. Immunodepletive Protocol in the Vascularized Skin Allograft Model.* Our other approach to tolerance induction via chimerism in VCA models was to test the efficacy of a short-term immunodepletive protocol using anti-$\alpha\beta$-TCR monoclonal antibody in combination with calcineurin inhibitors, either CsA or tacrolimus, in assessment of the vascularized skin allograft transplantation level [41]. The groin flap was used as an experimental model of VSA and was transplanted across full MHC barrier between ACI donor and Lewis recipients. In this model, immunosuppressive therapy was given for 7 days only, and the vascularized skin allograft was transplanted, without recipient conditioning. Under this protocol of anti-$\alpha\beta$-TCR/CsA, extension of skin allograft survival was observed up to 84 days posttransplant and was associated with the presence of donor chimerism of T-cell origin (4.7% in CD4 and 1.4% in CD8). Lifelong tolerance to the skin allograft was not confirmed; however, this observation indicates that the skin allograft, when transplanted alone, requires stronger immunosuppression than that when it constitutes a structural component of complex VCA, such as face or limb transplant [45].

The methods of manipulation of immune system which are applied for tolerance induction of vascularized skin components using donor hematopoietic cell transplantation and nonhematopoietic approaches, via T-cell depletion or costimulatory blockade, are reviewed by Horner et al. [24].

*3.2.2. Immunodepletive Protocol in Vascularized Bone Marrow Transplants (VBMTs) of a Single Component of VCA Containing Bone Marrow Cells.* Experimental limb allograft and face transplant models carrying bone component containing bone marrow cells (BMCs) are examples of vascularized bone marrow transplants (VBMTs). These models function as vascularized carrier of donor BMC, providing a continuous source of donor hematopoietic cell delivery, and are contributing to chimerism development and maintenance [46, 47].

The contribution of a vascularized bone marrow component in chimerism induction was investigated under our tolerogenic 7-day protocol of $\alpha\beta$-TCR mAb and CsA which was previously tested successfully in limb allograft transplants across an MHC barrier [44]. In this study, we documented that our protocol facilitated development of multilineage hematolymphoid chimerism via trafficking of the immature (CD90+) bone marrow cells (BMCs) between donor and recipient compartments. Early engraftment of donor BMCs into the recipient BM compartment was achieved at one week posttransplant and this was associated with active hematopoiesis within allografted bone and correlated with chimerism maintenance in the hematolymphoid organs in the thymus, spleen, and lymph nodes. Two-way trafficking between donor and recipient BM compartments was confirmed by presence of recipient MHC class I cells ($RT1^l$ cells) within the allografted bone up to three weeks posttransplant. At ten weeks posttransplant, decline of BMC viability in allografted bone corresponded with bone fibrosis and lack of hematopoiesis, and further studies documented that this was associated with osteopontin overexpression [48]. In contrast, active hematopoiesis was present in the recipient bone with predominance of donor-specific, immature (CD90/$RT1^n$) cells of B-cell lineage, which correlated with chimerism maintenance. The proliferative potential of donor-origin cells ($RT1^n$) was confirmed by clonogenic activity confirmed *ex vivo* by colony forming units assay. These results confirm that hematolymphoid chimerism develops early after-VBMT and is supported by T-cell lineage and, despite allografted bone fibrosis, chimerism maintenance is supported by B-cell lineage and presence of active hematopoiesis of donor-origin cells in bone marrow environment of allograft host [44, 48].

To enhance chimerism induction and maintenance, a bilateral VBMT rat model was created [42, 43]. The kinetics of peripheral blood chimerism revealed that presence of donor-specific cells showed a peak at 3 weeks aftertransplantation. The chimerism was characterized by the prevalence of donor-origin B cells which ranged from 15.7% to 26.9% (mean 24.2%). In the bone marrow compartment, 28.2% of donor-derived cells were detected, and most of the donor-origin cells (24.1%) revealed an immature phenotype (CD90 + /RT1$^n$+) which represents varying stages of bone marrow cell differentiation [43].

Two months after transplantation, peripheral blood chimerism declined to 1% for T lymphocytes and 1.5% for B lymphocytes, and these levels were maintained during the entire follow-up period of over 100 days posttransplant. In the host femoral bone marrow cavity, chimerism level was assessed at 10.4% and 3.7% of cells presented an immature phenotype of CD90 + /RT1$^n$+ which was associated with maintenance of stable donor-specific chimerism [43].

The studies on VBMT tested under immunodepletive protocols have proven the beneficial effect of donor bone marrow cells for chimerism induction in VCA transplants containing bone component with viable hematopoietic cells of donor origin. The coexistence of donor and recipient hematopoietic cells within the recipient bone marrow compartment leads to lifelong mixed chimerism maintenance in all hematopoietic cell lineages and permits the lifetime presence of antigen-presenting cells of both the donor and recipient origins supporting tolerance to newly developed lymphocytes.

In clinical experience, vascularized bone constitutes a structural segment of hand, arm, and some of the complex face transplants. In humans, macrochimerism after VCA transplants has never been reported and only transient microchimerism has been detected in the early posttransplant period, both in hand [49] and face transplant recipients receiving donor BMC as part of the posttransplant therapeutic protocol [50].

## 4. Protocols Supported with Donor Origin-Hematopoietic Cells for Chimerism Induction

Hematopoietic chimerism was first introduced by Owen when Freemartin cattle (fraternal twins sharing a placental circulation) were shown to be chimeric and tolerant to each other [51]. Acquired tolerance to allogeneic skin via chimerism induction by hematopoietic cell transplantation into neonatal mice was first reported by Billingham et al. [52]. Since that time, different strategies for tolerance induction via donor BMT have been developed in experimental studies and in clinical practice [53–59].

Vascularized bone with BMC was not yet clinically introduced as a supportive therapy of donor hematopoietic stem cells except in the cases where vascularized bone is an integral part of VCA (e.g., hand or face allograft). Based on the observation that VCA containing a bone with viable bone marrow compartment could function as a vascularized carrier of donor-origin bone marrow cells, providing a continuous source of donor hematopoietic cells, many experimental and clinical studies were developed for tolerance induction via chimerism. To test the beneficial effect of donor BMC for chimerism induction, we developed a new technique of intraosseous hematopoietic stem cell transplantation [53, 54].

In one study, we investigated the effect of intraosseous delivery of the selected population of donor-derived hematopoietic stem cell (HSC) CD90+ in rat hind-limb transplant model between Lewis-Brown-Norway and Lewis rats without immunosuppressive therapy [53]. Extended survival was achieved up to 15 days and was associated with 3.4% of donor-origin chimerism. In contrast, the control group without hematopoietic cell therapy rejected the limb allograft within 7 days [53].

The goal of donor BMT-based strategies for induction of transplant tolerance is to achieve the state when donor hematopoietic cells may reach the recipient thymus and promote negative selection of newly developed donor-reactive T cells [55]. We have tested, across the MHC barrier, the beneficial effect of intraosseous BMC delivery when compared with standard intravenous (i.v.) BMC transplantation. We discovered that hematopoietic recovery and efficacy of donor-cell engraftment into the BM and lymphoid organ compartments resulted in higher chimerism after intraosseous BMT (7.9% ± 1.3%) under $\alpha\beta$-TCR/CsA and $70 \times 10^6$ BM cells, whereas lower chimerism (4.2% ± 1.4%) was observed after intravenous BMT [54]. The seeding efficacy of donor cells into lymphoid tissues, including thymus, was greater after intraosseous BMT when compared with standard i.v. transplantation ($P = 0.007$) [54].

These observations indicate clinical applicability of a short-term immunodepletive protocol supported with donor bone marrow cells as a tolerance-inducing strategy in VCA.

Recently, the role of donor bone marrow cells for chimerism induction was reported in a rat heterotopic osteomyocutaneous flap model transplanted to a mixed allogeneic chimera [56]. Mixed allogeneic chimeras were created 4 to 6 weeks before osteomyocutaneous flap transplantation. Rats were subjected to total body irradiation with 600 to 300 cGy and transplantation of $100 \times 10^6$ T cells depleted with anti-$\alpha\beta$-TCR mAb bone marrow cells (day 0), followed by an 11-day course of tacrolimus and ALS (day 10) therapy. The long-term VCA survival was significantly better (57.1%) in chimeras receiving more than 300 cGy TBI and anti-$\alpha\beta$-TCR mAb as no long-term VCA acceptance was observed in animals treated with 300 cGy TBI without anti-$\alpha\beta$-TCR mAb preconditioning. Higher levels of chimerism, from 38.6% to 45.2%, were associated with VCA acceptance; however, the majority of flap acceptors lost peripheral blood chimerism within 6 months, but donor-origin cells were still present within the transplanted bone.

Clinical application of protocol utilizing hematopoietic cells for HLA-mismatched kidney transplantation underlines an immunologic benefit of donor bone marrow cells for transient chimerism induction and tolerance development to renal allograft [57]. However, this protocol requires conditioning therapies prior to donor BMT in order to induce chimerism and, clinically, is applicable only for living organ

donors. A recent report introduced simultaneous kidney and bone marrow transplantation from 5 HLA haploidentical living-related donors under modified nonmyeloablative conditioning [58]. In all patients, transient multilineage chimerism was observed up to two weeks after transplantation, but rapid development of tolerance to the kidney allograft was achieved in one of these patients [58]. In the VCA protocol, pretransplant donor-specific chimerism creation will never be applicable in clinic; however, simultaneous or posttransplant supportive therapy with donor bone marrow cells is clinically relevant, as demonstrated by its introduction during the first face transplant performed in clinic [50].

To reduce maintenance immunosuppression, infusion of unmodified donor hematopoietic cells has been recently introduced for hand transplant recipients at the University of Pittsburgh [59]. Long-term clinical and immunological results of the Pittsburgh protocol are awaited.

## 5. Immunosuppressive versus Immunodepletive Protocols and Chimerism Induction

The differences in chimerism levels observed in rodents, large animals, and humans are based on biologic variation between the species and are attributable to their genetic and developmental differences, which can involve innate and adaptive immunologic function and metabolic responses to various treatment protocols [6].

CsA monotherapy protocol induces chimerism in all types of VCA; however, over time, chimerism declines and this is usually associated with allograft rejection. Moreover, discontinuation of calcineurin inhibitor monotherapy always leads to allograft rejection 2-3 weeks after immunosuppression withdrawal as confirmed in rodent and large-animal experimental models [12, 13, 26]. Immunosuppressive protocol with calcineurin inhibitors is associated with donor cell engraftment in the spleen and the lymph nodes but not in the thymus of the recipients, even when VBM was a part of VCA [11, 23, 60]. The lack of donor-origin cells in the thymus of CsA-treated VCA recipients may reflect CsA-mediated lymphokine downregulation, and disruption of thymic function, which is essential for cell-homing and engraftment [61]. CsA therapy induces changes within the thymic microenvironment leading to a reduction in the size of the thymic medulla, decreasing the number of interdigitating cells, and changing morphology of the epithelial cells [61]. All these changes limit donor cell engraftment and thymic chimerism development. Lack of thymic chimerism under CsA protocol prevents development of tolerogenic T cells among newly developing lymphocytes, and a low dose of CsA maintenance therapy is necessary to prevent alloreactivity and to maintain allograft survival. However, a low, nontoxic dose of CsA maintenance protocol, used in VCA, is permissive for "prope" tolerance induction as reported in solid organ transplantations [62]. The pharmacologic result is an altered immune response, inhibiting the activation process of T cells by IL-2 production and by downregulating surface costimulatory molecule expression on rodent and human dendritic cells [63, 64].

However, chronic immunosuppression with calcineurin inhibitors is associated with a risk of leucopenia, nephrotoxicity, or infectious complications. Based on tacrolimus monotherapy applied in a heterotopic face allograft study in nonhuman primates, rejection-free allograft survival ranging from 60 to 177 days was reported. A major limitation of this immunosuppressive approach was that 5 of 6 animals developed a posttransplant lymphoproliferative disease (PTLD) without clinical evidence of graft rejection [65].

When comparing the chimerism level of different VCA models performed under CsA protocol, VCA tissue complexity and its immunogenicity should be considered [66]. We found that the hemiface model presented the highest chimerism level when compared to the total abdominal wall or vascularized skin allograft models. Skin is a major component of all facial VCA and serves as an abundant source of donor immunocompetent cells which migrate into the recipient periphery. The face-and-neck region in rats is very rich in lymph nodes, and we suggest that the presence of lymph nodes within VCA contributes to a high chimerism level in the peripheral blood and lymphoid organs of recipients. Moreover, in the rat facial allograft model, donor-origin hematopoietic cells present in the vascularized bone of mandible or calvaria actively participate in chimerism induction. Finally, mucosal tissue, combined with salivary glands, in the face transplant model, is also a rich supply of donor-origin cells represented by T cells, B cells, and NK cells, which are distinct from the cells present in peripheral lymphoid organs and, after transplantation, may support chimerism induction and maintenance [21, 23]. Thus, facial VCA differs significantly from a total abdominal wall or vascularized skin allograft models since these allografts include only skin and subcutaneous fat tissue components but lack the mucosal component. We found that the level of chimerism in skin allograft models correlates proportionally with skin allograft dimensions [9].

Multilineage, mixed hematopoietic chimerism is associated with lifelong central, deletional T-cell tolerance, permitting acceptance of any allograft of donor origin without immunosuppression [67]. In limb allograft and VBMT models performed under an anti-$\alpha\beta$-TCR/CsA immunosuppressive protocol, we have observed engraftment of donor-origin cells into spleen, lymph nodes, and thymus [33, 44]. These observations suggest that a short-term immunodepletive protocol facilitates development of intrathymic microchimerism, which may be permissive for tolerance induction [33]. Moreover, the beneficial effect of selective depletion with anti-$\alpha\beta$-TCR mAb is accomplished due to the lack of cytokine-release syndrome after drug administration and faster T-cell recovery, which reduces the chance of development of complications inherent to immunodepletive agents [28].

The immunodepletive induction protocol with antithymocyte globulin (ATG), methylprednisolone, and maintenance therapy with FK506 and rapamycin was used for heterotopic transplantation of facial allografts in cynomolgus monkeys [68]. Under this protocol, long-term facial VCA survival ranging from 6 to 129 days posttransplant was achieved but tolerance was not induced, indicating that further development of immunosuppressive protocols is needed

for nonhuman primate VCA models. In clinic, benefits of induction therapy with ATG or Campath-1 outweigh the adverse effects, especially when induction therapy is supported with calcineurin inhibitors or IL-2 signaling inhibitor [69]. However, these immunodepletive agents induce profound and durable lymphopenia that can be associated with adverse effects and immunodeficiency complications such as viral infections, CMV or EBV, or development of PTLD [70].

It is clear that none of the immunodepletive agents, neither nonselective nor selective T-cell depleters, are capable of acting as a single immunosuppressive agent. In our limb and face VCA models, induction therapy with an immunodepletive antibody, combined with CsA, significantly prolonged allograft survival and induced full or "prope" tolerance. Current experience with donor bone marrow transplantation, used as a supportive therapy in experimental VCA models, introduces viable strategies for tolerance induction which can be further refined and introduced to clinical cases of VCA such as hand or face transplants. The field of VCA transplantation is still open for introduction of innovative protocols such as our stem cell and chimeric cell therapies which have recently shown promising results in the face allograft model in rodents. These therapies may, in the near future, revolutionize the entire field of transplantation, including broad application of VCA.

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*Research Article*

# Heart Allograft Tolerance Induced and Maintained by Vascularized Hind-Limb Transplant in Rats

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Organ/tissue transplantation has become an effective therapy for end-stage diseases. However, immunosuppression after transplantation may cause severe side effects. Donor-specific transplant tolerance was proposed to solve this problem. In this study, we report a novel method for inducing and maintaining heart allograft tolerance rats. First, we induced indefinite vascularized hind-limb allograft survival with a short-term antilymphocyte serum + Cyclosporine A treatment. Peripheral blood chimerism disappeared 6-7 weeks after immunosuppression was withdrawn. Then the recipients accepted secondary donor-strain skin and heart transplantation 200 days following vascularized hind-limb transplantation without any immunosuppression, but rejected third party skin allografts, a status of donor-specific tolerance. The ELISPOT results suggested a mechanism of clone deletion. These findings open new perspectives for the role of vascularized hind-limb transplant in the induction and maintenance of organ transplantation tolerance.

## 1. Introduction

Organ/tissue transplantation has become a standard and, under certain circumstances, the single effective therapy for patients with otherwise incurable diseases, such as renal or heart failure. Due to the severe side effects caused by life-long nonspecific immune suppression required to [1, 2] maintain the allograft function, such as nephrotoxicity, infection, and tumor, donor-specific transplant tolerance was proposed to obviate these problems.

After Owen discovered the phenomenon that fraternal bovine twins who share the same placenta are born with and tolerant to erythrocyte from each other [3], Billingham and coworkers provided evidence for the feasibility of "actively acquired tolerance" with the experiments in mice and chickens, demonstrating that animals inoculated with homologous cells as fetuses are tolerant to the skin graft from the same donor in adulthood [4]. The phenomenon described previously is not a feature of only animals; human blood-group chimera in a twin was also reported [5].

Based on these studies, hematopoietic chimerism, which designates the coexistence of the hematopoietic cells from both recipient and donor after bone marrow transplantation, has been studied as a means to induce transplant tolerance. At the present, transplant tolerance of certain organs or tissues has been induced successfully by the induction of hematopoietic chimerism with different recipient conditionings (myeloablation or cytoreduction) or a "megadose" bone marrow transplantation in animals or humans [6–9]. However, although full chimerism in which donor-derived hematopoietic cells completely replace the counterpart of the host [10] always leads to transplant tolerance [11], dissociation between transplant tolerance and mixed hematopoietic chimerism has been reported [12, 13]. Additionally, "split

tolerance" has also been documented [14, 15]. In this type of tolerance, recipients are tolerized of a certain type of allograft while reject another type from the same donor, indicating an incomplete central tolerance of the donor pan-antigens.

Limb transplants usually include vascularised bone marrow intact with the hematopoietic microenvironment and stem cell niche. Emerging evidence indicates that sustained self-renewal of donor stem cells can enable protolerogenic mechanisms allowing successful weaning from immunosuppression under cover of short course regimens. In this study, we show that a short-term antilymphocyte serum + Cyclosporine A (ALS + CsA) treatment enabled indefinite vascularized hind-limb allograft survival, which induced secondary donor-strain skin and heart allograft tolerance.

## 2. Materials and Methods

*2.1. Animals.* Male Brown Norway (BN, $RT1^n$), Lewis (LEW, $RT1^l$), and August Copenhagen Irish (ACI, $RT1^{av1}$) rats, weighing 200 to 250 g, were purchased from Harlan Laboratories and used as donors, recipients, and third-party donors, respectively. Animals were housed under pathogen-free conditions at the University of Pittsburgh Animal Facilities according to NIH guidelines.

*2.2. Transplant Surgeries.* Orthotopic hind-limb transplantation (HLT) in this study was performed on day 0 as previously described [16]. Briefly, the hind-limb of BN rats was amputated at the middle level of the femur. Removal of the LEW recipients corresponding hind-limb was performed in a similar fashion. The donor and recipient femurs were joined with a 16-gauge needle as an intramedullary rod. The femoral artery and vein were anastomosed with 10-0 nylon. Vascularized skin/muscle (part of hind-limb with the bone component removed) transplantation was performed in a similar manner. The LEW recipients, whose primary hind-limb transplant survived over 150 days, underwent a secondary full-thickness skin transplant from both BN and ACI donors. Hind-limb, vascularized skin/muscle, and skin grafts were monitored daily after surgery for signs of rejection such as edema, change of color, and necrosis of the skin. Symptoms of graft-versus-host disease (GVHD) were also followed up. Rejection of hind-limb and skin allografts was defined as the necrosis of the tissue.

Intra-abdominal heart transplantation was performed as described by Ono and Lindsey [17] 50 days following secondary skin transplant. No immunosuppressive therapy was applied following skin and heart transplantation. Function of the transplanted heart was assessed by daily palpation of graft contractions through the abdominal wall. Rejection was defined as the complete cessation of myocardial contractions, which was confirmed at laparotomy.

*2.3. Experimental Groups.* For vascularized hind-limb transplant, five groups were employed: Group 1: ALS, day −4 and day +1, 0.5 mL, i.p.; Group 2: CsA, day 0–20, 3 mg/kg, ip; Group 3: CsA, day 0–44, 3 mg/kg, ip; Group 4, ALS + 21-day CsA, and Group 5, ALS + 45-day CsA. For vascularized skin/muscle transplant (VSMT), LEW recipients were treated with ALS + 45-day CsA as Group 6.

*2.4. ELISPOT.* To analyse the direct pathway response, purified splenic T cells (enrichment columns, R&D Systems) from LEW recipients were incubated with CD3-depleted, gamma-irradiated, splenic LEW, BN, or ACI APCs ($3 \times 10^4$ T cells + $2.5 \times 10^5$ APCs/well) in 96-well ELISPOT plates coated with IFN-gamma antibody. ELISPOT plates were developed 36 hours later following manufacturer's instruction (BD Biosciences).

*2.5. Flow Cytometry.* To monitor peripheral multilineage chimerism, blood cells were depleted of erythrocytes and incubated with PE-CD11b/c, FITC-RT1Ac (OX-27, Serotec), APC-CD3, and PE/Cy5-CD45RA.

*2.6. Histology.* Heart allografts were harvested 150 days after transplant. Tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) using standard techniques.

*2.7. Quantification of Donor-Derived Hematocytes by PCR Analysis.* For microchimerism analysis, peripheral blood was collected from the tail vein in ethylenediaminetetraacetic-acid-(EDTA-) containing tubes. Genomic DNA was prepared with DNeasy Blood and Tissue Kit (Qiagen, California, USA) following the manufacture's protocol. Specific primers (5′-CGCAGGGGATTTCGTATT-3′ p1; 5′-GGTGGGGACCTCCGTCT-3′) were used as described previously [18].

*2.8. Statistics.* GraphPad Prism Version 5 was used for statistical analyses. Results are expressed as mean ± SD. Unpaired 2-tailed Student's *t*-test and log-rank (Mantel-Cox) test were used for the statistical analysis. A $P$ value less than 0.05 was considered significant.

## 3. Results

*3.1. ALS Is Not Sufficient for but Essential to Long-Term Vascularized Hind-Limb Transplant Acceptance in This Model.* It has been well established that ALS achieves its immunosuppressive effect by bringing out a selective ablation of the population of recirculating lymphocytes, and the anti-lymphocytic antibodies in ALS are eliminated from the recipients rapidly. Based on our previous studies with ALS in rat vascularized hind-limb transplant, we injected ALS on days −4 and +1, whereby limb grafts were best protected. Since the efficacy of ALS on LEW recipients was different from batch to batch, we examined the absolute number of peripheral lymphocytes 3 days after injection. Only the batches that decreased lymphocyte number by >70% were used in this study. The mean survival time (MST) of vascularized hind-limb transplant in each group was as follows: Group 1: 9.3 ± 1.5 days; Group 2: 44.8 ± 5.5 days; Group 3: 77.0 ± 6.3 days; Group 4: 52.5 ± 5.7 days; Group 5: long-term acceptance (>350 days);

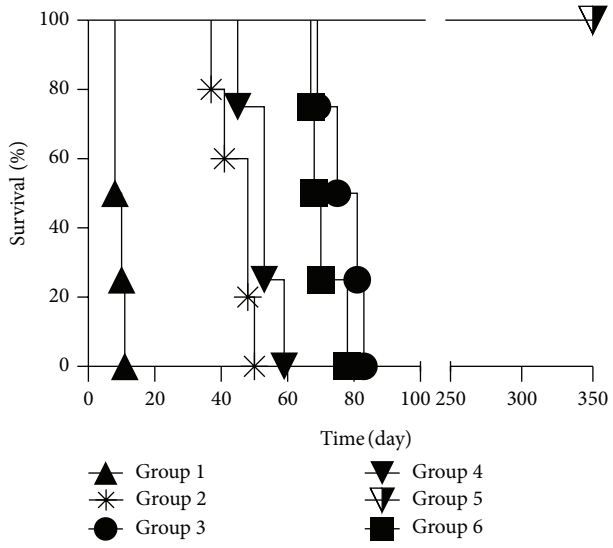



Figure 1: Vascularized hind-limb transplant survival time after surgery. ALS + 45-day CsA treatment induced vascularized hind-limb transplant tolerance. Other treatments failed to achieve long-term hind-limb transplant survival.

and Group 6: 70.8 ± 5.0. Two doses of ALS at days −4 and +1 were insufficient to induce long-term acceptance since ALS monotherapy could hardly prolong the vascularized hind-limb transplant survival. However, ALS was indispensable for vascularized hind-limb transplant tolerance induction as shown in Figure 1. Although CsA could suppress the rejection throughout the therapy, allograft necrosis due to rejection occurred shortly after the immunosuppressant was withdrawn (Figure 1).

*3.2. Bone Marrow in the Vascularized Hind-Limb Transplant Promotes Tolerance with a Short-Term Low-Level Peripheral Multilineage Chimerism.* Hind-limb is composed of skin, skeletal muscles, bone and bone marrow, and other soft tissues. Bone marrow cells, especially the stem cells, of donor origin are believed to give rise to peripheral hemocytic chimerism, which in turn promotes allograft tolerance. ALS + 45-day CsA treatment induced vascularized hind-limb transplant tolerance (Group 5) while failed in the case of vascularized skin/muscle transplant with MST of 70.8 ± 5.0 days (Group 6). Peripheral blood multilineage chimerism persisted for 12-13 weeks at a low level in Group 5 (Figure 2). At 13 weeks after vascularized hind-limb transplant, we could hardly detect peripheral blood multilineage chimerism by flow cytometry in 5 of 6 tolerant recipients in Group 5. At this point, we employed PCR to detect peripheral blood microchimerism. It turned out that even microchimerism had evanesced (data not shown). The only tolerant recipient with detectable peripheral blood chimerism at 13 weeks after transplant was completely dissociated from peripheral blood chimerism at 18 weeks after transplant (data not shown). After vascularized hind-limb transplantation, there were no clinical signs of GVHD in all groups until the end of this study.

*3.3. Duration of Immunosuppressive Therapy Plays a Critical Role in Vascularized Hind-Limb Transplant Tolerance Induction.* CsA has been widely used in transplantation medicine. It binds to cytoplasmic cyclophilin. Resulting complexes inactivate calcineurin, a crucial enzyme in T-cell receptor signalling. Calcineurin inhibition suppresses interleukin-2 (IL-2) gene transcription and thus inhibits IL-2 production of T cell. In this study, no clinical rejection turned up during CsA treatment. However, if CsA was administered from day 0 to 20, vascularized hind-limb allograft was rejected shortly after CsA was withdrawn, irrespective of ALS (Figure 1). In contrast, 45-day CsA + ALS induced long-term acceptance of the vascularized hind-limb allograft.

*3.4. Vascularized Hind-Limb Transplant Tolerance Induces Recipient Tolerance to a Secondary Skin and Heart Transplant from the Same Donor Strain.* To test the tolerance specificity, a secondary full-thickness skin transplant from both BN and ACI strains was performed when the vascularized hind-limb transplant survived >150 days. All BN skin grafts ($n = 6$) survived indefinitely while all ACI skin grafts ($n = 6$) were acutely rejected 10.0 ± 0.6 days after transplant, which proved the immunocompetence of the tolerant LEW recipients in Group 5.

To test the principle that vascularized hind-limb transplantation is capable of inducing tolerance to solid organs such as the heart from the same donor strain, we performed heart transplant from BN strain to tolerant LEW recipients in Group 5 when the vascularized hind-limb transplant survived >200 days. All heart allografts presented good function with minimum cellular infiltrate and no haemorrhage, edema, myocardial damage or signs of cardiac allograft vasculopathy (CAV) >150 days following transplant (Figure 3).

*3.5. Clonal Deletion Is a Potential Mechanism of Tolerance Maintenance in This Model.* To investigate the possible mechanisms by which tolerance to vascularized hind-limb, secondary skin, and heart allograft was achieved, we performed ELISPOT to detect clonal deletion. ELISPOT is a highly sensitive assay for detecting frequency of cytokine secreting cells, which are IFN-gamma secreting T cells in this study. The ELISPOT showed a significantly reduced frequency of donor-reactive T cells in the spleen of tolerized LEW recipients, compared with that of sensitized LEW recipients and naïve LEW rats as shown in Figure 4.

## 4. Discussion

Heart allograft rejection and lifelong nonspecific immunosuppression used to restrain the alloreaction remain the major obstacle to long-term survival subsequent to clinical heart transplantation [2]. Thus, donor-specific tolerance is deemed as the "holy grail" in the field of transplant. To our knowledge, we are the first to report successful heart graft tolerance induced and maintained by vascularized hind-limb transplant in a fully MHC mismatched rat model (BN to LEW). Tolerance induced in this study is donor specific and is associated with significantly decreased antidonor

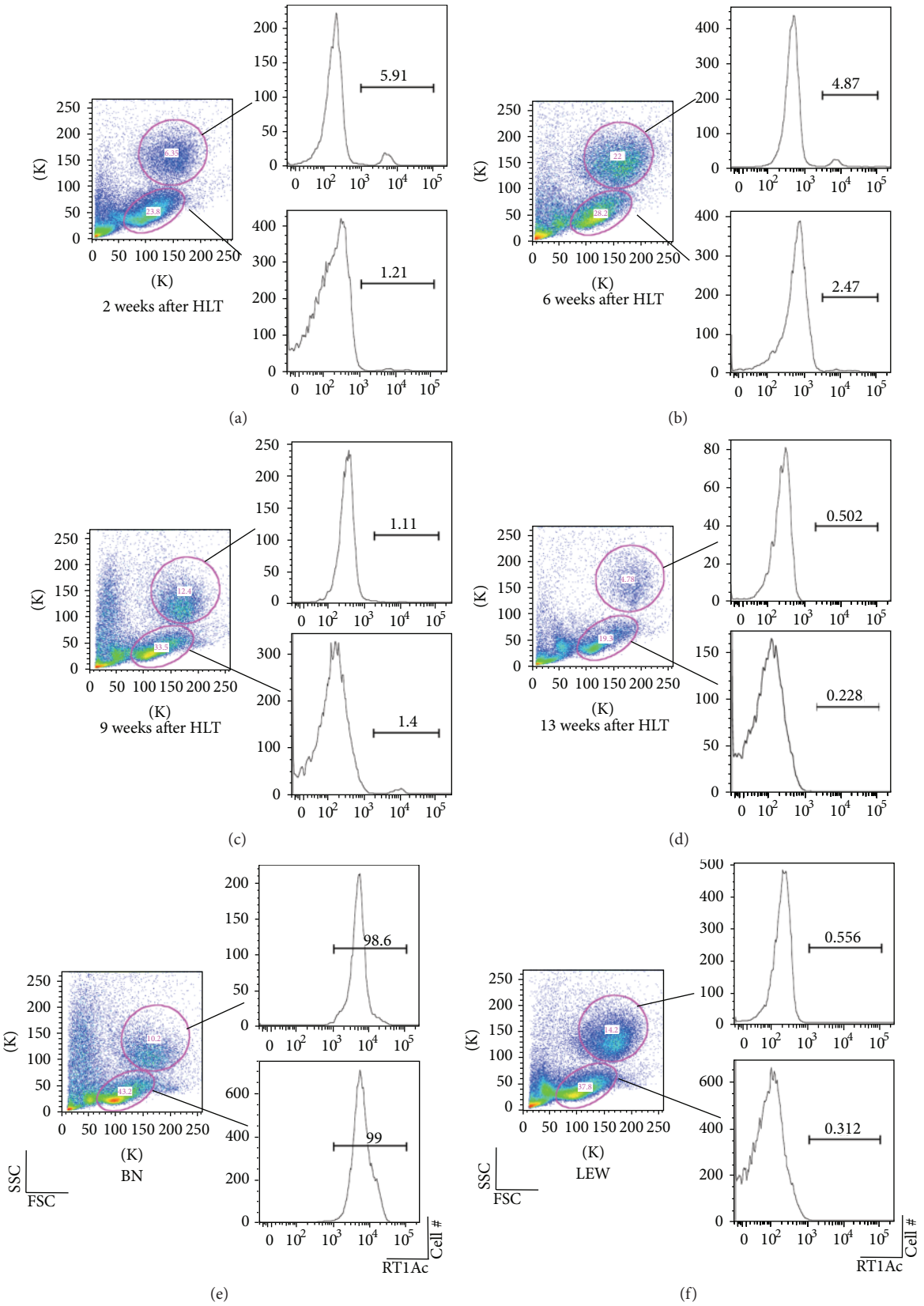
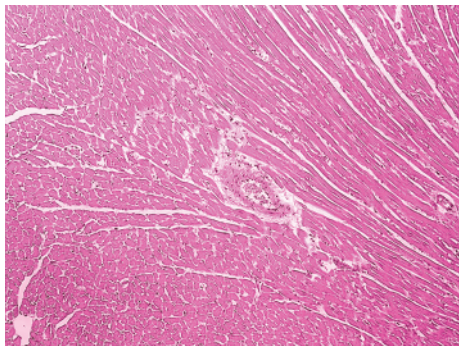
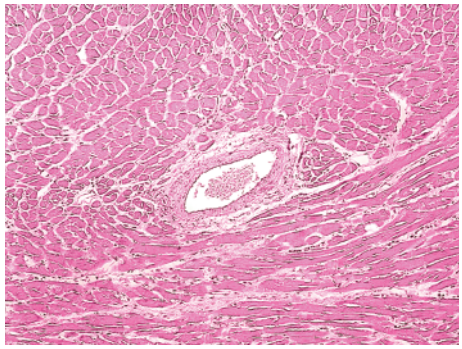



Figure 2: Peripheral blood multilineage chimerism in Group 5. Multilineage peripheral blood persisted for 12-13 weeks following vascularized hind-limb transplant, carried out by hind-limb transplant (HLT), at a low level in Group 5. At 13 weeks following vascularized hind-limb transplant, peripheral blood chimerism could hardly be detected by flow cytometry in 5 of 6 tolerant recipients in Group 5.



Naïve BN heart 100x

(a)



Tolerant heart allograft 100x

(b)

Figure 3: Histology of the secondary heart allograft. Secondary heart transplant from the same donor strain to tolerant LEW recipients in Group 5 was performed after the vascularized hind-limb transplant survived >200 days. Secondary heart allografts showed minimum cellular infiltrate and no haemorrhage, myocardial damage, or signs of cardiac allograft vasculopathy (CAV) >150 days following transplant. 100x.

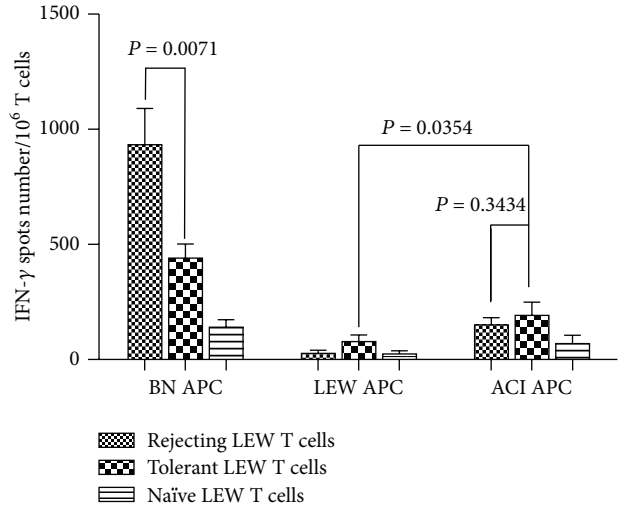



Figure 4: ELISPOT showed a significantly reduced frequency of BN-reactive IFN-$\gamma$ secreting T cells in the spleen of tolerized LEW recipients, compared with that of rejecting LEW recipients (933.3 ± 90.76 versus 440.1 ± 35.17, $n = 3$, $P = 0.0071$). In contrast, rejecting and tolerant LEW had similar frequency of ACI-reactive splenic T cells (151.1 ± 17.78 versus 191.1 ± 32.74, $n = 3$, $P = 0.3434$).

alloreactions. The most important difference between the current study and others' is that we achieved transplant tolerance without myeloid ablation in a more stringent and clinically relevant model.

Perisurgical lymphocyte ablation is thought to diminish the donor-reactive lymphocyte clones [19]. The fact that absence of ALS abolished tolerance induction, even though CsA was used 45 days after transplant, suggests alloreactive lymphocyte reductive conditioning is crucial for achieving the tolerant state in this experimental model. However, the diminished lymphocyte clone level needs to be maintained for a sufficient period of time by CsA. If no CsA or 21-day CsA was used in the current model, acute rejections displayed shortly after CsA withdraw, implying a pivotal role of this prolonged time window (21-day versus 45-day) for the recipients to be tolerized. Evidence emerged recently that peripheral plasmacytoid dendritic cells are able to contribute to immune tolerance through CCR9-dependent transport of peripheral antigens to the thymus and subsequent deletion of antigen-reactive thymocytes. However, this thymic clonal deletion may be prevented by infectious signals (toll-like receptor signals) [20]. It has also been demonstrated that donor-derived dendritic cells contribute to the central deletion of donor-reactive thymocytes in the recipient thymus [8, 21]. Our ELISPOT assay results are consistent with this concept. In addition, it has been well established that both early and late inflammations promote allograft rejection [22–25]. Thus, a 45-day CsA therapy may have provided sufficient time needed for inflammation caused by transplant surgery to dissolve and central deletion to happen in this study.

In conclusion, we established a novel method for inducing and maintaining heart allograft tolerance in the rat. The findings in this study may open new perspectives for the role of vascularized hind-limb transplant, especially vascularised bone marrow transplant, in the induction and maintenance of organ transplantation tolerance.

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*Review Article*

# Site-Specific Immunosuppression in Vascularized Composite Allotransplantation: Prospects and Potential

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Skin is the most immunogenic component of a vascularized composite allograft (VCA) and is the primary trigger and target of rejection. The skin is directly accessible for visual monitoring of acute rejection (AR) and for directed biopsy, timely therapeutic intervention, and management of AR. Logically, antirejection drugs, biologics, or other agents delivered locally to the VCA may reduce the need for systemic immunosuppression with its adverse effects. Topical FK 506 (tacrolimus) and steroids have been used in clinical VCA as an adjunct to systemic therapy with unclear beneficial effects. However, there are no commercially available topical formulations for other widely used systemic immunosuppressive drugs such as mycophenolic acid, sirolimus, and everolimus. Investigating the site-specific therapeutic effects and efficacy of systemically active agents may enable optimizing the dosing, frequency, and duration of overall immunosuppression in VCA with minimization or elimination of long-term drug-related toxicity.

## 1. Introduction

Since 1998, more than 200 patients have received vascularized composite allografts (VCAs). VCAs such as hand, face, or abdominal wall transplants are unique from solid organ transplants (SOTs) because of their heterogenous tissue composition that may include skin, muscle, vessels, tendon, nerve, lymph nodes, cartilage, bone, and bone marrow. Importantly, skin has been shown to be the most immunogenic constituent of certain VCA [1] mandating long-term immunosuppression for graft survival. However, transplantation of a whole limb allograft interestingly elicits a lower immune response than transplantation of individual tissue components, such as skin in the form of vascularized or nonvascularized grafts [1].

Despite evolving clinical experience and progress in the understanding of the biology of VCA, one of the main factors preventing wider acceptance or routine clinical application is the associated adverse effects of long-term immunosuppression. Antirejection therapy can lead to diabetes mellitus, nephrotoxicity, osteonecrosis, leukopenia, hypertension, hyperlipidemia, and opportunistic bacterial and viral infections [2, 3]. Since VCAs are non-life-saving procedures, the risks and toxicity of immunosuppression must be carefully balanced against their potential life enhancing benefits in recipients.

Unlike most SOTs, VCAs offer unique opportunities for local delivery of immunosuppressive medications directly to the graft. The rationale for such site-specific, transplant-targeted delivery of immunosuppression is to reduce systemic exposure and global collateral or end-organ adverse effects. Hypothetically, site-specific graft immunosuppression could facilitate minimization of overall dosing, frequency, and

duration of systemic immunosuppression and also help reduce the number of systemic drugs required for desired efficacy and improved graft survival.

Like in SOT, noncompliance with immunosuppressive medication is emerging as an imminent threat to long-term graft survival in clinical VCA. There has been at least one report of a VCA loss (1 hand has been explanted) due to confirmed medication noncompliance [4]. It may be argued that reducing the overall number or frequency of administration of systemic agents for graft maintenance by adjunctive use of site-specific graft immunosuppression may improve compliance with oral medications. The VCA graft is the trigger and the target of the recipient immune response. Advances in systemic immunosuppression have been based on insights into key pathways in donor or recipient allorecognition including adaptive or innate responses. Based on the specific cellular and molecular targets and mechanisms of action of various immunosuppressive agents, it may be expected that different agents (used topically or systemically) affect different pathways, components, or cascades of the immune response after VCA. In some cases, this may result in more specific immunosuppression, whereas in others it may result in diversion of the immune response from the allograft while preserving systemic immunity. Such innovation in maintenance immunosuppression has resulted in dramatic reductions in AR and improvement in short- and long-term patient and graft outcomes in SOT. Although true tolerance is still a holy grail, the reduction or elimination of some immunosuppressive agents as part of multidrug regimens with their long-term toxicities is a potential near term achievable goal. Such a goal could be realized in VCA by graft delivered therapies used adjunctively with systemic drugs. Site-specific delivery of agents that individually and selectively inhibit the immune trigger processes such as APC activation, T-cell priming, B-cell help, transendothelial migration of activated T or B cells, and regional lymph nodal mechanisms including early or ongoing antigen presentation could enable reductions in need for systemic immunosuppression by complementary, synergistic, or additive effects. Local agents may also prevent ischemia-reperfusion injury, in which leukocyte-endothelial cell interactions (as in rejection) are thought to play a key role. Optimal graft tissue concentrations/bioavailability of locally delivered drugs (mono or combination therapy) may indeed allow rejection control with lower systemic troughs compared to similar antirejection efficacy with higher overall systemic troughs secondary to mono- or multidrug maintenance therapy.

## 2. Route of Application

Several strategies exist for local delivery of immunosuppressive/immunomodulatory therapies in VCAs: (1) topical therapies are applied to the surface of the skin but do not effectively overcome the skin barrier, for example, therapies for psoriasis or atopic dermatitis; (2) transdermal therapies such as patches are more successful in overcoming the skin barrier and promote a local or systemic effect; (3) subcutaneous or intradermal (e.g., insulin injection, TB vaccination); and (4) intravascular delivery of the immunosuppressive drugs directly into the transplanted allograft [5]. In VCA, all of these strategies might be of potential interest and of significant relevance, depending on the immunosuppressive regimen and the predicated clinical situation. They can be also considered as adjunct or alternative to systemic treatment [6].

It may be a challenge to distinguish the unique effects of topical versus transdermal therapy. In certain cases, topical delivery can result in systemic levels that are higher or lower than expected. Topical calcineurin inhibitors and glucocorticoids have been the focus of several such studies. However, conclusions from these and other similar studies are limited by confounding effects of increased systemic absorption of the locally applied agents (due to locally inflamed or abraded skin) or failure to routinely monitor blood levels or the drug toxicity [7]. In studies with topical cyclosporine, low systemic levels have been reported [8].

*2.1. Topical Therapy.* The classical formulations used for topical therapy are ointments, crèmes, lotions, gels, and powders, which are widely used in the treatment of dermatological disorders. Topical calcineurin inhibitors were initially developed for the treatment of atopic dermatitis [9]. FDA-approved commercial formulations available include tacrolimus (Protopic), which is available as an ointment, pimecrolimus (Elidel), which is a cream, and clobetasol propionate, which is also a cream. Different reports suggest a beneficial effect in conditions such as seborrheic dermatitis, vitiligo, atopic dermatitis, lichen planus, or psoriasis [10–12].

Sirolimus (rapamycin) is a macrolide antibiotic, structurally similar to tacrolimus. It binds to FKBP-12 and affects the $G_1$ phase of the cell cycle by acting on a unique cellular target called mammalian target of rapamycin (mTOR) [13]. Sirolimus has been mainly used systemically in SOT, but topical treatment has also been reported in an experimental/preclinical setting in dermatology. It has been shown to have beneficial effects in conditions such as facial angiofibromas, psoriasis, and lichen planus, with either undetectable or insignificant blood levels [14–16]. Sirolimus lotion [16] has been used in oral lichen planus.

Mycophenolate mofetil (MMF) is an ester of mycophenolic acid (MPA). It selectively blocks proliferation of T cells and suppresses antibody formation by B cells [17]. There is only limited data that is available on topical MPA. Different MMF formulations as eye drop solutions and aqueous suspensions have been evaluated. In a corneal allograft model topical MPA showed no significant difference compared to vehicle only or no treatment [18]. However, Shoji et al. showed that topical MPA may be effective in an experimental model of allergic contact dermatitis [19]. None of the above-mentioned agents have been systematically evaluated as a topical therapy for VCA.

*2.2. Transdermal Therapy.* Transdermal patches that have been used for the delivery of drugs include but are not limited to nicotine, nitroglycerin, fentanyl, or estrogen. Different methods to optimize systemic exposure of other drugs have also been described. Prausnitz and Langer [20] proposed a categorization of transdermal delivery systems into three

generations: first-generation transdermal delivery systems are used for the delivery of small, lipophilic, low-dose drugs and have been clinically implemented [20]. Drug transport relies on diffusion through the stratum corneum and the epidermis into the dermis. Second-generation delivery systems increase skin permeability and driving forces to improve systemic delivery. Different methods have been introduced such as chemical enhancement [21], noncavitational ultrasound and iontophoresis [22]. Third-generation delivery systems rely on disruption of the stratum corneum and include combinations of chemical enhancers, biochemical enhancers, transfollicular strategies, microneedles [23], thermal ablation, microdermabrasion, laser electroporation (microporation), and cavitational ultrasound [20]. In this context numerous different patents have also been reported. To our knowledge, none of these novel methods of transdermal application have been evaluated either in translational models or clinical settings involving VCA.

*2.3. Intradermal/Subcutaneous Injections.* Several agents (drugs, antibodies) have been evaluated in dermatology for therapeutic efficacy via the intradermal/subcutaneous administration. The intradermal route is the preferred route for some vaccinations (such as BCG), while subcutaneous delivery is superior for some therapies (such as insulin). Efomycine M is a novel small molecule that blocks E- and P-selectin [24]. It has shown therapeutic efficacy in mouse models of psoriasis [25]. Hautz et al. could significantly prolong hind limb allograft survival in rats with weekly subcutaneous injections of efomycine M with short-course systemic antilymphocyte serum (ALS) and tacrolimus [24]. Local intragraft delivery of other leucocyte migration blockers such as anti-ICAM-1, anti-LFA-1, and the fibrin derivative B $\beta$ 15–42, which blocks VE-cadherin, has also been shown to prolong VCA survival with or without short-course systemic therapy.

*2.4. Local Therapy.* Local therapy aims at the utilization of drug administration systems to establish a more selective delivery of currently available nonspecific immunosuppressive agents directed at the vascular supply of the transplanted organ/graft through the spatial and temporal control of drug delivery, for example, via use of catheter delivery or Alzet miniosmotic pump systems [26].

## 3. Site-Specific Immunosuppression in Solid Organ Transplantation

Limited studies have evaluated the application of intravascular administration of immunosuppressive drugs in SOT [26–28], demonstrating a beneficial outcome of site-specific immunosuppression by delivering the immunosuppressive agents directly to the graft. Ruers et al. [29] introduced an implantable osmotic minipump delivering prednisolone with an arterial catheter to the renal allograft. Local application was superior to systemic application at a dosage of 4 mg/kg body weight per day, whereas i.p. or i.v. administration was ineffective at this dose.

Yano et al. [30] compared the effects of tacrolimus administered via different routes (hepatic artery, portal vein, and systemic circulation) in a rat liver allotransplantation model. In addition to systemic application of tacrolimus (0.08–1.28 mg/kg) for 7 days, a low dose of tacrolimus (0.32 mg/kg) was infused into the hepatic artery or the portal vein for three days. In contrast to additional infusions of systemic tacrolimus, local delivery led to dramatically improved allograft outcomes. Further, local delivery of immunosuppressants was superior to systemic delivery in cardiac [31] and pancreatic islet cell [32] transplantation models.

The lung offers a unique opportunity of directed local application of drugs, as the highly antigenic airway epithelium of the bronchial/bronchiolar passages is readily accessible for drug delivery. Nebulized cyclosporine delivered via inhalation to the lung has been used to treat episodes of AR as well as chronic rejection (CR) in lung transplants at the University of Pittsburgh. The beneficial effect of local delivery has also been shown with aerosolized cyclosporine as a rescue therapy for refractory AR [33, 34]. A randomized trial over a two-year period showed lower rates of CR and improved survival of lung grafts, but no difference in the incidence of AR [35].

Inhaled cyclosporine is associated with airway irritation clinically, requiring the addition of lidocaine to the preparation. Tacrolimus is more potent then cyclosporine, thus allowing for similar or superior efficacy with twenty to forty times less drug. Deuse et al. [36] presented a study aiming at elucidating the mechanism of inhaled tacrolimus using in vivo and in vitro models. Human airway epithelium was grown in vitro at an air-liquid interface in order to simulate inhaled and systemic application of tacrolimus. The aerosolized tacrolimus was capable of inducing higher tissue concentrations and lower blood concentrations than the systemic application of tacrolimus. Concentrations of tacrolimus in tissue from the trachea and the lung showed higher peak values after 1 hour if the animals had received aerosol treatment compared with oral gavage. The peaks, however, were followed by a rapid decline in tissue drug concentrations, and 24-hour trough tissue levels were approximately 2 times lower after TAC inhalation. In vitro IL-1$\beta$ was used to simulate an inflammation similar to AR or ischemia reperfusion. The inhibitory effects of tacrolimus on NF-$\kappa$B phosphorylation and nuclear translocation of human airway epithelial cells in the presence of IL-1$\beta$ could be clearly demonstrated, and a reduction of inflammatory cytokine secretion was observed after aerosolized tacrolimus. Airway epithelium absorbed tacrolimus from the aerosol and enabled effective inhibition of subepithelial lymphocyte activation.

To our knowledge, there is only one report of intra-arterial administration of immunosuppressive drugs in humans. Furtado et al. [37] reported two patients who received local immunosuppression. The first patient suffered a small bowel rejection after combined liver and small bowel transplantation. At 3 months after the initial transplantation a second small bowel transplant was performed from a cadaveric ABO identical, two-HLA-match donor. A 15 cm

segment of the middle colic artery was isolated in continuity with the superior mesenteric artery, allowing insertion of a catheter. The immunosuppressive treatment consisted of methylprednisolone and tacrolimus (0.12–0.06 mg/kg per day) via catheter for 3 weeks. The second patient received a small bowel transplant from a cadaveric ABO-identical, total HLA-mismatched donor. The catheter was inserted in the same way as mentioned into the middle colic artery and maintained for 7 weeks. Two major episodes of acute cellular rejections were treated with 250 mg boluses of methylprednisolone and elevated doses of tacrolimus (dosage 0.08–0.02 mg/kg per day). Both patients could be discharged from the hospital and continue work and education. These two cases illustrate that in very critical patients local intraarterial immunosuppressive treatment is feasible.

## 4. Site-Specific Immunosuppression in VCA

The skin is the largest organ in the body [1, 38] and is a physical and immunological barrier [39, 40]. It is the most immunogenic component of a VCA [1]. In hand transplantation the early signs of clinical rejection are seen in the skin. Pathologically, this may correlate with differential degrees of cellular infiltrates in the epidermal-dermal or adnexal structures as classified by the Banff system for VCA. [41, 42]. The phenomenon of "split tolerance" is associated with indefinite survival of the musculoskeletal portion, but rejection of the epidermis of VCA and has been reported in animal models [43]. It seems to be logical therefore to locally target the skin and avoid high degree of overall systemic immunosuppression [44]. However, the cumulative experience on the use of graft-delivered therapies in clinical VCA such as hand or face transplants is varied and inconsistent, leading to debate on the practical utility of such strategies.

Over 90 upper extremities and 20 face transplantations have been performed worldwide in the past decade [45]. Most AR episodes have been amenable to bolus systemic steroids, changes to systemic immunosuppression with or without interventions such as extracorporeal phototherapy and topical tacrolimus ointment, and/or steroid (clobetasol) creams used PRN as adjunctive therapy [45]. In hand transplantation, there have been reports of topical tacrolimus and clobetasol resulting in adequate to complete response during Banff Grade 1 and 1-2 rejection episodes [46]. Despite their frequent use, the efficacy of local immunosuppression currently remains unproven in clinical VCA, due to the challenge of designing carefully controlled studies or implementation of such treatment per established protocols that rely on clinicopathologic correlation [47].

In preclinical studies, topical tacrolimus has been shown to prolong graft survival in hind limb allotransplantation models as well as in face transplantation models [7, 48]. Topical tacrolimus did not lead to elevated blood concentrations, but a 100-fold higher concentration of the drug was observed in the skin versus underlying tissues [8]. Compared to topical steroids, which can result in collagen atrophy and skin thinning, tacrolimus has few local adverse effects [49]. Compared to cyclosporine, which has also been evaluated in topical treatment, tacrolimus has been shown to have superior effect due to its higher potency. Different mechanisms of action of topical tacrolimus and pimecrolimus have been suggested in the literature. There may be a depletion of inflammatory dendritic epidermal cells [50], or a reduction of costimulatory molecules on dendritic cells and alteration of their function [51, 52]. Additional mechanisms include apoptosis-induced depletion of T cells [53] and reduced expression of adhesion molecules [54].

Shirbacheh et al. [55–57] were the first to describe intraarterial delivery of immunosuppressive drugs in VCA. These pioneering studies involved calcineurin inhibitors in an experimental large animal VCA limb transplant model to correlate tissue and local pharmacokinetics with systemic trough levels and adverse effects [55, 58]. The conclusion was that tacrolimus is pharmacokinetically inferior to cyclosporine. Despite its demonstrated efficacy in experimental and clinical transplantation, our Findings suggest tacrolimus would not be an appropriate immunosuppressant to be delivered via the i.a. route for prevention of limb allograft rejection.

The monitoring of a clinical VCA such as a hand or face transplant has conventionally relied on protocol skin biopsies and those biopsies mandated by clinical signs of rejection. Deeper tissues (muscle, nerve, vessel and bone) have not been biopsied on a regular basis in VCA. Based on these paradigms, there are several questions that remain to be answered in VCA that could impact the overall relevance of topical immunosuppression in VCA.

(1) What are the temporal kinetics and dynamics of rejection of the various tissue components of VCA after transplantation?

(2) Is clinical and histopathologic resolution of rejection in skin (upon systemic or topical intervention) associated with similar outcome in underlying tissues?

(3) Is continuous topical treatment superior to systemic therapy by augmenting local tissue concentrations at the trigger site of the immune response?

## 5. Development of New Topical Formulations: Promise and Potential

Tacrolimus and clobetasol are available for topical administration as they are commercially available with FDA approval for other indications. Other immunosuppressive drugs do have applications in dermatology and have only been used systemically, or in experimental skin formulations. With the intention to develop new immunosuppressive formulations, we started an in vitro study comparing novel formulations of topical MPA and sirolimus to commercially available tacrolimus and clobetasol preparations. Our goal was to evaluate the local release, tissue bioavailability, and pharmacokinetics of these immunosuppressive drugs. For the in vitro studies, a semipermeable membrane simulating the skin barrier was used in Franz Diffusion Cells. Preliminary results showed that less than 10% of the original dose of tacrolimus penetrates the membrane. In contrast, approximately 10% of the initial dose of sirolimus diffuses across the membrane.

With MPA, 38% of the initial applied dose diffuses into the recipient compartment. This diffusion was time dependent and continued to occur past 24 hrs. Based on in vitro data, we hypothesize that if used at the right dosing and frequency, both sirolimus and tacrolimus could achieve good local concentrations in the skin, with lower levels in deeper tissues and minimal systemic drug exposure. MPA on the other hand appears to have the greatest potential for diffusion across the membrane in vitro. This suggests that topical application of MPA will result in higher concentrations of the drug in muscle and other local sites with possibly some increase in systemic concentrations as well. Controlling the concentration of MPA in the formulation and alterations in the frequency of dosing could further minimize systemic drug exposure after topical application of MPA.

## 6. Conclusion

Graft-targeted and delivered site-specific immunosuppression is uniquely suited for VCA due to direct accessibility to such interventions. Local graft manipulation with targeted preloading of immunosuppression may also have potential benefit in supplementing or reducing the intensity of systemic induction therapy. Such a strategy may be customized to deliver high or sustained concentrations of drugs, antibodies, biologics, and other molecules, which may not be feasible via the systemic route due to short half-life, inactivation due to first pass hepatic metabolism, or enzymatic degradation. The additive, adjuvant, complementary, or synergistic role of locally administered immunosuppression in conjunction with systemic delivery cannot be underestimated in VCA with their accessible cutaneous or mucosal components amenable to such innovative delivery techniques. The minimization of need for multiple drugs, and their dosing, frequency, and duration of treatment with concomitant toxicity of such systemic therapy by site-specific therapies needs further investigation and collaborative inquiry especially in VCA which are not life-saving grafts such as SOT, but life-enhancing procedures. Potentially, targeting distinct mechanistic pathways and molecular targets in the skin immune system with combination of site-specific immunosuppression may facilitate an immunomodulatory environment that may indeed induce a permissive milieu for the development of tolerant VCA.

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*Review Article*

# Review of the Early Diagnoses and Assessment of Rejection in Vascularized Composite Allotransplantation

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The emerging field of vascular composite allotransplantation (VCA) has become a clinical reality. Building upon cutting edge understandings of transplant surgery and immunology, complex grafts such as hands and faces can now be transplanted with success. Many of the challenges that have historically been limiting factors in transplantation, such as rejection and the morbidity of immunosuppression, remain challenges in VCA. Because of the accessibility of most VCA grafts, and the highly immunogenic nature of the skin in particular, VCA has become the focal point for cross-disciplinary approaches to developing novel approaches for some of the most challenging immunological problems in transplantation, particularly the early diagnoses and assessment of rejection. This paper provides a historically oriented introduction to the field of organ transplantation and the evolution of VCA.

## 1. Organ Transplantation

The concept of replacing organs or limbs that have become diseased or damaged is a deeply rooted human dream so old that it has been incorporated into our mythology in chimeric beings like the Hindu Ganesha [1]. The oldest recorded attempted transplant was the use of skin from a donor to conduct a reconstructive rhinoplasty on another man, performed by the classical Indian surgeon Sushruta, sometime between 1000 and 600 BCE [2–4]. Throughout the ages surgeons have attempted transplantation time and again, but it was not until key contributions from Medawar, Brent, and Billingham at the turn of the 20th century that real progress in understanding the biology underlying host-allograft interactions was made [5–7]. At approximately the same time, important insights into the circulation and role of lymphocytes in immunologic response were being made [8–12]. This essential work came on the heels of important early descriptions of lymphocyte activity in inflammation [13–16].

As the scientific foundations for transplant biology rapidly evolved, the first successful kidney transplant between identical twins was conducted in 1954 [17, 18]. Although a surgical success, little immunologic information was generated because the transplant was not an allograft (or homograft). The monozygotic twins were genetically identical and therefore shared the same major histocompatibility complex (MHC). Rejection rarely occurs in such cases. The identical twin transplant of 1954 was an isograft, immunologically closer to an autograft than an allograft, and the potent issues of allogenicity were left unresolved. It would not be until the 1960s that appreciable graft survival was achieved in MHC mismatched patients [19–22].

Throughout the 1960s and 1970s, attempts to control rejection included irradiation of the recipient to neutralize the host immune system [12, 23–27], the administration of azathioprine [19–22], and eventually treatment with antilymphocyte globulin (ALG/ALS) [28–32]. Although these were shown to have beneficial effects on graft survival, morbidities were extensive [33–35], rejection was still a threat [36], and graft-versus-host disease would sometimes overtake patients [37–44].

With the arrival of cyclosporine in the late 1970s, a new era in the clinical viability of transplantation as a therapeutic intervention dawned. Significant improvements in outcome and graft survival were achieved first in liver [45], then in kidney [46] patients. A new class of immunosuppressant cyclosporine was powerful enough to provide the high levels of immunosuppression required for managing transplants, with fewer of the morbidities associated with prior treatment regimens.

However, these improvements came with a price. Cyclosporine was shown to be nephrotoxic over time [47–49], and care still had to be taken to avoid the morbidities associated with a suppressed immune system, such as infection [50]. Despite these drawbacks, the level of clinical improvement cyclosporine offered over previous methods was very compelling, and cyclosporine fueled much of the explosive growth in transplantation during the 1980s and beyond [51–53].

In late 1987 a report from Japan introduced FK-506 (Tacrolimus) as a new and potent immunosuppressive agent [54]. Additional studies rapidly followed in more animal models, confirming FK-506's effectiveness in suppressing and rescuing grafts from rejection [55–61]. Synergistic effects with cyclosporine were also observed [56, 62]. The potency of FK-506, and its synergistic effects with other drugs, would open the door for future therapeutic strategies to leverage immunosuppression dosage as a controller for modulating the tolerance/rejection balance in transplants [63].

The search for cyclosporine's mechanism of action began almost immediately after it was shown to have clinical promise, but it was not until after the introduction and clinical adoption of FK-506 in the early 1990s that both FK-506 and cyclosporine were discovered to inhibit the calcineurin phosphatase pathway [64–67]. Further studies rapidly elucidated additional mechanism details in subsequent years.

Although mainstream clinical practice had vigorously adopted high-dose combination immunosuppression therapy as the treatment of choice because of the specter of rejection, in 1992 the notion that more immunosuppression was not necessarily better emerged. A group of patients were discovered to have become chimeric or developed tolerance towards their allograft [68], helping to elucidate the fact that allografts carried passenger leukocytes that conducted an immune response against the host; much as the host carries out an immune reaction against the allograft [69]. This became known as the double-immune response or clonal exhaustion and deletion [70]. Further investigation of these cases revealed that moderate levels of immunosuppression, carefully timed and tailored to each individual, were at least partially successful in eliminating patient dependence on lifelong immunosuppression [71]. Prior to these observations the clinical view was that the immune response needed to be quashed as early and completely as possible, in order to prevent the leviathan of rejection from emerging. However after the chimeric patients were discovered, the door to the consideration of more nuanced application of immunosuppression was opened.

Organ transplantation has evolved from an essentially nonexistent field to one of the most prominent disciplines in medicine over the last sixty years.

## 2. Vascularized Composite Allotransplantation

In 1998 the first human hand transplant under current clinical standards of immunosuppression was conducted, making vascularized composite allotransplantation (VCA) a performed clinical reality. Over the past decade it has become a treatment option for the many patients suffering from complex tissue injuries or defects not amenable to conventional reconstruction [72]. More than 60 hand/forearm and most recently arm transplants as well as 90 hands and over 20 face transplants performed throughout the world have also shown that allograft survival with good functional outcomes can be routinely achieved after VCA [73–77]. However, despite the fact that surgical procedures and functional outcomes are highly successful, the need for long-term and high-dose immunosuppression to enable graft survival and to treat/reverse acute rejection episodes are the remaining and pace-limiting obstacles to widespread application [78, 79]. The toxicity profile of such drug treatment is considerable and includes serious side effects, such as opportunistic infections, malignancy, and end organ damage [80–83].

VCA recipients are unique in that they undergo a transplant procedure for what is considered to be a nonlife-threatening condition. Therefore, there is a critical need to develop immunosuppression minimization strategies to reduce the risks of chronic immunosuppression.

The skin is the principal target of rejection after VCA transplantation, making it an obstacle to tolerance induction or minimizing immunosuppression. On the other hand, due to its external location, the skin provides a unique clinical opportunity for monitoring, early diagnosis, prevention, and treatment of VCA rejection, including the possibility of therapies applied directly/topically to the skin.

Acute rejection in hand transplantation appears with maculopapular skin lesions, which can be limited to a small area of the skin or can spread over large parts of the transplant [74, 75, 84–87].

Clinical macroscopic manifestations can range from mild pink discoloration or erythema to lichenoid papules, edema, and onychomadesis. The main histological feature of acute rejection is a mononuclear cell infiltrate. It first appears in the perivascular space of the dermis and then spreads to the interface between dermis and epidermis and/or adnexal structures. A perivascular, cellular infiltrate within the epidermis is typical for a moderate grade of rejection with the immunologic response reaching the outermost

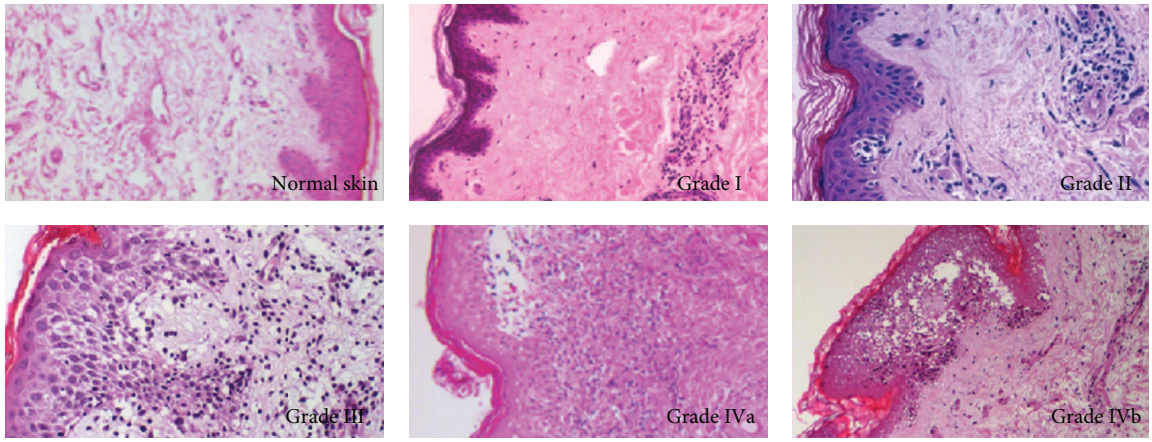


Figure 1: Banff Grading of Acute Skin Rejection in VCA; Allograft histology rejection grades. Grade 0: no or rare inflammatory cells, Grade I: mild perivascular infiltration. No involvement of overlying epidermis, Grade II: moderate. Perivascular inflammation with/without mild epidermal or adnexal involvement (limited to spongiosis and exocytosis). No epidermal dyskeratosis or apoptosis, Grade III: dense inflammation and epidermal involvement with apoptosis, dyskeratosis, and/or keratinolysis, Grade IV: necrotizing acute rejection. Frank necrosis of epidermis or other skin structures.

layer. If rejection is not successfully treated at that stage, necrosis of single keratinocytes can be observed, resulting in focal dermal-epidermal separation and significant graft damage [84, 86, 87]. If rejection progresses further, necrosis and loss of the epidermis, as the ultimate stage of skin rejection, are considered irreversible. However, very limited information is available on the involvement of components other than the skin in this acute rejection process [86]. The histological findings in VCA patients are in line with results from experimental studies indicating that the skin is highly immunogenic and hence the primary/sentinel target for rejection. This is further substantiated by the fact that immunological tolerance can be achieved towards all components of a VCA experimentally except the skin. It was also shown that skin alterations in a VCA are not exclusively limited to alloimmune-mediated injury. The clinical and histopathological features of immune-related and nonrejection processes are potentially overlapping or may coincide with acute rejection. The underlying mechanisms are largely unknown and represent a current major clinical challenge in differentiating between acute rejection and other forms of skin inflammation.

## 3. Cytokines in the Study of Skin Rejection

Skin rejection is becoming increasingly useful as a platform to study rejection because it is easy to access and can be monitored more consistently than internal organs during the process of rejection. Because of its high immunogenicity skin is a VCA that is prone to frequent and sudden episodes of rejection, much more so than other tissues such as muscle, making it a clinically important tissue to investigate from the perspective of VCA. Insight and understanding of the dynamics of rejection in skin will likely be elucidative for other tissues and lead to a more complete picture of immune system function under conditions of rejection.

The Banff 97 Working Classification of Renal Allograft Pathology [88] provided a uniform basis for the grading rejection in allograft biopsies. It has been subsequently updated most recently by Banff 07 Classification of Renal Allograft Pathology: Updates and Future Directions [89]. Grading schemes relevant to skin and VCA were also defined in The Banff 2007 Working Classification of Skin-Containing Composite Tissue Allograft Pathology (Figure 1) [85].

Interestingly, in our recent unpublished study, in a rat hind limb allograft model we observed a differential rejection pattern in the animals receiving a long-last form of IL-2, IL-2/Fc fusion protein, in combination with antilymphocyte serum and cyclosporine A. Despite all animals undergoing early acute rejection, approximately 55% of them spontaneously recovered and went on to long-term survival for more than 200 days. Moreover, the cytokine and FoxP3 gene expression profiles from the skin biopsy at the earliest sign of rejection revealed a significant increased ratio of FoxP3 expression versus Granzyme, IFN-$\gamma$, and Perforin in the animals that spontaneously recovered (benign rejection) as against the animals who had a lower FoxP3 expression that went onto grade 4 rejection (progressive rejection). It suggested that, based on cytokine gene expression profiles from skin biopsy at the earliest sign of rejection, it may be possible to predict the ultimate course of the rejection and provide evidence for a proper treatment (paper in preparation).

## 4. Similarities in Early Skin Rejection and Other Sources of Skin Inflammation

Skin rejection in VCA is presented with erythematous macules that may progress if not treated to infiltrated scaly violaceous lichenoid papules covering the complete surface of the graft [90]. These alterations are not specific for rejection and may mimic inflammatory dermatoses. Kanitakis et al. emphasized the diagnostic challenges in early or mild skin rejection. Early rejection (grades 1 and 2) can be especially

difficult to differentiate from contact dermatitis, insect bites, or dermatophyte infections. It is notable that histologic lesions such as eosinophilia, leukocytoclastic vasculitis, and demonstration of infectious antigens can indeed lend specificity to pathologic diagnoses. While the geographic limitation of lesions to the skin of the allograft can be an important and helpful hint, atypical cases of skin rejection with regard to the anatomical site, progression, or the clinical manifestation have been described [91] and the location alone cannot be considered proof. Early and accurate diagnoses, however, are critical to either prevent progression of rejection or incorrect treatment of the patient.

Parallels between acute skin rejection and inflammatory dermatoses (e.g., contact dermatitis, psoriasis, and atopic dermatitis) also exist on the molecular and cellular levels. Allergic contact dermatitis, for example, is a T-cell-mediated-delayed-type hypersensitivity reaction that occurs upon hapten challenge in sensitized individuals [92]. Therefore, the differentiation mainly based on histological and macroscopic criteria can be difficult. It has been demonstrated that T cells (CD4+ and CD8+ cells) are critical effectors and that elements of the innate immune system (e.g., natural killer cells) may play a key role [93]. Epidermal Langerhans cells as the most powerful antigen presenting cells in skin as well as keratinocytes are regulating this inflammatory process. Cytokines derived from Langerhans cells (e.g., IL-12) and from T-cells (IFN-gamma, IL-4, and IL-10) play a pivotal role in the induction and initiation of this common skin disease [92, 94].

In recently collected unpublished data, cytokine expression patterns associated with rejection-associated inflammation versus non-rejection-associated inflammation in full thickness skin (FTS), vascularized heterotopic skin-muscle-bone (SMB) composite allografts, and hind limb composite allografts are consistently and significantly different. In this model SMB can be engrafted under routine continuous immunosuppression; however, FTS will still be acutely rejected. Through multivariate analysis it was clearly observed that distinct immune signaling patterns mediate rejection in SMB versus FTS. Specific cytokines were observed as the primary drivers of these distinct patterns, and the biological functions of those cytokine ensembles were then elucidated and correlated with the numeric analysis to reveal that rejection-associated inflammation followed clearly different patterns in SMB and FTS [95] (Figure 2, paper in preparation).

## 5. Alternative and Experimental Methods for Detecting Rejection

Interest in finding a better means of detecting or predicting rejection has spawned a range of research approaches. Although these methods have not yet found widespread clinical adoption, the approaches and technical innovations are informative with regards to how challenges faced by the field are being overcome.

Utilizing little or no tissue data, the psychiatric analysis described by [96] concluded that although the features measured could be used to identify certain risk factors for rehospitalization, they were not predictive of rejection specifically. Rehospitalizations were due to a variety of causes, including immunosuppression-associated infection. One of the most predictive factors for rehospitalization included patient noncompliance with medication instructions.

A significant amount of ongoing research is being invested in finding genetic markers for rejection. The most promising results to date have come from [97] showing correlation between miRNA coding for cytotoxic proteins and rejection as well as [98] showing strong correlation between donor gene fragments in circulating blood and the progression of rejection. However, in the presented results there is a high degree of variance in key metrics measured, and the detection of rejection is thought to occur at the onset of graft damage. This may eventually provide an improvement over current clinical standards by reducing unnecessary biopsies and may eventually become a platform for more advanced miRNA-based analytical methods. Additional work in the area of genetic rejection detection has been done by [99, 100].

Cellular analysis is perhaps the most popular alternative approach to assessing rejection. A large number of biomarkers have been identified and catalogued [101] however, *in-vivo* most biomarkers suffer from high false positive rates or are not cost-effective to assess. For kidney transplant cases, [102] describes a method that is a reliable indicator in about 62% of studied cases. [103] identifies cells associated with rejection in circulating blood, but like [98], these cells provide limited predictive value beyond what may be achieved by pathologist examination of a biopsy.

Doppler tissue imaging as described by [104] may eventually provide a noninvasive alternative to heart biopsy. As described, the system is capable of providing 82% sensitivity and 53% specificity, although it does not confer predictive power.

Significant recent advances in proteomic analysis have been made by [105] who proposed a breath-test for heart transplant rejection that is capable of providing 71.4% sensitivity and 62.4% specificity. Excellent performance in predicting corneal transplant rejection was shown in [106] with the application of linear discriminant analysis to selected cytokines, reinforcing the potential clinical or diagnostic utility of computational and statistical inference methods.

## 6. Computational and Statistical Inference Literature

The analysis of systems that contain multiple dependent variables, unknown influencing factors, and context dependence presents an especially challenging problem to traditional methods of analysis such as ANOVA or other univariate methods of analysis. To elucidate the actual behavior of complex systems, and to build models with predictive power, more advanced methods of computational and statistical analysis are required. Concise and thorough coverage of the statistical inference and modeling methods that are extensively used in medicine and computational methods of

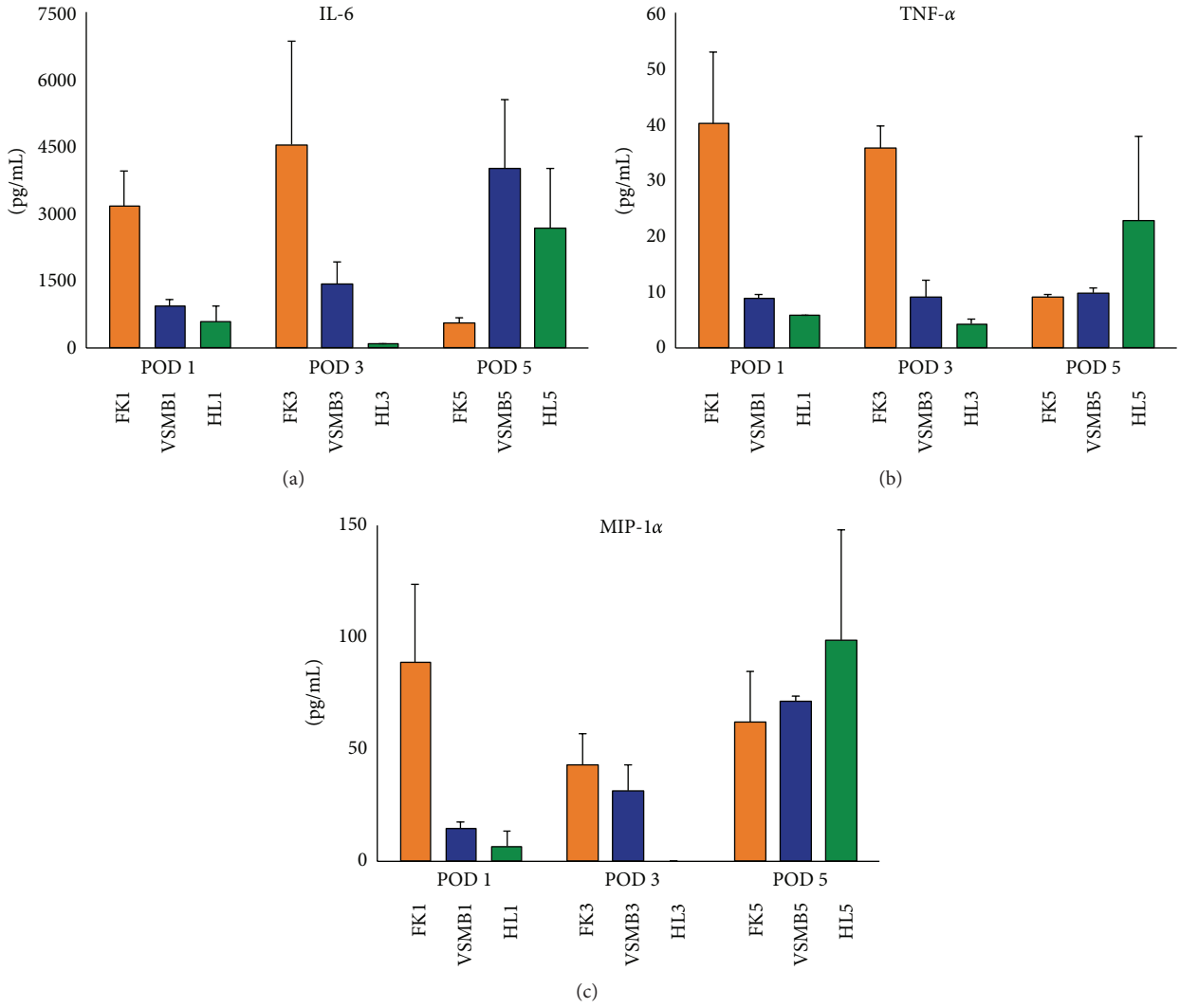


Figure 2: IL-6, TNF$\alpha$, and MIP-1$\alpha$ are highly expressed from fullness skin (FS) allografts in comparison with that from hind limb (HL) and vascularized skin muscle bone (VSMB) allograft at POD 1 and POD 3.

biological analysis is given in [107–110]. Both discriminative and generative methods are important analytical tools in analyzing biological data. Discriminative methods are often able to produce classifiers that have superior performance in predicting class membership than their generative counterparts; however, generative methods allow data to be generated from the model, effectively allowing *in silico* simulation of system behavior through changes in model parameters. Agent-based models provide a means of understanding complex phenomenon by simulating the behavior of actors within the system, a technique that holds promise for demystifying many biological processes where simulation results can be appropriately constructed, evidentially linked to the biological reality, and experimentally verified. The construction and analysis of this class of computational models are discussed in [111, 112].

Many of the most promising methods and approaches that have the potential to improve the widespread adoption of VCA are at the intersection of medicine, immunology, mathematics, and computer science. By leveraging the strengths and capabilities of each field to solve problems that have been resistant to analysis in another, more rapid progress can be made in delivering novel and clinically relevant findings, diagnostics, or therapeutic compounds.

Approaches that take a cross-disciplinary approach and seek to synthesize the strengths of diverse fields, such as mathematics, computer science, and immunology, are providing new methods and insights that may help to advance the state of the art as well as the development of novel and clinically relevant technologies or therapies for VCA.

## Authors' Contribution

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*Review Article*

# Tolerance Induction Strategies in Vascularized Composite Allotransplantation: Mixed Chimerism and Novel Developments

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Since the start of the clinical vascularized composite allotransplantation (VCA) era over a decade ago this field has witnessed significant developments in both basic and translational research. Transplant tolerance, defined as rejection-free acceptance of transplanted organs or tissues without long-term immunosuppression, holds the potential to revolutionize the field of VCA by removing the need for life-long immunosuppression. While tolerance of organ and vascularized composite transplants may be induced in small animal models by a variety of protocols, only mixed-chimerism-based protocols have successfully bridged the gap to preclinical study and to clinical trial in solid organ transplantation to date. In this paper we review the mixed-chimerism approach to tolerance induction, with specific reference to the field of VCA transplantation, and provide an overview of some novel cellular therapies as potential adjuvants to mixed chimerism in the development of tolerance induction protocols for clinical vascularized composite allotransplantation.

## 1. Introduction

The challenges of transplantation have engaged plastic and reconstructive surgeons since the early years of the specialty [1]. During the past decade, since the start of the clinical vascularized composite allotransplantation era, progress in this field has accelerated, with significant developments in both basic and translational research [2, 3].

Currently, vascularized composite allotransplantation remains dependent on long-term immunosuppression in order to prevent graft rejection. While modern immunosuppressive medications are effective in controlling acute rejection, they have little impact on chronic rejection; an incompletely understood phenomenon, observed in all branches of solid organ transplantation, which leads to a progressive decline in transplant function [4]. The requirement for life-long immunosuppression, and the attendant risks and side effects of current regimens which include metabolic disorders, renal impairment, infectious complications and an increased risk of tumor development, present a major cause for concern in the treatment of conditions which are not, in contrast to solid organ transplantation, immediately life threatening.

Induction of donor specific transplant tolerance, defined as the specific absence of a destructive immune response to a transplanted tissue in the absence of immunosuppression, is a primary goal of transplantation research, and holds the potential to avoid the risks posed by long-term immunosuppressive regimens. Tolerance would also overcome chronic rejection, the impact of which on VCA has not yet become clear, but which should be considered a real possibility as follow up continues into the long term [5, 6]. Unfortunately, while tolerance of a variety of organ and composite tissue transplants may be reliably induced in small animal models, translating these findings to large animal preclinical models and clinical trials has proved challenging, and to date only protocols utilizing lymphohematopoietic mixed chimerism have proved successful at inducing tolerance across genetic disparities at these levels [7]. The term mixed chimerism has been subjected to a widely encompassing definition,

ranging from donor hematopoietic stem cell engraftment with stable long-term multilineage contribution, to regimens using donor bone marrow infusion to achieve transient mixed chimerism that may or may not be followed by a state of microchimerism. These various states of chimerism and the relative role they play in the induction and maintenance of tolerance of transplanted tissues have been demonstrated to vary across models and target organs [8].

These results provide proof of concept for clinical transplantation tolerance, and it can be hoped that further development of tolerance protocols will overcome the stringent challenge posed by composite transplants including skin, and lead to development of clinically applicable protocols for VCA tolerance. In this paper we will review progress in the development of mixed-chimerism-based tolerance protocols, and outline encouraging areas of research with potential for development of novel alternatives to current immunosuppressive regimens in vascularized composite allotransplantation.

## 2. Mixed Chimerism and Transplant Tolerance

In immunological terms, a chimera is an individual in whom a proportion of the hematopoietic cell population can be identified as originating from another individual. This may occur naturally, as in the case of Owen's freemartin cattle [9], but is most usually the result of hematopoietic cell transplantation [10]. In the context of tolerance induction, it is also important to differentiate between full and mixed chimerism. Full chimerism describes the complete replacement of an individual's hematopoietic system with donor cells. This is commonly seen following treatment for hematological malignancy, but is associated with reduced immunocompetence and a significant risk of graft versus host disease (GvHD) [11]. Mixed chimeras, as the name suggests, possess a mixture of recipient and donor hematopoietic cells. Mixed chimerism requires less stringent conditioning of the recipient, maintains immunocompetence and has a lower risk of graft versus host disease, and as such is preferable as a potential tolerance induction strategy [12].

The use of hematopoietic cell transfer in production of chimeras and hence induction of donor specific tolerance has been known for many years, and this approach remains at the forefront of attempts to develop clinically applicable tolerance induction strategies for all forms of surgical transplantation. Interest in this approach has been maintained by reports of patients accepting organ transplants without chronic immunosuppression, having previously received bone marrow transplants from the same donors [13]. Early experimental protocols in mice relied on myeloablative conditioning and complete reconstitution with a donor hematopoietic stem cell graft in order to achieve engraftment and chimerism [14]; however such protocols carry significant morbidity and a risk of impaired immunocompetence which would not be considered acceptable outside the field of hematological malignancy. The development of non-myeloablative conditioning regimens achieving engraftment and mixed chimerism with significantly reduced morbidity represents a key step in the search for clinically relevant tolerance induction regimens and has been demonstrated in small animals [15], large animals [16], and in clinical protocols [17].

It has been proposed that donor stem cell engraftment, resulting in maintenance of donor cell lineages within the recipient, provides continued presentation of donor antigen, facilitating donor-specific tolerance through the same central and peripheral mechanisms responsible for self-tolerance (Figure 1) [13]. The presence of antigen presenting cells of both donor and recipient origin within the thymus will facilitate clonal deletion of both donor and recipient-reactive thymocytes, as reviewed by Sykes [12]. It is presumed that alloreactive thymocytes escaping deletion will be controlled by T regulatory cells, which may be educated either in the thymus or in the periphery within transplanted tissues.

Progressive clonal deletion of peripheral T cells has been identified as an important mechanism [18]. However, non-deletional mechanisms have also been shown to play a role, as donor specific tolerance can be demonstrated before clonal deletion is complete [19–21]. The balance of regulatory and deletional mechanisms in tolerance is complex, but there is evidence in mild regimes, achieving low level chimerism, that regulatory mechanisms predominate [13, 22]. Regulatory mechanisms appear to retain an important role during the induction of tolerance by more stringent regimes, but this gradually declines with time, as progressive deletion of donor-reactive T cells continues [21, 23].

Interestingly, the requirement for the establishment of stable mixed chimerism may not be applicable for the long-term rejection free survival of various organs and tissues. In both preclinical studies in nonhuman primates, and the MGH clinical trial, transient mixed chimerism was found to be sufficient for induction of renal allograft tolerance [24, 25]. It is hypothesized that T regulatory cells, educated during the period of chimerism, are responsible for maintenance of tolerance in this scenario. Pronounced $FoxP3^+$ T cell infiltration in the absence of signs of inflammation or tissue damage was observed within the kidneys tolerated by recipients in the nonhuman primate studies, suggesting local regulation of the immune response within the donor organ [24]. In contrast to these findings of organ tolerance despite loss of detectible chimerism, Leventhal and colleagues have recently reported establishment of durable chimerism and kidney transplant tolerance, without graft versus host disease. Non-myeloablative conditioning and transplantation of an enriched hematopoietic stem cell graft in combination with an infusion of graft-facilitating cells, composed primarily of plasmacytoid precursor dendritic cells, achieved chimerism and permitted weaning of immunosuppression in five of eight patients [26].

In contrast to kidneys, studies in large animal recipients of bone marrow and vascularized composite allografts suggest that these transplants may have more stringent requirements. A series of studies in MGH Miniature Swine indicate that while tolerance of the musculoskeletal components of vascularized composite allografts may be induced by protocols achieving transient chimerism, tolerance of skin requires engraftment of donor hematopoietic stem

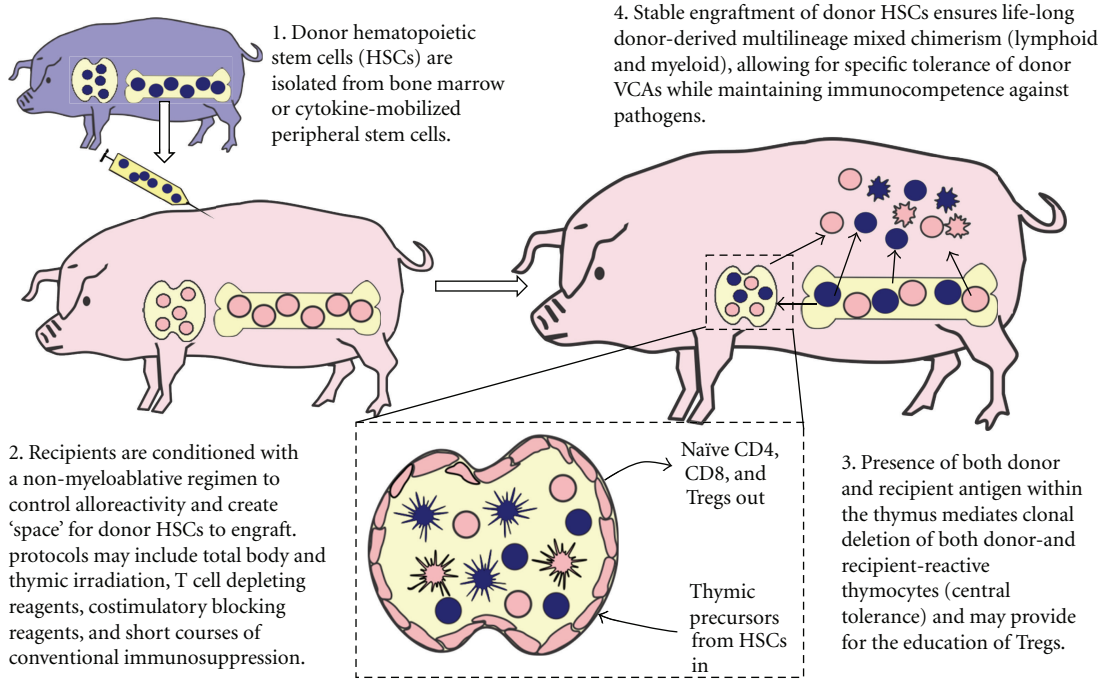


Figure 1: Mechanisms of tolerance in mixed chimerism.

cells and persisting mixed chimerism [27–29]. Skin has long been regarded as the most robust test of transplant tolerance [30]. Initial studies utilized a heterotopic hind limb model, with or without skin paddle, transplanted between MHC-matched, minor antigen mismatched animals, treated with a 12 day course of Cyclosporine A. All animals in both groups accepted the musculoskeletal components of the transplanted limb long-term. Those in the skin-free transplant group subsequently received frozen donor split thickness skin grafts, which rejected [31]. Recipients of skin-bearing transplants demonstrated prolonged skin survival, in one case to 180 days, but in all cases epidermal rejection eventually occurred [27]. This state of tolerance to one organ or tissue, while simultaneously rejecting another, has a long historical record, as it was first described by Billingham and Silvers and termed "split tolerance" [32]. These findings are consistent with previous studies demonstrating that skin is consistently more prone to rejection than other tissues, but that primarily vascularized skin appears to enjoy a survival advantage over conventional skin grafts. While tissue specific antigens are often offered as a potential explanation for the difficulty in achieving skin acceptance, a definitive skin specific antigen is yet to be identified, and other factors including graft size, the skin immune system and the inflammatory milieu resulting from a period of relative ischemia in the absence of primary vascularization have all been implicated [33–35].

Subsequent studies addressed the important step of transplantation across major histocompatibility barriers, once again utilizing the skin-bearing heterotopic limb model. In this series, the musculoskeletal components of the limb were once again uniformly tolerated, across both single haplotype and full class I and class II MHC barriers in recipients of hematopoietic stem cell transplantation using either cytokine-mobilized peripheral blood mononuclear cells or bone marrow cells. Prolonged skin survival to between 35 and 50 days was observed but tolerance of skin was not demonstrated [28]. Animals receiving cytokine mobilized cells received a significantly higher dose of hematopoietic cells than those receiving bone marrow, and demonstrated detectable albeit progressively declining peripheral blood mixed chimerism, while those receiving bone marrow did not. Regardless, these studies further illustrate that while kidney tolerance may be achieved by similar protocols in the context of both transient and long-term chimerism, induction of tolerance of skin components of VCAs will require more robust induction mechanisms. The relationship between chimerism and tolerance has often been controversial [36, 37], and in this model it appears that stable chimerism is not necessary for tolerance of musculoskeletal components of the allografts.

In 2009 Horner et al. published a preliminary report of the successful induction of tolerance to skin across a major histocompatibility barrier in the MGH miniature swine model in which stable mixed chimerism was established using a non-myeloablative conditioning regimen and cytokine mobilized hematopoietic stem cells. Following confirmation of donor stem cell engraftment, primarily vascularized skin flaps and conventional skin grafts were transplanted, and in this experiment one animal demonstrated tolerance of its flap for over 300 days of follow up. This tolerance was robust, as demonstrated by the acceptance of a subsequent donor split thickness skin graft placed 124 days following the original skin flap [29]. Recently,

similar results in a canine model of vascularized composite allotransplantation have been reported by Mathes et al., with long-term graft survival and stable mixed chimerism in a MHC-matched, minor antigen mismatched model [38]. These studies support the hypothesis that engraftment of donor hematopoietic stem cells, and persisting mixed chimerism are required for tolerance of skin in these large animal models.

## 3. Cellular Therapies in Mixed Chimerism and VCA Tolerance

Considerable reductions in the toxicity and morbidity of conditioning regimens have been achieved since the initial studies utilizing myeloablative protocols, although the majority of these have been described in small animal models. Thus, achieving mixed chimerism while minimizing the adverse effects of conditioning remains a challenging balance and a variety of novel strategies have been investigated as potential adjuncts in an effort to enhance engraftment and mitigate complications such as GvHD.

*3.1. T Regulatory Cells.* Regulatory cells have been extensively studied in the context of transplantation tolerance. The existence of a population of lymphocytes capable of suppressing immune responses was first described 40 years ago, and shortly thereafter these cells were demonstrated to facilitate tolerance of non-self antigens in a murine skin transplant model, demonstrating the potential importance of these cells in achieving tolerance of the skin component of VCA [39, 40]. The characterization and diverse functions of the canonical $CD4^+CD25^+FoxP3^+$ Treg cell population has been extensively reviewed [41]. The ability of these cells to enhance engraftment following allogeneic bone marrow transplantation was described by Joffre et al. who subsequently demonstrated that $CD4^+CD25^+FoxP3^+$ T regulatory cells could prevent both acute and chronic rejection of skin and cardiac allografts [42, 43].

Direct evidence for cellular regulation by $CD4^+CD25^+$ T cells has been demonstrated in some murine models of bone marrow transplantation. Bigenzahn and colleagues found that depletion of $CD25^+$ cells at the time of bone marrow transplantation and costimulatory blockade (anti-CD154 and CTLA4Ig) blocked development of tolerance, but that late depletion of $CD25^+$ cells failed to abrogate tolerance, demonstrating that, in this model, the role of Tregs was most prominent during induction rather than maintenance phases [44]. Pilat and colleagues recently demonstrated that recipient T regulatory cells, administered in conjunction with anti-CD40L mAb and CTLA4Ig costimulatory blockade and a short course of Rapamycin, could achieve engraftment and stable multilineage chimerism, and subsequent skin tolerance, following radiation-free conditioning and conventional dose bone marrow transplantation in a fully mismatched murine model [45]. Interestingly, Rapamycin was found to be an essential component of this protocol, which is consistent with other studies finding that Rapamycin facilitates selective expansion of T regulatory cells while inhibiting clonal proliferation of effector cells [46].

Issa and colleagues in Oxford have reported the development of a humanized mouse model, in which they have extensively investigated the potential of human Tregs as a tolerance induction strategy for transplantation. They recently demonstrated the ability of these cells to prevent transplant arteriosclerosis (a hallmark of chronic graft rejection), and uniquely, to induce tolerance to human skin allografts [47]. There is also evidence that, in the context of bone marrow transplantation, donor $CD4^+CD25^+$ cells may protect against acute graft versus host disease (GvHD) [48, 49]. Taken together and in combination with the work done by many other groups, these experiments are certainly encouraging, but further work is required to refine the specificity of Treg markers, and to provide evidence of efficacy in large animal models prior to considering the therapeutic application of T regulatory cells in clinical composite tissue allotransplantation.

*3.2. Dendritic Cells.* The traditional view of dendritic cells (DCs) as potent inducers of immune reactivity has been augmented in recent years by the recognition that, as specialized antigen presenting cells, they have the ability to facilitate immunologic tolerance [50, 51]. It is logical that dendritic cells exert their tolerogenic effects through interaction with T cells, and indeed, they have been shown to suppress $CD4^+$ and $CD8^+$ T cell proliferation [52], and to control activation and function of $CD4^+CD25^+Foxp3^+$ Tregs [53].

The accepted paradigm states that immature dendritic cells (characterized by low expression of cell surface MHC II and costimulatory molecules) induce tolerance upon interaction with T cells, while mature dendritic cells (high expressers of MHC II and costimulatory molecules) induce an effector response from T cells [54]. However, it has been demonstrated that dendritic cells of both immunogenic and tolerogenic phenotypes may be directed toward a change of phenotype by ligation of specific cell surface receptors [55]. Therefore, while the existence of functionally distinct subsets of dendritic cells is being continuously defined and expanded (reviewed in [56]), it seems likely that the distinction between tolerogenic and immunogenic roles does not lie along simple divisions between subsets, and that dendritic cells demonstrate functional plasticity, although the discriminating factor in this plasticity remains controversial.

Attempts have been made to exploit the tolerogenic potential of dendritic cells in animal models of transplantation with some success. Tolerance of cardiac transplants has been achieved in rodent models following administration of donor allopeptide-pulsed DCs in combination with a short course of antilymphocyte serum [57]. Long term survival of skin allografts and hind-limb composite tissue allotransplants, has also been demonstrated, in some cases with demonstrable expansion of Tregs [58–62]. In the context of mixed chimerism protocols, it has been demonstrated in a murine model that cotransplantation of bone marrow

with immature dendritic cells lead to engraftment and stable multilineage chimerism without cytoreductive conditioning and with no evidence of graft versus host disease. These chimeras accepted cardiac allografts long-term, and while skin tolerance was not achieved, challenge skin grafts did enjoy significantly prolonged survival [63].

There have been some early studies in nonhuman primates which have demonstrated the presence of tolerogenic dendritic cells with the ability to modulate the T cell response to alloantigens [64], and, it has been demonstrated that dendritic cells are able to induce tolerance of model antigens and facilitate expansion of T regulatory cells in human volunteers [65, 66]. These findings point to the possibility of "negative cellular vaccines" as a potential route to tolerance in composite tissue allotransplantation, but further preclinical work is required [67].

*3.3. Mesenchymal Stem Cells.* Mesenchymal stem cells (MSCs) are a component of the bone marrow stroma, and play homeostatic roles important for hematopoiesis through synthesis of numerous cytokines and growth factors including granulocyte colony stimulating factor (G-CSF), stem cell factor (SCF), Flt-3 ligand and members of the interleukin family [68, 69]. MSCs have been shown to lack expression of costimulatory molecules and consequently to have limited capability for stimulating alloreactive T cells [70–72]. Furthermore, MSCs have been demonstrated to possess immunological inhibitory potential, suppressing proliferation in mixed lymphocyte cultures and prolonging skin graft survival in a rodent allotransplant model [73, 74].

The natural physiologic role of MSCs within bone marrow stroma has been exploited in the successful treatment of GvHD in patients following bone marrow transplantation [75], and recently to facilitate engraftment and induction of tolerance to limb composite tissue allografts in animal models. Using a rat model, Pan and colleagues performed hind-limb allotransplantation after having induced chimerism 30 days previously with a regime of antilymphocyte serum, rapamycin, 3 Gy total body irradiation, bone marrow cells and ex vivo expanded MSCs. Rapamycin was continued for 100 days, and following cessation of treatment, animals exhibited stable chimerism, tolerated their transplanted limb for greater than 100 days without any exogenous immunosuppression, and showed no evidence of GvHD [76].

MSCs have also been reported to be a useful adjunct to bone marrow transplantation for tolerance induction in a large animal model. In an outbred miniature swine model, Kuo and colleagues demonstrated survival of heterotopic hind-limb allotransplants for greater than 200 days in animals treated with irradiation, bone marrow transplantation, 28 days of cyclosporine, and three doses of donor MSCs (each of $1 \times 10^7$ cells, on days 7, 14, and 21 post limb transplant). Interestingly, while no signs of GvHD were observed, the same regime in the absence of MSCs resulted in a maximal allograft survival of 57 days and symptoms of severe GvHD ultimately resulting in death [77]. This study provides proof of principle that MSCs may be an effective adjunct to bone marrow transplantation in tolerance induction, and taken together with previously reported clinical use of MSCs for treatment of GvHD, indicates that this may be a useful and interesting avenue for further research in composite tissue allotransplantation.

## 4. Conclusions and Future Directions

The shared history of reconstructive and transplant surgery have, over the past 15 years, witnessed a new chapter with the emergence of vascularized composite allotransplantation as a viable option for the treatment of patients with severe, complex extremity and craniofacial defects for which the outcomes of conventional reconstructive techniques remain suboptimal. While clinical data demonstrate the short to medium term efficacy of these procedures, the decision to prescribe life-long immunosuppression in the treatment of non-life-threatening condition remains an ethical dilemma.

The induction of donor specific tolerance holds the potential to avoid both the risks of life-long immunosuppression and to prevent chronic rejection. A clinically applicable tolerance strategy for vascularized composite allotransplantation would fundamentally alter the risk-benefit analysis for potential recipients and could expand availability of these procedures to patients currently considered high risk or unsuitable candidates, for example those requiring restoration of congenital anomalies or following oncological resection. Skin remains a particularly stringent test of any transplant tolerance protocol, and while tolerance of skin has been reported in preclinical studies further work is required to demonstrate that this can be reliably achieved by a clinically applicable protocol.

While there is encouraging evidence from small animal models for a wide variety of tolerance strategies, mixed chimerism is the only approach to prove successful in large animal studies, or to reach clinical trials in organ transplantation, and is the established frontrunner in preclinical studies of composite tissue tolerance. While it has been shown clinically that transient mixed chimerism and other immunomodulatory approaches are sufficient for induction of tolerance of other organs, and can play an important role in minimization of immunosuppression for VCA [25, 78], to move toward the goal of true tolerance and immunosuppression free VCA acceptance, stable mixed chimerism appears to have the most promise at this time. Despite a steady reduction in the toxicity of experimental regimens, the morbidity associated with the conditioning regimes required to permit engraftment of hematopoietic stem cells remains a concern. Despite success in small and now large animal models translation to clinical application remains challenging. In addition to the combined kidney and bone marrow transplantation studies reported by several centers [25, 26], recent work by Bolaños-Meade et al. [79] in the field of HLA mismatched bone marrow transplantation demonstrates progress toward establishment of chimerism across MHC barriers with a low incidence of GvHD suggesting that the goal of inducing VCA tolerance with mixed chimerism remains highly possible. In this paper we have reviewed

a number of adjuvants to the mixed chimerism approach, which appear to have the potential to enhance engraftment and to mitigate complications such as GvHD. Some of these strategies have already been tested in preclinical models with encouraging results, and it can be hoped that further translational studies will result in development of safe, effective tolerance induction protocols for clinical trial in vascularized composite allotransplantation.

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*Research Article*

# Vascularized Composite Allograft Rejection Is Delayed by Intrajejunal Treatment with Donor Splenocytes without Concomitant Immunosuppressants

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*Background*. Mucosal or oral tolerance, an established method for inducing low-risk antigen-specific hyporesponsiveness, has not been investigated in vascularized composite allograft (VCA) research. We studied its effects on recipient immune responses and VCA rejection. *Methods*. Lewis rats ($n = 12$; TREATED) received seven daily intrajejunal treatments of $5 \times 10^7$ splenocytes from semiallogeneic Lewis-Brown-Norway rats (LBN) or vehicle ($n = 11$; SHAM). Recipients' immune responses were assessed by mixed lymphocyte reaction (MLR) against donor antigen and controls. Other Lewis ($n = 8$; TREATED/VCA) received LBN hindlimb VCA and daily intrajejunal treatments of $5 \times 10^7$ LBN splenocytes, or LBN VCA without treatment ($n = 5$; SHAM/VCA), until VCAs rejected. Recipients' immune responses were characterised and VCAs biopsied for histopathology. Immunosuppressants were not used. *Results*. LBN-specific hyporesponsiveness was induced only in treated Lewis recipients. Treatment significantly reduced MLR alloreactivity, significantly reduced VCA rejection on histopathology, and significantly delayed clinical VCA rejection ($P < 0.0005$; TREATED/VCA mean 9.6 versus 6.0 days for SHAM/VCA). Treatment significantly increased immunosuppressive IL-10/IL-4/TGF-$\beta$ production and significantly decreased proinflammatory IFN-$\gamma$/TNF-$\alpha$. *Conclusion*. Jejunal exposure to antigen conferred donor specific hyporesponsiveness that delayed VCA rejection. This method may offer a low-risk adjunctive treatment option to help protect VCAs from rejection.

## 1. Introduction

The technical feasibility of transplanting vascularized composite allografts (VCA) such as of hand/forearm, larynx, partial face, and others is not disputed [1–5]. However, reconstructive VCA is unlikely to become widely available until either the risk profiles of lifelong immunosuppressant drugs become more acceptable or a safe method of donor-specific VCA tolerance induction applicable to humans is devised [6, 7]. Although transplantation tolerance has been established in many experimental models and anecdotal incidents of tolerance in humans can be found in the literature, efforts to replicate the state safely and reliably in humans have proven futile [8, 9].

Another method to reduce the attendant risks of nonspecific immunosuppression may be to induce donor-specific hyporesponsiveness [7, 10]. Although this is not transplantation tolerance, such a state may decrease the dosages required

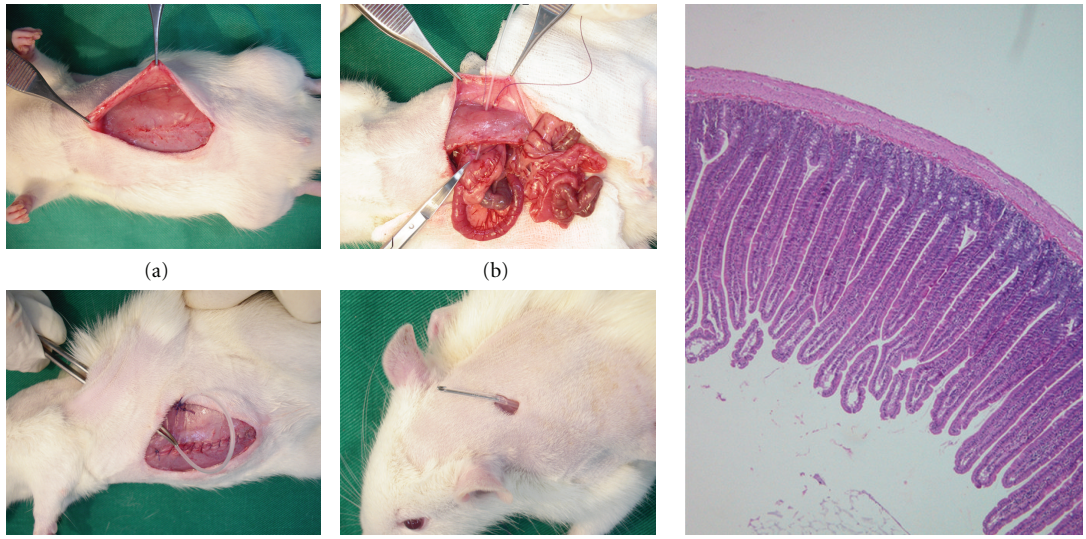



Figure 1: Percutaneous gastroduodenojejunostomy to deliver FDS or vehicle directly to the jejunal mucosa of Lewis rats. (a) Midline laparotomy. (b) Silicone tubing was delivered through the anterior abdominal musculature into the stomach and passed distal to the Treitz ligament. (c) The abdomen was closed and the tubing secured to abdominal wall musculature and tunneled to the posterior neck. (d) The tube was additionally secured at the posterior neck and sealed with a stopper that could be removed and replaced for intrajejunal administrations. (e) Characteristic jejunal histological appearance of intestinal tissue 5 mm distal to the tube end at time of sacrifice confirmed correct intrajejunal placement in all recipients.

to maintain the allotransplant. Enteral (usually oral) administration of appropriate antigens can specifically suppress development or progression of experimental autoimmunities such as experimental autoimmune encephalitis, collagen-induced arthritis, nickel hypersensitivity and others [11, 12]. Human trials of oral tolerance have been conducted to treat allergies, rheumatoid arthritis, uveitis, diabetes, and other immunological diseases [11]. Others have shown that delivery of alloantigen to nongastrointestinal mucosa may be superiorly tolerogenic through avoidance of gastric acid and proteolytic enzymes [13–15]. Thus, Ishido et al. compared orally with intrajejunally administered donor splenocytes in a cardiac allotransplantation rat model and concluded from *in vitro* and *in vivo* evidence that jejunal mucosal tolerance was significantly more tolerogenic [16]. Importantly, their treatment protocol was commenced after transplantation and was donor specific since third party allotransplants were rejected normally [16, 17].

The effects of mucosal tolerance induction methods have not previously been assessed in a VCA model. Based on Ishido et al.'s investigations, we studied jejunal in preference to oral tolerance induction in a rat model of hindlimb VCA [16]. In contrast, to avoid counteraction with possible tolerization mechanism(s), we never administered immunosuppressive drugs; for this reason, we chose a semiallogeneic mismatch instead of a full mismatch.

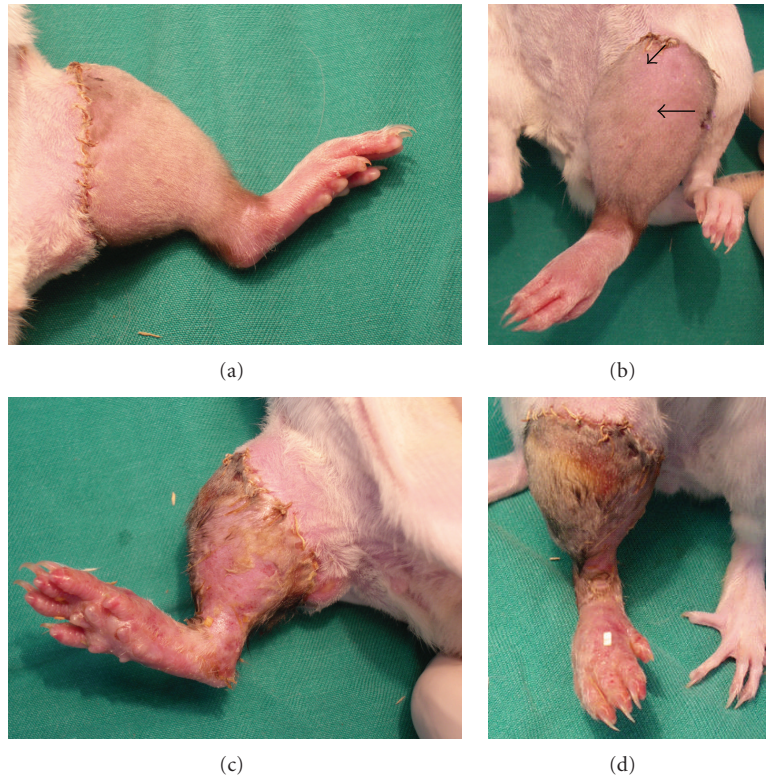
In this study, we aim firstly to confirm *in vitro* that intrajejunal treatments with donor splenocytes could render recipients immunologically hyporesponsive in a donor-specific manner. Second, we test this regimen *in vivo* to see if the commencement of rejection of semiallogeneic hindlimb VCAs could be significantly delayed. Third, further *in vitro* and *in vivo* analyses provide potential explanations for the underlying mechanisms of donor-specific hyporesponsiveness induced in recipients.

## 2. Materials and Methods

*2.1. Animals.* Adult (8–12 weeks old; 180–220 g) male inbred recipient Lewis ($RT1^l$) and donor Lewis-Brown-Norway (LBN; $RT1^{l+n}$) rats, representing a semiallogeneic mismatch, were obtained from the National Laboratory Animal Centre (Education Research Resource, Taiwan). They were housed individually in pyrogen-free conditions under controlled temperature and 12 hourly light/dark cycles, with water and commercial rat chow freely available at the Chang Gung Memorial Hospital Animal Centre. All experiments were authorised by and performed under instruction from the institution's Animal Care and Ethics Committee.

*2.2. Study Design.* Resealable percutaneous gastroduodenojejunostomies (Figure 1) were sited in all Lewis rats on Day −12 to establish direct access for intrajejunal administrations (Day 0 denoted the time of VCA or of animal sacrifice for one-way mixed lymphocyte reaction (MLR), depending on the Group to which the animal belonged). Two experimental ("TREATED" and "TREATED/VCA") and two control ("SHAM" and "SHAM/VCA") Groups were created as follows.

Lewis rats received $5 \times 10^7$ LBN fresh donor splenocytes (FDS) in 0.2 mL HBSS intrajejunally (TREATED Group; $n = 12$), or vehicle alone (0.2 mL HBSS; SHAM Group; $n = 11$), everyday on Days −9 through −3 (7 doses) and were



(a) (b) (c) (d)

Figure 2: TREATED/VCA (panels (a) and (b)) and SHAM/VCA ((c) and (d)) on post-transplant Day +10. Arrows highlight the commencement of shedding of VCA hair in a TREATED/VCA rat. In contrast, recipients in the SHAM/VCA group ((c) and (d)) had commenced rejection at least three days previously and by now rejection was advanced.

sacrificed on Day 0 for one-way MLR. This optimal dose of FDS for inducing hyporesponsiveness was characterized by preliminary studies that compared the effect of various doses (ranging between $1 \times 10^7$ and $2 \times 10^8$ FDS) administered for the same duration on Day 0 MLR responses versus vehicle-treated controls (data not shown).

Other Lewis rats received heterotopic LBN hindlimb VCAs on Day 0 and $5 \times 10^7$ LBN FDS in 0.2 mL HBSS intrajejunally (TREATED/VCA Group; $n = 8$), or vehicle alone (0.2 mL HBSS; SHAM/VCA Group; $n = 5$), everyday from Day −9 until VCAs rejected. Importantly, immunosuppressive drugs were never administered.

*2.3. Intrajejunal Access and Transplant Procedures.* All operative procedures were performed aseptically with the animal deeply anaesthetized by intraperitoneal sodium pentobarbital (induction: 50 mg/kg; maintenance: 10 mg/kg/hr).

Resealable percutaneous gastroduodenojejunostomies were sited via standard midline laparotomies, securing the silicone tubing (model reference 806700; Shineteh Instruments Co. Ltd., Taiwan) palpably 1 cm distal to the ligament of Treitz. All tubes maintained this position as confirmed at animal sacrifice. The bowel 5 mm distal to the tube end was histologically confirmed in all sacrificed animals to be jejunum, confirming that treatment delivery was specifically to the jejunal mucosa and not more proximally (Figure 1).

Heterotopic hindlimb VCAs (Figure 2) were performed essentially as previously described [18]. All VCAs were revascularized after exactly 45 min of ischemia time by releasing both arterial and venous microvascular clamps at the designated time. All microanastomoses were complication-free and all VCAs maintained normal vascularity postoperatively. Donors and recipients were weight-matched to within 15 grams.

*2.4. Preparation of Fresh Donor Splenocytes.* Freshly harvested LBN whole spleens were gently mashed within serum-free RPMI-1640 and passed through nylon mesh (Millipore; 100 $\mu$m pores) to produce single cell suspensions. Cells were washed with HBSS once and resuspended in ACK buffer for 5 min to lyse red blood cells. Cells were washed two times further with HBSS and resuspended at $5 \times 10^7$ cells/0.2 mL HBSS. Splenocyte viability was >95% according to trypan blue dye exclusion.

*2.5. One-Way Mixed Lymphocyte Reaction.* MLR responses were assessed at the peak of the reaction as determined by preliminary study data (not shown); semiallogeneic MLR consistently provide counts that are not as high as fully allogeneic mismatched models.

One-way MLR was used to determine evidence of donor-specific hyporesponsiveness *in vitro*. On Day 0, spleens were freshly harvested for splenocytes from TREATED and

SHAM Group recipients (responders), as well as naïve LBN (stimulator), and the animals sacrificed.

Freshly harvested whole LBN spleens were gently mashed within serum-free RPMI-1640 and passed through nylon mesh (Millipore; 100 $\mu$m pores) to produce single cell suspensions. Cells were washed with HBSS once and then resuspended in ACK buffer for 5 min. Cells were washed two times further with HBSS.

At this point, responder cells were resuspended in complete RPMI-1640 at $1 \times 10^6$ cells/mL. Stimulator cells instead were treated with 5 $\mu$m/mL mitomycin C in complete RPMI-1640 for 30 min at 37°C, washed twice with HBSS, and resuspended in complete RPMI-1640 at $1 \times 10^6$ cells/mL.

Responder cells ($1 \times 10^5$ cells/100 $\mu$L/well) were cultured in 96-well round-bottomed plates in triplicate with either: (1) equal numbers of mitomycin-C-treated stimulator cells ($1 \times 10^5$ cells/100 $\mu$L/well; "SHAM + LBN Stim" and "TREATED + LBN Stim"); or (2) equal numbers of mitomycin-C-treated syngeneic cells ($1 \times 10^5$ cells/100 $\mu$L/well; "TREATED Alone" and "SHAM Alone"); or (3) conclavulin A (ConA; 2 $\mu$g/mL; "SHAM + ConA" and "TREATED + ConA"). Plates were maintained at 37°C in a 5% $CO_2$ incubator for five days, consistent with the peak of the reaction according to preliminary studies. At 96 hr, cultures were pulsed with [$^3$H]-thymidine (1 $\mu$Ci/well) for 24 hr and harvested. Cell proliferation was assayed by ($^3$H)-thymidine incorporation measured by $\beta$-scintillation counter. Two independent experiment repeats yielded essentially identical results.

*2.6. Monitoring of Transplants and Recipients.* Lewis rats were inspected daily for signs of graft-versus-host disease (GvHD): diarrhea, rash to the paws and/or ears, unkempt appearance, failure to thrive, and lack in weight gain. VCAs were monitored daily for markers of rejection (edema, erythema, desquamation, hair loss, epidermolysis, exudation, pustulation, and skin necrosis/escharification). Rejection was rigidly defined as "the first change in the skin after erythema and edema but before progressing toward epidermolysis, desquamation, or even eschar formation" [19]. The first specific sign was almost invariably the sudden shedding of hair specifically on the hindlimb VCA; this sign was both binary in its presence/absence and easily diagnosed with clarity. Rejection is defined by other authors as necrosis of 70–90% of the skin paddle of a VCA (which represents a nonsalvageable transplant), but we find this assessment to be more subjective in its interpretation than the presence/absence of hair shedding. Furthermore, in the clinical setting, acute rejection would be treated immediately rather than delayed in an attempt to reverse imminent VCA loss, and hence we believe the shedding of hair to be a more clinically relevant sign of rejection (and survival) than near-total necrosis.

*2.7. Measurement of Cytokine Levels in MLR Supernatants and Day +7 Sera.* Day +7 blood was obtained from each recipient's tail vein when VCA biopsies were performed. All sera and 96 hr MLR supernatants were stored at −80°C before assays, at which point they were gradually thawed. IFN-$\gamma$, TNF-$\alpha$, IL-4, and IL-10 concentrations were measured by flow cytometric bead array (flow-CBA), whilst TGF-$\beta$ concentrations were measured by enzyme-linked immunosorbent assay (ELISA).

*2.7.1. Flow-CBA for Quantifying IFN-γ, TNF-α, IL-4, and IL-10 Concentrations.* IFN-$\gamma$, TNF-$\alpha$, IL-4, and IL-10 concentrations were quantified in multiplexed fashion in individual 96 hr MLR supernatant and Day +7 sera samples using the respective BD Biosciences CBA Flex Sets (Category Numbers: IFN-$\gamma$—558305; IL-10—558306; IL-4—558307; TNF-$\alpha$—558309) according to the manufacturer's instructions. Briefly, 50 $\mu$L of unknown samples, or standards, were added to premixed microbeads (50 $\mu$L) in 12 × 75 mm Falcon tubes. After adding 50 $\mu$L of a mixture of PE conjugated antibodies against the cytokines, the mixture was incubated for 3 hr in the dark at room temperature. This mixture was washed and centrifuged at 200 ×g for 5 min and the pellet resuspended in 300 $\mu$L of wash buffer. The BD FACSCalibur flow cytometer was calibrated with setup beads and 1200 events were acquired for each sample. Individual cytokine concentrations were indicated by their fluorescent intensities (FL-2) and calculated using FCAP Array Software. Representative results from two independent experiments for sera and supernatants, respectively, which yielded identical results, were presented.

*2.7.2. ELISA for Quantifying TGF-β Concentrations.* TGF-$\beta$ concentration was quantified in 96 hr MLR supernatants and Day +7 sera by ELISA with specific antibody to TGF-$\beta$ according to the manufacturer's protocol (Biosource International; Catalog no. KAC1688/KAC1689). For both cell culture media and sera, a sample extraction step was required to release TGF-$\beta$ from latent complexes, making it accessible for measurement in the immunoassay. Representative results from at least two independent experiments (each performed in triplicate) for sera and supernatants, respectively, which essentially yielded identical results, were presented.

*2.8. Histopathology of Rejecting Tissues.* On Day +7, VCA-Muscle (9 mm$^3$) and VCA-Skin biopsies (16 mm$^2$) were obtained from the lateral aspect of the transplanted hindlimb and the wound closed (5/0 Vicryl; Ethicon). Biopsies were stored in 10% formalin for 36 hr, then embedded in paraffin, cross-sectioned, and stained with hematoxylin and eosin as standard. Lymphocyte counts per 0.1 mm$^2$ field were assessed in quadruplicate per sample in muscle and at the dermal-subcutis interface in skin [20]. Histopathological analyses were performed, blinded by an independent pathologist.

*2.9. Statistical Methods.* Data were expressed as mean ± standard deviation unless otherwise indicated. Statistical differences between groups were examined by Mann-Whitney *U*-Test, one-tailed (MWT-*ot*) or two-tailed (MWT-*tt*), and *t*-test as appropriate. Timing of VCA rejection was presented by survival curve using the product limit method of Kaplan-Meier and compared for differences using the logrank test.

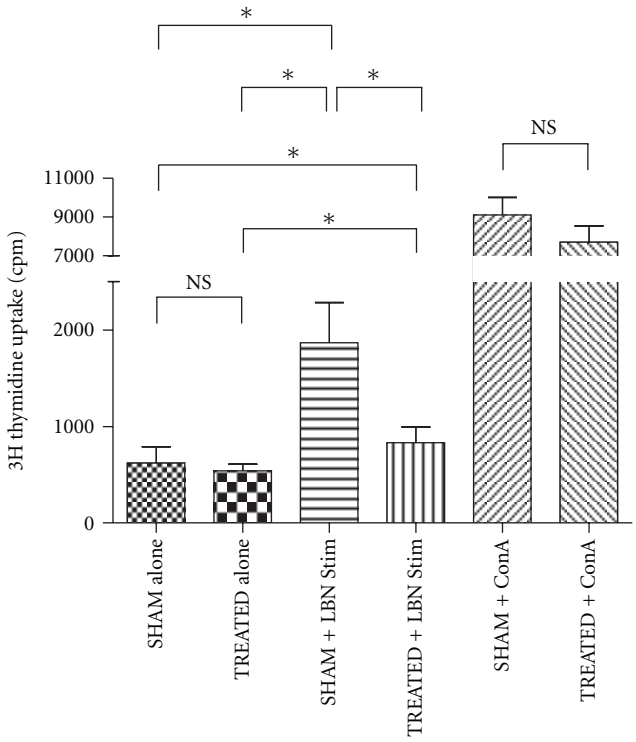



Figure 3: One-way MLR demonstrating significant donor-specific hyporesponsiveness conferred by seven consecutive daily intrajejunal doses of $5 \times 10^7$ LBN FDS. "$*$" $P < 0.05$; "NS" $P < 0.05$.

Statistical analyses were performed using SPSS software (Version 15). A statistically significant difference was indicated by a $P$ value less than 0.05.

## 3. Results

*3.1. Animal Monitoring.* All animals remained entirely healthy throughout. In particular, no clinical evidence of GvHD was noted on daily evaluation and animals thrived and gained weight normally. All operations were complication-free.

*3.2. Donor-Specific MLR Responses Reduced by Intrajejunal Administration of FDS.* Splenocytes from TREATED rats showed suppressed MLR responses when used as responder cells with LBN as a specific stimulator ($P < 0.05$; MWT-*tt*; TREATED versus SHAM; Figure 3). Splenocytes from both SHAM and TREATED rats proliferated equally strongly against nonspecific ConA stimulation ($P > 0.05$; MWT-*tt*; SHAM + ConA versus TREATED + ConA). Splenocytes from SHAM and TREATED rats proliferated equally against syngeneic mitomycin-C-treated splenocytes ($P > 0.05$; MWT-*tt*; SHAM Alone versus TREATED Alone).

*3.3. Cytokine Profiles in MLR Supernatants.* Concentrations of IFN-$\gamma$ ($P < 0.0001$), TNF-$\alpha$ ($P < 0.0001$), and TGF-$\beta$ ($P < 0.001$) in 96 hr supernatants were significantly reduced when splenocytes from TREATED rats were used as responder and LBN splenocytes as stimulator, whereas concentrations of IL-10 ($P < 0.0001$) and IL-4 ($P < 0.0001$) were significantly increased (each statistical comparison is TREATED versus SHAM using MWT-*tt*; Figure 4). When ConA was used as a non-specific stimulator of TREATED and SHAM splenocytes, 96 hr MLR supernatants did not reveal differences in IL-4, IL-10, TNF-$\alpha$, TGF-$\beta$, or IFN-$\gamma$ concentrations ($P > 0.05$; MWT-*tt*; TREATED Alone versus SHAM Alone). There were no differences in IL-4, IL-10, TNF-$\alpha$, TGF-$\beta$, or IFN-$\gamma$ concentrations when SHAM or TREATED splenocytes were cultured with syngeneic mitomycin-C-treated splenocytes ($P > 0.05$; MWT-*tt*; TREATED Alone versus SHAM Alone).

*3.4. Onset of Rejection Delayed by Intrajejunal Administration of FDS.* Lewis VCA recipients were monitored daily for signs of rejection. When Lewis recipients were treated everyday by intrajejunal LBN FDS starting Day $-9$ before LBN hindlimb VCA (TREATED/VCA Group), VCA rejection was significantly delayed compared to untreated (SHAM/VCA) Lewis ($P < 0.0005$; mean 9.6 days versus mean 6.0 days; Figure 5).

*3.5. Cytokine Profiles in Day +7 Sera of VCA Recipients.* When Lewis recipients were treated everyday by intrajejunal LBN FDS starting Day $-9$ before LBN hindlimb VCA, concentrations of IFN-$\gamma$ ($P < 0.005$; MWT-*ot*; TREATED/VCA versus SHAM/VCA) and TNF-$\alpha$ ($P < 0.05$; MWT-*ot*; TREATED/VCA versus SHAM/VCA) in Day +7 sera were significantly reduced compared to Day +7 sera from untreated Lewis recipients, whereas concentrations of IL-10 ($P < 0.05$; MWT-*ot*; TREATED/VCA versus SHAM/VCA) and IL-4 ($P < 0.05$; MWT-*ot*; TREATED/VCA versus SHAM/VCA) were significantly increased. Differences in TGF-$\beta$ concentration, however,did not reach statistical significance ($P > 0.05$; MWT-ot; TREATED/VCA versus SHAM/VCA; Figure 6).

*3.6. Histopathology of VCA-Muscle and VCA-Skin.* Biopsies from all recipients in TREATED/VCA and SHAM/VCA Groups were obtained from transplanted LBN hindlimbs on Day +7 and analyzed by an independent pathologist in blinded manner. VCA-Skin from SHAM/VCA all showed essentially the same characteristics: severe papillary edema with epidermal detachment, early necrotic changes in the superficial epidermis, and severe diffuse lymphocytic infiltration into all layers of the cutis and subcutis. VCA-Skin from TREATED/VCA rats, in contrast, revealed only mild papillary edema, essentially normal epidermal and dermal cytoarchitecture, properly adherent epidermis-dermis junction, and only mild focal lymphocytic infiltrates. Quantitatively, lymphocyte infiltration into the dermis-subdermis interface was significantly increased in SHAM/VCA compared with TREATED/VCA ($P < 0.001$; $t$-test; mean $60.6 \pm 22.0$ versus $23.9 \pm 2.12$ lymphocytes/0.1 mm$^2$ resp.). VCA-Muscle from SHAM/VCA rats revealed generalized haphazard cytoarchitecture and severe diffuse lymphocytic infiltrate. VCA-Muscle from TREATED/VCA rats instead all revealed largely normal cytoarchitecture and only mild perivascular lymphocytic infiltrates. Quantitatively, lymphocyte infiltration into

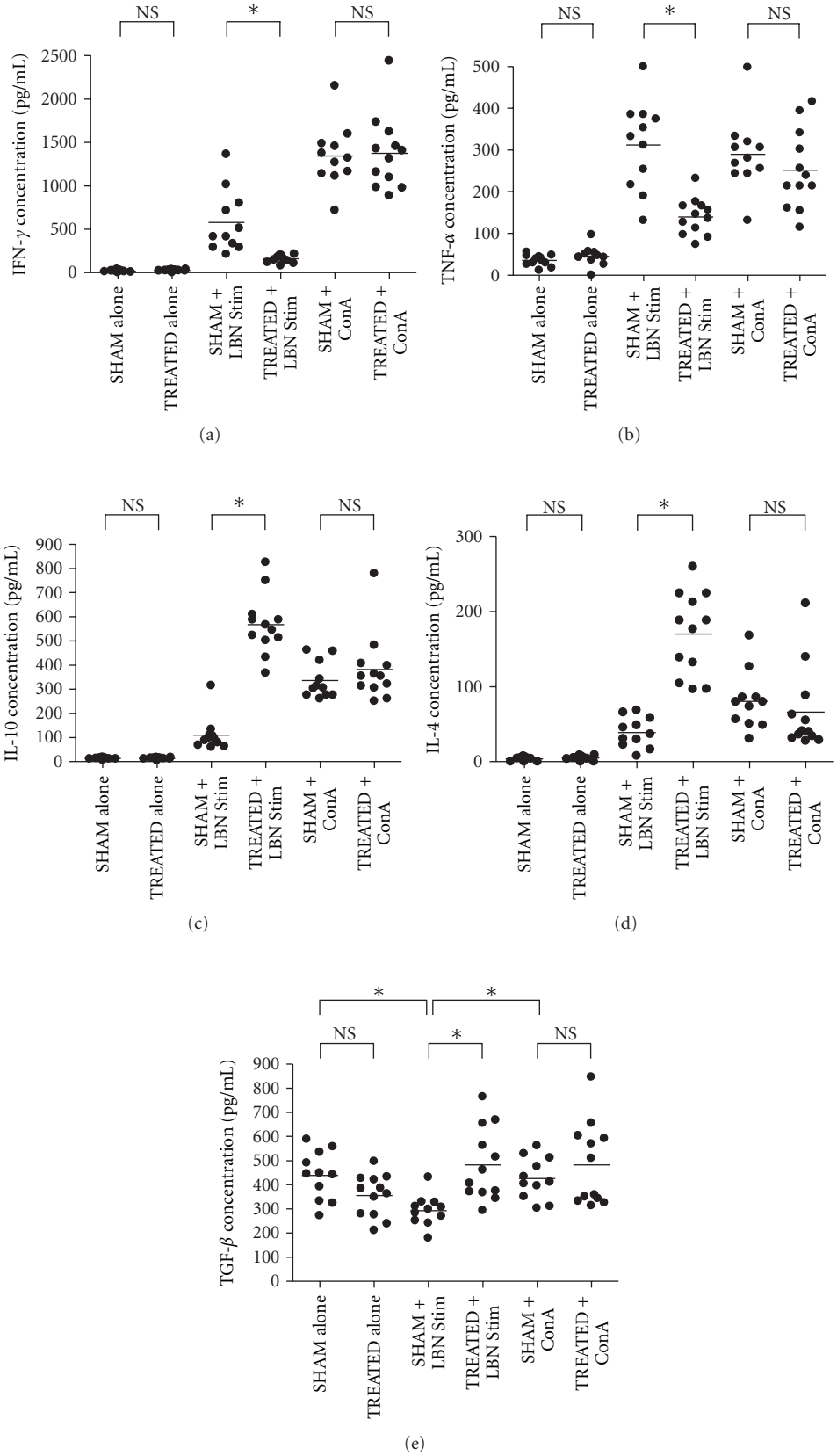



Figure 4: Cytokine concentrations in one-way MLR supernatants according to flow cytometric bead arrays ((a) IFN-$\gamma$; (b) TNF-$\alpha$; (c) IL-10; (d) IL-4) and ELISA ((e) TGF-$\beta$) presented as scatter plots with mean bar. "$*$" $P < 0.05$; "NS" $P < 0.05$ (specific $P$ values provided in the text).

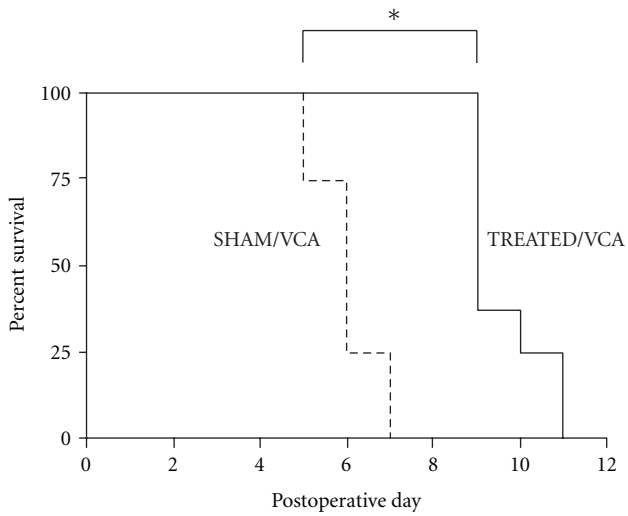



Figure 5: VCA rejection presented as survival curves using the product limit method of Kaplan and Meier. (dotted line—SHAM/VCA; solid line—TREATED/VCA; "$*$" $P < 0.0005$).

muscle was significantly increased in SHAM/VCA compared with TREATED/VCA ($P < 0.001$; $t$-test; mean $38.1 \pm 11.7$, versus $16.9 \pm 1.8$ lymphocytes/0.1 mm$^2$ resp.). Representative samples are shown (Figure 7).

## 4. Discussion

This study demonstrated for the first time that daily intrajejunal administration of donor antigen could delay the rejection of hindlimb VCA despite the absence of immunosuppressant drugs. MLR demonstrated that recipient treatment with daily intrajejunal mucosal exposure to donor FDS suppressed alloimmune responses in a donor-specific manner. *In vivo*, this hyporesponsiveness manifested in delayed hindlimb VCA rejection as determined clinically and histopathologically. Cytokine concentrations in supernatants and recipients' sera showed decreased levels of proinflammatory IFN-$\gamma$ and TNF-$\alpha$ and increased levels of immunosuppressive IL-10 and IL-4 in treated animals, but no differences in ConA non-specifically stimulated conditions in MLR, further suggesting the induction of a LBN-specific cytokine response. Although significantly elevated TGF-$\beta$ levels in MLR supernatants suggested an immunosuppressive role for this cytokine in this model, this was not supported by *in vivo* data from sera. Further investigations into the role of TGF-$\beta$ are warranted as cells predominantly secreting this cytokine may traffic extravascularly. Although this regimen did not produce tolerance, the hyporesponsiveness that was demonstrated was clinically relevant to VCA survival and was achieved purely by mucosal exposure to donor antigen.

Our findings support those of Ishido et al. who used a jejunal mucosal tolerization protocol in a cardiac allotransplantation model, although the immunogenicity of non-skin-bearing transplants (such as cardiac and renal) and skin-bearing VCA are known to be different [16, 21, 22]. Another critical difference between Ishido et al.'s and the present study was that the former used concurrent CsA therapy [16, 17]. Immunosuppressants such as CsA were not used in the present study because we hypothesized that nonspecific immunosuppression might interfere with the mechanistic establishment of hyporesponsiveness. Several mechanisms have been proposed to explain mucosal tolerance, including anergy, deletion of antigen-specific T cells, and induction of regulatory T cells (T-regs) [11]. Many different T-reg subtypes have been implicated in oral tolerance and in gastrointestinal immunoregulation, including: Th3, Tr1, $CD4^+CD25^+$ T, $CD4^+CD45Rb^{low}$, and $CD4^+LAP^+$ T cells [11, 23]. However, recent independent experiments in experimental and human transplant recipients have provided a strong evidence base that suggests calcineurin inhibition by CsA interferes with T-reg production, notably of $CD4^+CD25^+FoxP3^{High}$ T-regs and the highly suppressive subset that are additionally $CD27^+$ [24–29]. One such study suggested calcineurin-dependent IL-2 production was critically required for T-regs *in vivo*; the functional defect of T-regs after CsA exposure could be reversed by exogenous IL-2 [29]. Thus, CsA was omitted from our investigations in case antigen-specific T-reg production might also be important in this model and yet be abrogated by calcineurin inhibition. If our further investigations reveal a role for T-regs in this model, it will be important to determine whether they are spared by concurrent subtherapeutic rapamycin therapy instead of using CsA [24–29].

In the present study, it was demonstrated *in vitro* that intrajejunal LBN FDS caused upregulation of immunosuppressive cytokines IL-10, IL-4, and TGF-$\beta$ with concomitant downregulation of proinflammatory IFN-$\gamma$ and TNF-$\alpha$. These findings were reflected in Day +7 sera from VCA recipients *in vivo*, except TGF-$\beta$ levels at this time point were not significantly affected by treatment. These findings were largely in agreement with those of Ishido et al.; however they found no changes in TGF-$\beta$ production in MLR supernatants, probably related to CsA administration [16, 27, 29]. Taken collectively, it seems likely that the present findings reflect the dominant involvement of donor-specific T helper-2 (Th2) cells although it is not yet possible to exclude secondary involvement of IL-10/TGF-$\beta$ secreting T-regs in this system. In either circumstance, optimum dose, duration, and effective formulation of enteral antigen administration need to be determined to induce maximum antigen-specific immunosuppression and clarify the dominant mechanism [11, 30, 31].

Importantly, donor alloantigens can be delivered to the gastrointestinal mucosa in different forms, such as allogeneic cells or synthesized MHC proteins. Splenocytes from Lewis rats that had been fed donor splenocytes, their lysates, or synthesized donor MHC determinants, exhibited significant antigen-specific reduction of MLR responses *in vitro* and delayed-type hypersensitivity responses *in vivo* compared to unfed controls [19, 32–34]. Oral administration of a synthetic peptide (B7.75–84), corresponding to residues 75–84 of the human HLA-B7 molecule, to ACI rat recipients together with subtherapeutic cyclosporine A (CsA) caused Lewis cardiac allotransplants to survive indefinitely

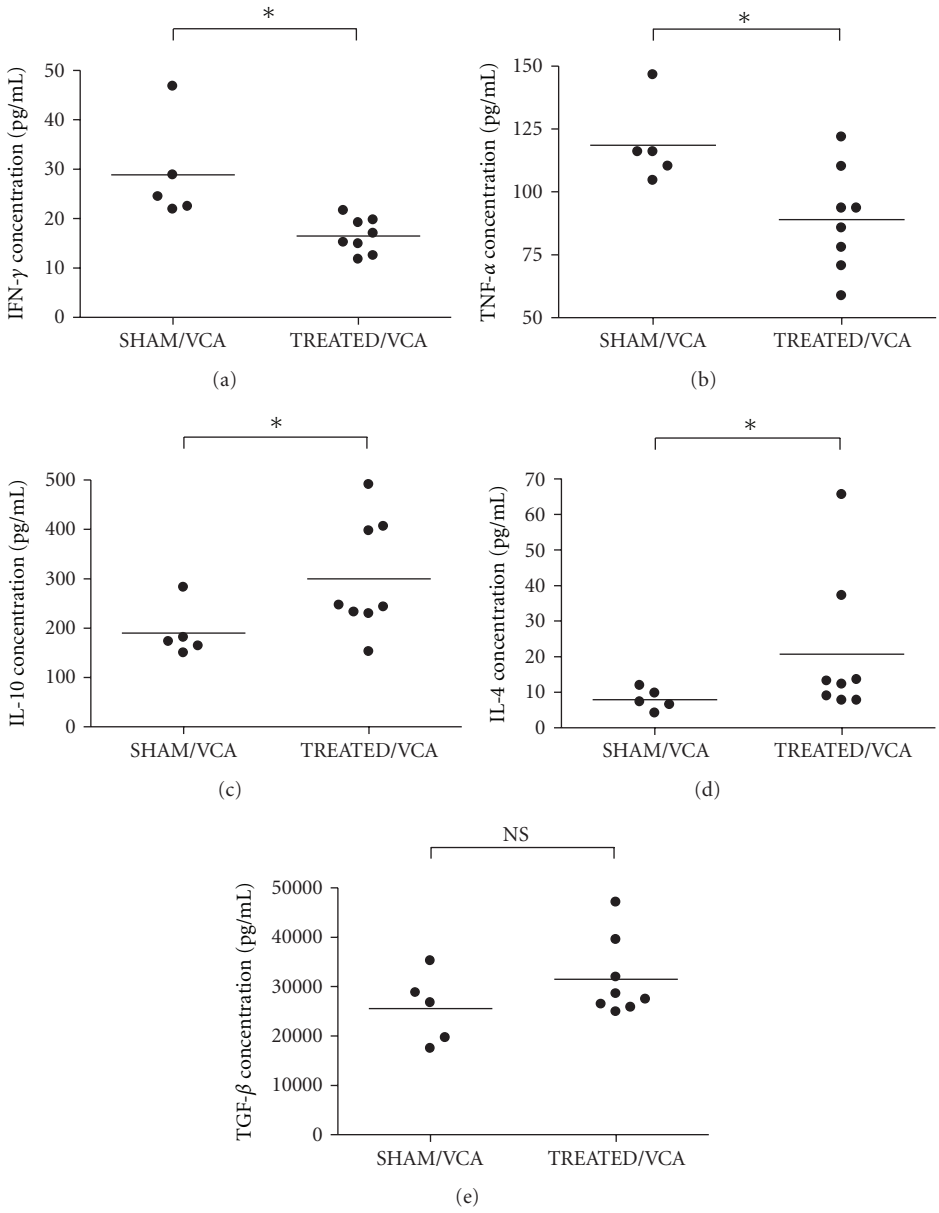



Figure 6: Cytokine concentrations according to flow-CBA ((a) IFN-$\gamma$; (b) TNF-$\alpha$; (c) IL-10; (d) IL-4) and ELISA ((e) TGF-$\beta$) in Day +7 sera from TREATED/VCA and SHAM/VCA Groups presented as scatter plots with mean bar. "$*$" $P < 0.05$; "NS" $P < 0.05$ (specific $P$ values provided in the text).

(>200 days) in 75% of recipients whilst third party skin allografts were rejected normally [35]. In a rat model of second-set rejection, oral administration of donor splenocytes prolonged semiallogeneic and fully allogeneic cardiac allotransplant survival times in an antigen-specific manner [19, 34]. Fully mismatched (BN to Lewis) renal allotransplant first-set survival times were significantly prolonged in recipients that had been prefed donor splenocytes and were prolonged further by donor cell feeds before and after transplantation [36, 37]. This prolongation was alloantigen specific and was accompanied by generation of intragraft $CD8^+$ regulatory cells in tolerized animals [37, 38]. Adoptive transfer of these $CD8^+$ cells to naïve rats transferred allotransplant tolerance observed in the original fed rats [38]. Other oral tolerization protocols have significantly prolonged survival times for nonvascularized allografts, including skin [39–44].

For a tolerance induction regimen to be applicable in humans, it must be safe. Intrajejunal access by endoscopically or fluoroscopically guided nasoenteric catheterization can be safely achieved in humans for up to 30 days [45]. Alternatively, donor antigens could be delivered in gastric-acid protected capsule form. Furthermore, no human trials of mucosal tolerance, which have tested a wide variety of antigens, have demonstrated toxicity from treatment or worsening of disease [11]. Since the dosage, type (e.g., whole cell or peptide; soluble or insoluble), and route of

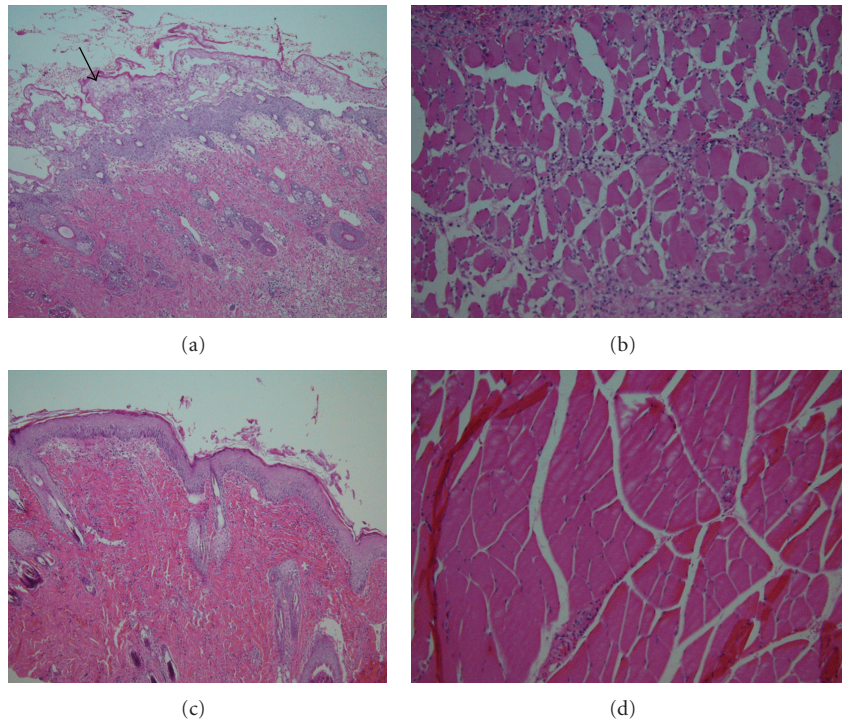



Figure 7: Photomicrographs (original mag: 200×) of Day +7 VCA-Skin ((a) and (c)) and VCA-Muscle ((b) and (d)) biopsies from SHAM/VCA ((a) and (b)) and TREATED/VCA ((c) and (d)) rats.

antigen delivery are critical to the mechanism and success of mucosal tolerance induction, variations in each of these and other parameters may be important in improving the tolerogenicity of mucosally delivered antigen whilst maintaining its safety [11, 30, 31].

It is conceivable, if further experiments confirm the safety, reliability, and underlying mechanisms of the approach, that early intrajejunal access and jejunal tolerance induction in human VCA recipients could be used to boost peripheral mechanisms of tolerance. Thereafter, although not yet investigated, it appears plausible that oral delivery of capsule-protected (against gastric acid) donor antigens could be used to maintain donor-specific hyporesponsiveness and allow reductions in immunosuppressive drug therapies. Additionally, antigen delivery as synthesized MHC peptides would likely be more acceptable to patients than enteral delivery of cell matter and requires further investigation.

## 5. Conclusion

The present study showed that intrajejunal administration of donor antigen induced donor-specific hyporesponsiveness with Th2 dominant status and delayed VCA rejection without concomitant immunosuppressants. Tolerance was not achieved but the hyporesponsiveness was clinically relevant and significant. Further investigations are warranted to optimize the administration (e.g., dose, form, and treatment duration) of alloantigens to maximize donor-specific hyporesponsiveness, changes to which may cause variations in the dominant mechanism(s) involved. Finally, the role, if any, and identification of various T-reg subtypes in this system invite clarification.

## Abbreviations

CBA: Cytometric bead array
ConA: Conclavulin A
CsA: Cyclosporine A
ELISA: Enzyme-linked immunosorbence assay
FDS: Fresh donor splenocytes
GvHD: Graft-versus-host disease
HBSS: Hanks balanced salt solution
IFN: Interferon
IL: Interleukin
LBN: Lewis-Brown-Norway
MHC: Major histocompatibility complex
MLR: Mixed lymphocyte reaction
MWT-*ot*: Mann-Whitney test one-tailed
MWT-*tt*: Mann-Whitney test two-tailed
TGF: Transforming growth factor
Th2: T helper-2
TNF: Tumor necrosis factor
T-reg: Regulatory T cell
VCA: Composite tissue allotransplantation.

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*Review Article*

# Mesenchymal Stem Cells as Immunomodulators in a Vascularized Composite Allotransplantation

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Vascularized composite allotransplantations (VCAs) are not routinely performed for tissue reconstruction because of the potentially harmful adverse effects associated with lifelong administration of immunosuppressive agents. Researchers have been eagerly seeking alternative methods that circumvent the long-term use of immunosuppressants. Mesenchymal stem cells (MSCs) show promise as an immunomodulatory therapeutic agent and are currently being tested in preclinical and clinical settings as therapies for autoimmune disorders or transplant rejection. The mechanisms by which MSCs modulate the immune response are still under thorough investigation, but these most likely involve expression of local factors influencing T-cell regulation, modulation of cytokine expression (e.g., IL-10, TGF-$\beta$, TNF-$\alpha$, INF-$\gamma$, etc.), and interactions with dendritic or antigen presenting cells. In this paper, we summarize the current understanding of immunomodulation achieved by MSC therapies and introduce a possible outline for future clinical applications in VCA.

## 1. Introduction

Avascularized composite allotransplantation (VCA) consists of various tissue combinations, including muscle, nerve, tendon, skin, bone, cartilage, and bone marrow. VCA serves as an ideal solution for the replacement or repair of certain tissues following traumatic loss, tumor resection, or repair of congenital abnormalities [1]. Recently, important advances have been made and new studies demonstrate that VCA is clinically feasible [2, 3]. Indeed, 78 successful human hand and 14 partial face allotransplantations have been performed [4–6]. However, VCA is not routinely performed for tissue repair and reconstruction because lifelong administration of immunosuppressive agents, which have potentially harmful side effects, is necessary to avoid rejection of the highly antigenic skin tissue [7–9]. Furthermore, even if patient compliance is excellent, conventional immunosuppressive protocols may not be sufficient to prevent chronic rejection [4, 10, 11]. Consequently, researchers have been eagerly seeking alternative methods of establishing lifelong tolerance while minimizing toxicity. Studies have reported numerous active clinical trials in which mesenchymal stem cells (MSCs) are used in the treatment of inflammatory diseases, such as graft-versus-host disease (GVHD) [12, 13], Crohn's disease [14, 15], ulcerative colitis [16], multiple sclerosis [17, 18], and systemic lupus erythematosus [19, 20]. Herein, we focus on the immunomodulatory effects of MSCs, provide a snapshot of the results from current *in vitro* and *in vivo* studies, and discuss future prospects in which these procedures can be made widely available in VCA.

## 2. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), which originate in the bone marrow, are multipotential nonhematopoietic progenitor cells capable of differentiating into various mesenchymal cell types. Bone marrow (BM) stromal cells were first identified by Friedenstein, who described an adherent fibroblast-like population, which was able to differentiate into bone, that he referred to as osteogenic precursor cells [21]. Subsequent studies demonstrated that these cells have the ability to differentiate into various other mesodermal cell lineages, including chondrocytes, osteocytes, tenocytes, and myoblasts, and this ability is currently used as a functional criterion in defining MSCs [22, 23]. Recent studies have identified pluripotent cells that not only differentiate into cells of the mesodermal lineage, but also into endodermal and neuroectodermal lineages, including neurons, hepatocytes, and endothelial cells [24]. Based on this multilineage differentiation capacity, Caplan introduced the term mesenchymal stem cells (MSCs) [25].

*2.1. Source and Characteristics of MSCs.* Although MSCs were originally isolated from BM, similar pluripotent cell types have been isolated from other tissues, including adipose tissue, placenta, amniotic fluid, and fetal tissues, such as lung [22, 26, 27]. They can also be isolated from cord blood, synovial tissue and, at extremely low frequencies, from adult peripheral blood [26, 28]. Currently, no specific marker or combination of markers have been identified that specifically defines MSCs. MSCs have been expanded in culture, *ex vivo*, and have been phenotypically characterized by flow cytometry. MSCs expressed CD44, CD73, CD90, MHC class I, CD105, and CD166, as determined by positive surface staining. MSCs are devoid of hematopoietic and endothelial markers such as MHC class II, CD11b, CD14, CD31, CD34, CD45, and CD80/B7-1 [26–28]. The capacity to differentiate into multiple mesenchymal lineages, including adipocytes, osteoblasts, and chondrocytes, is used as a functional criterion to define MSCs (Figure 1).

### 2.2. MSC-Mediated Immunosuppression In Vitro

*2.2.1. The Interaction between MSCs and T Cells.* Previous studies have revealed that MSCs do not express immunogenic costimulatory molecules such as B7-1, B7-2, or CD40. Therefore, it is likely that they are unable to stimulate alloreactive T cells [29, 30]. Glennie et al. suggested that bone marrow MSCs can arrest the division of activated T cells and induce T-cell anergy. Studies have demonstrated that MSCs can suppress T-lymphocyte activation and proliferation *in vitro*, and that this inhibition affects the proliferation of T cells induced by alloantigens and mitogens, as well as the activation of T cells by CD3 and CD28 antibodies [31]. Further study has indicated that soluble factors are involved, as the separation of MSCs and peripheral blood mononuclear cells (PBMCs) by a semipermeable transwell membrane does not prevent inhibition of proliferation [32]. Supernatants from human and mouse MSC cultures show no inhibitory effect unless MSCs have been cocultured with lymphocytes, suggesting that the suppressive factor(s) are not constitutively secreted by MSCs, but require dynamic cross-talk between MSCs and T lymphocytes [33].

Our previous study tested the effects of co-cultured adipose-derived mesenchymal stem cells (ASCs) and allogeneic T cells from a completely MHC-mismatched strain. BrdU proliferation assays have revealed a statistically significant reduction in the proliferation of T cells that were co-cultured with ASCs, compared to T cells that were cultured alone [34]. Our study further revealed a statistically significant reduction in the proliferation of T cells that were co-cultured with syngeneic MSCs [35]. These data indicate that the suppression of T-cell proliferation by MSCs has no immunological restriction, as similar suppressive effects were observed with cells that were autologous or allogeneic to the responder cells.

*2.2.2. MSC-Induced T-Cell Regulation.* It is commonly accepted that immunosuppression can be accomplished by lymphocyte populations that are known as regulatory T cells. The regulatory T-cell population resides mainly within the CD4+ T-cell subset; specifically, these cells are described as CD4+CD25+ forkhead box P3+ (Foxp3+) (Treg) cells [36, 37]. CD4+CD25+ regulatory T cells have emerged as a unique population of T cells that help to maintain a peripheral immune tolerance [38, 39]. In our study, we analyzed *in vitro* the percentage of CD4+/CD25+/Foxp3+ regulatory T cells and revealed that this population was significantly increased in MSC and T cell co-cultures, as compared to T cells cultured alone [34]. These results indicate that MSCs both suppressed T-cell proliferation and increased the number of regulatory T cells.

The mechanism by which the anti-proliferative effects of MSCs are delivered has not yet been elucidated, although several candidate molecules have been proposed [30, 40, 41]. Previous studies have indicated that MSCs actively inhibit the functions of several immune cells through enzymatic activity and the secretion of cytokines and growth factors [40–42]. The mechanisms underlying these effects are not fully understood, but they appear to involve both cell contact and a range of soluble factors, including transforming growth factor (TGF)-$\beta$, interleukin-10 (IL-10), interferon-$\gamma$ (IFN-$\gamma$), metabolites of tryptophan that are generated by the activation of indoleamine-2,3-dioxygenase (IDO), or prostaglandin E2 (PGE2) [33, 43–46]. Recent studies have elucidated an important and complex role for TGF-$\beta$ in regulatory T-cell biology [44, 47]. The disruption of TGF-$\beta$ signaling in T cells impairs the maintenance of regulatory T cells, which results in the expansion of activated effector T-cell populations [48]. Aggarwal and Pittenger showed that coculturing MSCs with differentiated effector T cells simultaneously led to a decreased release of the proinflammatory cytokine IFN-$\gamma$ from Th1 cells, an increase in IL-4 release from Th2 cells, and an increase in the proportion of regulatory T cells [42]. These data provide a strong evidence that MSCs can induce a shift from a pro-inflammatory to an anti-inflammatory state.

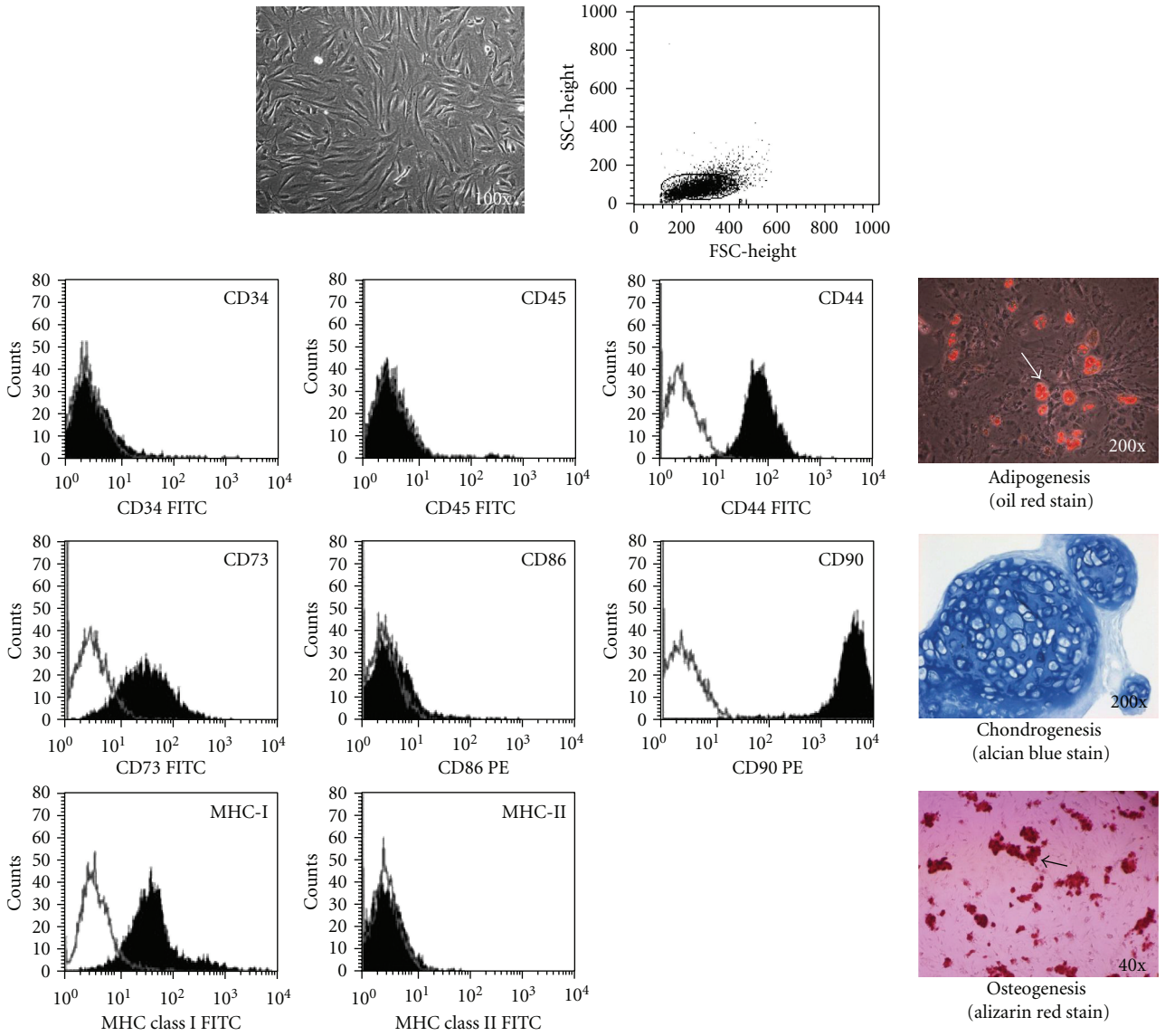


Figure 1: The phenotypic characterization and differentiation potential of mesenchymal stem cells (MSCs) *in vitro*. Mesenchymal stem cells were expanded in culture and demonstrated positive surface staining for CD44, CD73, CD90, and MHC class I, but not for CD34, CD45, MHC class II, and CD86 expression, as detected by flow cytometry. MSCs were further tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Osteoblasts were identified by Alizarin red staining, lipid droplets were identified by oil-red O staining, and chondrogenic differentiation was visualized by Alcian blue staining.

*2.2.3. MSCs Inherent Maturation Process of Dendritic Cells (DC).* Dendritic cells (DCs) play a key role in the induction of immunity and tolerance, depending on their activation and maturation stages and, as recently proposed, the cytokine milieu at the sites of inflammation [49, 50]. Mature DCs express high levels of MHC class II, CD80, and CD86, which are well described in antigen presentation to CD4+ T cells [51]. Thus, DC maturation plays a key role in initiating T cell responses to evade immunity. DCs have the ability to initiate a primary adaptive immune response through the capture, processing, and presentation of antigen to naive CD4+ T cells; however, differences in the capacities of DCs to initiate these responses are linked to the developmental maturation state of the DC [52]. Our study revealed that recipient immature DC pulsed alloantigen combined with a short-term immunosuppressant could significantly increase hind-limb allograft survival in rodents and could increase the percentage of regulatory cells *in vivo* [42, 53]. MSCs have been demonstrated to interfere with the differentiation and maturation of DCs by suppression of the expression of MHC class II, CD80, and CD86 [54, 55]. These data indicated that MSCs can modulate DC maturation and decrease T-cell activation.

## 3. Immunomodulatory Effects of MSCs in VCA

*3.1. MSCs Suppress GVHD in a VCA Model.* It has been previously demonstrated that the combination of bone marrow

Table 1: Pre-clinical allotransplant models utilizing MSC for immune modulation.

| Authors | Animal model | Allotransplant model | MSC source | Combined short-term immunosuppressant | Outcome |
|---|---|---|---|---|---|
| Kuo et al. (2009) [53] | Swine | Hind-limb VCA (allogeneic) | Donor bone marrow | CsA | MSC alone, prolong allograft survival; MSC + TBI + BMT + CsA, significantly prolong graft survival |
| Pan et al. (2010) [61] | Rat | Hind-limb VCA (allogeneic) | Allogeneic bone marrow | Rapamycin + ALG | MSC + rapamycin + ALG + TBI + BMT prolong allograft survival and induce mixed chimerism |
| Kuo et al. (2011) [35] | Swine | Hind-limb VCA (allogeneic) | Donor bone marrow | CsA | MSC + CsA + TBI prolong allograft survival |
| Kuo et al. (2011) [34] | Rat | Hind-limb VCA (allogeneic) | Allogeneic adipose tissue | ALS + CsA | MSC + ALS + CsA prolong allograft survival and induce immune tolerance |
| Kuo et al. (2012) [66] | Swine | Facial VCA (allogeneic) | Donor bone marrow | CsA | MSC + CsA prolong allograft survival |
| Itakura et al. (2007) [65] | Rat | Islet cell transplant (allogeneic) | Allogeneic bone marrow | CsA | MSC + BMT + CsA prolong islet allograft survival and induce immune tolerance |
| Kim et al. (2011) [43] | Rat | Islet cell transplant (allogeneic) | Autologous bone marrow | CsA | Prolong islet allograft survival |
| Casiraghi et al. (2008) [64] | Mouse | Heart (semiallogeneic) | Donor allogeneic bone marrow | — | Prolong heart allograft survival |
| Ge et al. (2009) [67] | Mouse | Heart (allogeneic) | Donor allogeneic bone marrow | Rapamycin | Prolong heart allograft survival |
| Sbano et al. (2008) [60] | Rat | Alloskin transplantation (allogeneic) | Donor allogeneic bone marrow | CsA | MSC + CsA prolong skin allograft survival |

transplantation (BMT) and immunosuppressant administration prolongs organ transplant survival [56]. Despite the promising potential of mixed allogeneic chimerism in the induction of VCA tolerance, graft-versus-host disease (GVHD), secondary to the introduction of donor BMT, and toxicity from ablative host conditioning are considered to be the main hurdles in the widespread acceptance of this technique [57, 58]. Studies have indicated that donor MSCs are potent inhibitors of T-cell proliferation in mixed lymphocyte cultures, thus preventing GVHD caused by total-body-irradiation-(TBI-) BMT and prolonging skin allograft survival in rodent models [59, 60]. Pan and colleagues have indicated a potential use of MSCs for the induction of stable and high level mixed hematopoietic chimerism and subsequent donor specific tolerance in a rat hind-limb VCA under a nonmyeloablative conditioning regimen [61]. In our study, we used a swine heterotopic hind-limb VCA model under TBI as a nonmyeloablative conditioning regimen [62]. The results revealed that TBI, combined with BMT and short-term cyclosporine-A (CsA) treatment, induced GVHD-related symptoms [63]. However, multiple rounds of donor MSC therapy combined with BMT, after TBI and short-term CsA treatment, appear to modulate GVHD and prevent graft rejection [63].

*3.2. MSCs Prolong VCA Survival.* The immunomodulatory effects and therapeutic potential of MSCs in organ transplantation have resulted in successful preclinical applications for composite tissue and organ allotransplantation (Table 1) [34, 35, 43, 53, 60, 61, 64–67]. We have investigated the effects of MSCs on prolonged VCA survival by measuring the immunosuppressive activity that was rendered by multiple injections of adipose-derived MSCs (ASCs, $2 \times 10^6$/dose on days 7, 14, and 21 after transplantation), short-term anti-lymphocyte serum (ALS) and CsA in a rodent hind-limb model [34]. The results revealed that ALS-CsA-ASCs significantly prolonged VCA survival without rejection, as compared to the results observed in ALS-CsA and untreated control groups [34]. We adjusted the protocol to be applicable to a large animal VCA model, and our results demonstrated that the administration of multiple injections of donor MSC injections ($2 \times 10^7$/dose on days 7, 14, and 21 post-transplantation) without BMT, combined with preoperative irradiation and short-term CsA, has similar results on allotransplant survival in the swine hind-limb VCA model [35]. Our results led us to speculate that BMT is unnecessary to prolong VCA survival if MSCs are used as an immunosuppressant. To reconfirm this hypothesis and to test another VCA model for pre-clinical study, we applied

MSCs in another large animal study, using a miniature swine hemifacial VCA model (consisting of skin paddle, muscle, ear cartilage, and lymphoid gland tissue) [68]. The difference between the hemi-facial VCA model and the hind-limb model is that the hemi-facial model does not contain donor vascularized bone, but does include more alloskin area and lymphoid gland tissue. Our results revealed that the MSC-CsA group had significantly prolonged hemi-facial VCA survival [66]. However, the survival of a VCA composite with vascularized bone in the hind-limb model is significantly longer than that of the hemi-facial model without vascularized bone. These results indicated that multiple injections of MSCs can prolong VCA survival, especially in a model of a hind-limb VCA composite with vascularized bone marrow.

*3.3. MSCs Modulate T-Cell Regulation in VCA.* To assess the regulation of T-cells in VCA that was treated with MSCs, regulatory-like T cells in circulating blood were detected by flow cytometry, and topical tissue expression of allotransplants was examined by immunohistochemical (IHC) staining in the rodent and miniature swine VCA models. In our rodent hind-limb VCA model, the flow cytometric analysis of recipient peripheral blood revealed that CD4+/CD25+/Foxp3+ regulatory T-cell populations were significantly increased at early time points (4–6 weeks after transplant) in the animals that were treated with multiple rounds of ASCs and short-term ALS-CsA, as compared to the ALS-CsA and control groups [34]. Furthermore, IHC staining of alloskin biopsies revealed significantly higher numbers of Foxp3+ T cells in the subcutaneous and dermis layers of skin from the animals that were treated with ASCs, CsA, and ALS, as compared to the other groups [34]. In our miniature swine hind-limb VCA study, flow cytometric analysis of recipient peripheral blood revealed that CD4+/CD25+ regulatory-like T-cell populations increased significantly in animals that were treated with MSCs, CsA, and irradiation at 2 weeks and 6 weeks following transplant, as compared to controls. Foxp3+ T cell populations increased significantly in the animals that were treated with MSCs, CsA, and irradiation at 2 weeks following transplant [35]. The percentages of regulatory-like T-cells gradually declined to a normal ratio by 300 days after transplant. In contrast, IHC staining of graft skin tissue biopsies revealed significant numbers of CD25+ T cells in the subcutaneous and dermis layers in animals treated with MSCs, CsA, and irradiation, as compared to the CsA alone and control groups [34, 35]. These results demonstrate that treatment with MSCs, combined with a short-term immunosuppressant regimen, increased the percentages of regulatory T-cell populations at the early time points after allotransplantation; however, this effect decreases with time.

*3.4. MSCs Induce Mixed Chimerism in VCA.* To evaluate the donor-specific chimerism in the rodent hind-limb VCA model (Brown-Norway (BN) to Lewis (LEW) strain), donor lymphoid cells ($RT1^n$ (+) cells) from the peripheral blood of the long-term survivors were examined by flow cytometric analysis. Our study revealed a significant increase in the population of RT1n-expressing BN donor cells in circulating blood from the recipients (LEW) that were treated with MSCs and short-term immunosuppressant therapy [34]. This study demonstrates that donor MSCs upregulate donor-cell microchimerism in recipients. These hind-limb allotransplants contain vascularized bone-marrow, which provides a constant resource of donor-specific progenitor cells and stromal cells, where the latter are essential for the proliferation and differentiation of BM-derived cells into hematopoietic progenitors.

*3.5. MSCs Modulate Cytokine Expression in VCA.* Studies have indicated that MSCs actively inhibit the functions of several immune cell types through enzymatic activity and the secretion of cytokines and growth factors such as TGF-$\beta$, IL-10, IFN-$\gamma$, and indoleamine-2,3-dioxygenase (IDO), PGE2 [44, 48, 69]. In our previous study, recipient peripheral blood serum was analyzed by ELISA following different treatments in a rodent hind-limb VCA model [34]. The data revealed that the circulating concentrations of both TGF-$\beta$l and IL-10, but not IFN-$\gamma$, increased significantly in VCA that were treated with multiple rounds of adipose-derived MSCs and short-term CsA-ALS at 4 weeks and 21 weeks following transplant, as compared to controls [34]. In our miniature swine hemi-facial VCA model, the concentrations of the soluble forms of TNF-$\alpha$, IL-10, and TGF-$\beta$l were determined by ELISA following the various treatments [66]. An analysis of recipient peripheral blood serum revealed that TNF-$\alpha$ levels were decreased significantly in the groups that were treated with MSCs alone or MSC-CsA at 2 weeks after transplantation, as compared to those in controls. Further analysis of recipient peripheral blood serum showed a trend toward increased TGF-$\beta$1 levels in MSC-treated groups, as compared to control groups. IL-10 levels were increased significantly in animals that were treated with MSC or MSC-CsA at 2 weeks after transplantation, as compared to controls [66].

In contrast, IHC staining of alloskin biopsies revealed significantly lower numbers of CD45 and IL-6 positive cells in the subcutaneous and dermis layers of skin from animals that were treated with MSC or MSC-CsA, compared to control animals [66]. The alloskin biopsies revealed a significant increase in the number of TGF-$\beta$l positive cells in the subcutaneous and dermis layers of skin from animals that were treated with MSC at 2 weeks after transplantation and MSC-CsA groups at 2 and 6 weeks following transplantation compared to controls [66]. These data indicate that regulatory-like T-cell subset-related cytokines were involved in MSC-induced immune tolerance and allotransplant survival.

*3.6. Homing of Exogenous MSCs in VCA.* Homing is the process by which cells migrate to, and engraft in, the tissue in which they can exert local and functional effects. To explore the mechanisms by which MSCs modulate allograft survival, the homing of MSCs in VCA recipients was tracked using BrdU-labeled donor MSCs [63]. BrdU-labeled donor MSCs were intravenously injected into the recipient swine in our large animal VCA model, followed by an investigation

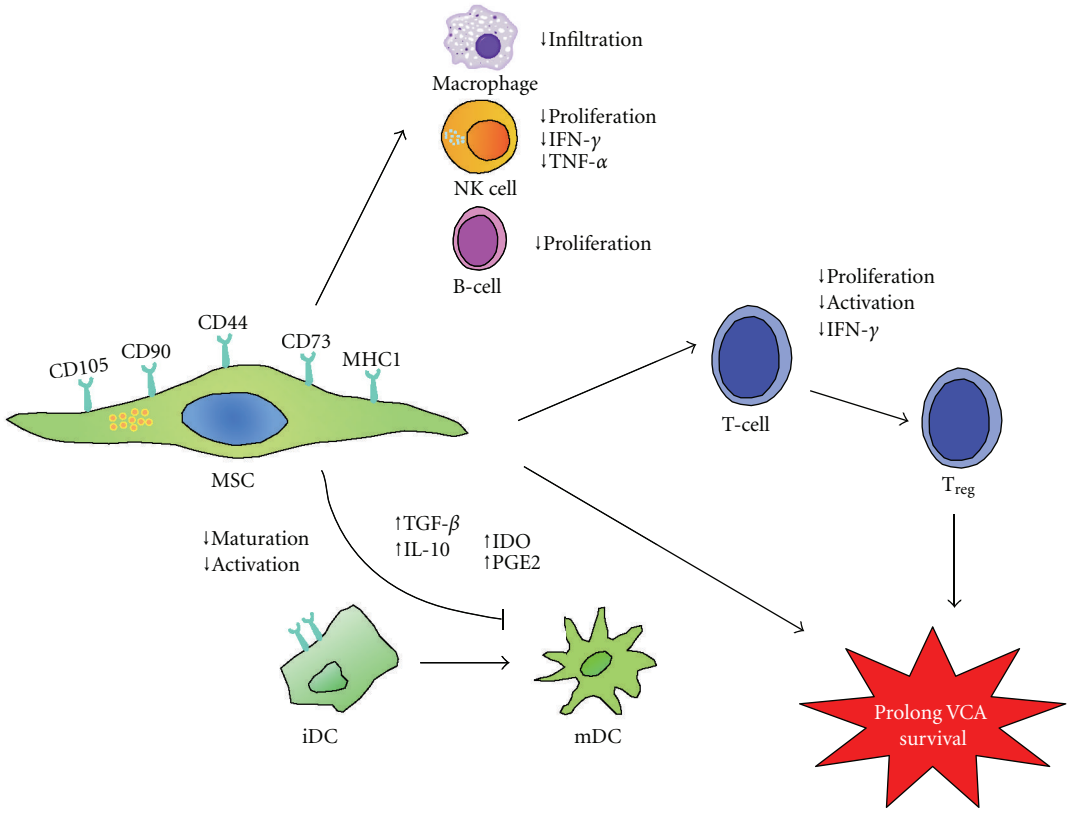


Figure 2: Proposed immunomodulatory mechanisms of MSCs in a vascularized composite allotransplantation (VCA). MSCs mediate their immunomodulatory effects by interacting with cells from both the innate (DCs, macrophages, NK cells) and adaptive immune (T cells and B cells) systems, particularly through the regulation of T-cell proliferation and the inhibition of DC differentiation. MSC inhibition of TNF-$\alpha$ and IFN-$\gamma$ secretion, promotion of IL-10 and TGF-$\beta$ secretion, and IDO and PGE2 expression may affect the maturation states and functional properties of DCs, resulting in skewing of the immune response toward the prolongation of VCA survival. DC: dendritic cells; IFN-$\gamma$: interferon (IFN)-$\gamma$; IDO: indoleamine-2,3-dioxygenase; NK: natural killer; PGE2: prostaglandin E2; $T_{reg}$: regulatory T-cells; TGF-$\beta$: transforming growth factor-$\beta$; TNF-$\alpha$: tumor necrosis factor (TNF)-$\alpha$.

of MSC homing and engraftment. Our data revealed a significant population of BrdU-labeled donor MSCs in the subcutaneous layers of both the donor and recipient skin and the perivascular parenchyma of the recipient liver, as detected by horseradish peroxidase-diaminobenzidine (HRP-DAB) staining [63]. This result indicated that the hematogenous spread of MSCs could enable engraftment and proliferation of these cells in the recipient tissue.

## 4. Conclusion

The therapeutic potential of MSCs in VCA has recently generated great interest and enthusiasm. The effects of MSCs can be exploited to produce a potent immunosuppressive response, as virtually all immune cells are susceptible, and much is expected from the use of these cells in allotransplantation. Multiple infusions of MSCs, combined with a nonmyeloablative preconditioning regimen (e.g., irradiation, antilymphocyte serum) and transient immunosuppression, could effectively prevent GVHD and prolong VCA survival. This prolongation of survival might occur because MSCs promote the engraftment of donor progenitor cells and modulate the host immune function. Based on our previous results and other reports, we suggest that the biomechanisms of MSC suppression involve the modulation of cytokine expression (e.g., IL-10, TGF-$\beta$, TNF-$\alpha$, INF-$\gamma$) and regulatory T-cell subsets, as we propose in Figure 2. Thus, MSC infusion is a potentially novel strategy for clinically improving VCA survival and inducing immune tolerance.

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*Review Article*

# The Need for Inducing Tolerance in Vascularized Composite Allotransplantation

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Successful hand and face transplantation in the last decade has firmly established the field of vascularized composite allotransplantation (VCA). The experience in VCA has thus far been very similar to solid organ transplantation in terms of the morbidity associated with long-term immunosuppression. The unique immunological features of VCA such as split tolerance and resistance to chronic rejection are being investigated. Simultaneously there has been laboratory work studying tolerogenic protocols in animal VCA models. In order to optimize VCA outcomes, translational studies are needed to develop less toxic immunosuppression and possibly achieve donor-specific tolerance. This article reviews the immunology, animal models, mixed chimerism & tolerance induction in VCA and the direction of future research to enable better understanding and wider application of VCA.

## 1. Introduction

Two areas of transplantation that posed significant obstacles to clinical application were vascularized composite allotransplantation (VCA) and donor-specific tolerance. However, over the last decade, it is heartening to note the progress that has been made in both of these fields. VCA has achieved acceptance in the field of transplantation [1] and promises to grow exponentially in the next few years. In the last 5 years there have been prospective investigational studies of donor bone-marrow infusion in living donor renal transplant recipients which have successfully induced donor-specific tolerance [2–5]. This new development has the potential for a wider application.

## 2. Immunology of VCA

Clinical feasibility of VCA has been established with the long-term success of hand and face transplantation. Over 50 hand and 14 face transplants have been performed worldwide with excellent outcomes [6]. The successful transplantation of these skin-bearing structures has been possible with the availability of potent immunosuppression. The vast majority of these recipients were managed with lymphocyte-depleting induction therapy [7] and triple drug maintenance immunosuppression (tacrolimus, MMF, and prednisone). T-cell depletion through antibody-mediated induction therapy is routinely used to promote long-term graft survival in solid organ transplantation. The most commonly used agents include antithymocyte globulin (ATG) and Campath-1H [8]. The majority of patients undergoing VCA have received T-cell depleting induction therapy [7]. Despite this aggressive immunosuppressive therapy, episodes of acute rejection have been recorded in 85% of hand and 54.5% of face transplant recipients in the first year after the transplant [9–11]. Thus the incidence of acute rejection following VCA transplantation is significantly higher than that seen currently with solid organ transplantation—the overall incidence of acute rejection within the first year after renal transplantation is now less than 15% [12].

*2.1. Immunology of VCA: VCA Is Not One Single Tissue.* VCA is composed of skin, muscle, vessels, nerves, tendon, bone, and so forth—each with differing immunogenic potential. Skin is probably the most immunogenic of all human tissues [13]. Lee et al. demonstrated that a whole limb allograft elicits a less intense alloimmune response as compared to each of its individual components [14]. This notion has been significant in the success of a whole limb allotransplantation compared to an isolated skin allotransplantation [15]. Several theories have been put forward to explain this and include (1) the vascularization of the skin arises from the donor in the whole limb versus the recipient in the isolated skin graft; (2) the occurrence of a consumption phenomenon when the host immune system is exposed to an excessive antigen load. A definitive immunological reason is yet to be elucidated [16].

In addition, the other theoretical advantage of VCA is the potential to transplant vascularized bone marrow present in the skeletal component of the allograft. The bone marrow is transplanted with its microenvironment. This has been postulated to confer an immunomodulatory effect that could lead to an improved long-term graft survival [17]. Although this concept has been established in experimental studies, there is paucity of data to support this in the clinical setting [18, 19]. Not surprisingly, graft-versus-host disease (GVHD)—a common occurrence with bone-marrow transplantation—has not been reported following VCA [7]. Notably, while VCA in the rat contains hematopoietic tissue, most bones in human VCA are not hematopoietic.

*2.2. Acute Rejection in VCA.* The high antigenicity of skin can be traced to the high proportion of potent antigen-presenting Langerhans cells. These and skin keratinocytes express MHC class I constitutively and upon stimulation present MHC class II, intercellular adhesion molecule 1 (ICAM-1), and proinflammatory cytokines. In addition, skin bears similarity with solid organs such as lung and intestine which have the highest rates of acute rejection [20, 21]. Skin biopsies from transplanted limbs have shown infiltration by CD3 positive T cells: both CD4 and CD8 subtypes and a minority of CD4 and CD8 negative cells [22]. During rejection, there is an increased expression of CD68, FoxP3, and indoleamine 2, 3 dioxygenase. Adhesion molecule expression is upregulated upon rejection—ICAM-1 and E-selectin correlated with severity of the rejection process [22].

Clinically, episodes of rejection are manifested by the appearance of characteristic cutaneous lesions—rash, edema, vesiculation, desquamation, necrosis, and ulceration [23]. Atypical rejection with reddening of palm and nail changes has occasionally been seen [24]. Biopsy of the skin (often protocol based without visual changes) remains the gold standard for detection of acute rejection. Acute rejection manifests initially as mild perivascular lymphocytic/mixed cellular infiltrate in the dermis. With an increase in the severity of rejection, there is an involvement of skin adnexal structures and epidermis that may lead to frank necrosis if left untreated. The Banff 2007 working classification is the currently used system to classify rejection in VCA [25], including Grade 0: no or rare inflammatory infiltrates; Grade I: Mild perivascular infiltration and no involvement of the overlying epidermis; Grade II: moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis) and no epidermal dyskeratosis or apoptosis; Grade III: Dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis, and/or keratinolysis; Grade IV: frank necrosis of epidermis or other skin structures.

There are two aspects that are well established in solid organ transplantation that are yet to be clearly delineated in VCA. These are the roles of HLA antibodies and the occurrence of chronic rejection. In renal transplantation, humoral rejection is diagnosed by the presence of (1) histological injury—neutrophils in capillaries, acute tubular injury and fibrinoid necrosis; (2) evidence of antibody interaction with tissue—C4d deposition in peritubular capillaries; (3) serological evidence of antibodies to donor HLA (DSA). The occurrence of this triad is clearly related to organ dysfunction. The incidence and occurrence of humoral rejection in VCA have not been studied. Although C4d deposition has been documented in VCA literature, it has been described in the absence of donor-specific antibodies and histological tissue injury [26].

Similarly, chronic rejection is poorly defined in VCA. Histological and clinical features indicative of chronic injury in VCA include vascular narrowing, loss of adnexa, skin and mucosal atrophy, fibrosis of deep tissue, myointimal proliferation, and nail changes [11]. Transplant vasculopathy and features suggestive of chronic rejection have been induced after multiple untreated episodes of acute cellular rejection in a rat hind-limb allotransplantation model [27]. Graft vasculopathy has been described in hand transplant recipients and has been associated with graft loss in one patient [28]. Novel methods such as the use of ultrasound biomicroscopy to evaluate vessel wall thickness in VCA grafts have been proposed to enable early detection of graft vasculopathy [28]. However, the etiopathogenesis, incidence, risk factors, and management of this entity in VCA remain to be defined.

*2.3. Clinical Results in VCA.* Functional outcomes after hand transplantation have been excellent. In the report of the international registry on hand and composite tissue transplantation [6], protective sensibility was restored in 100% patients, tactile sensibility in 90%, and discriminative ability in 84%. Most patients are able to perform daily activities one year after transplantation and their quality of life was significantly improved. The majority of them returned to work eventually.

In contrast, functional outcome assessment in face transplant recipients is more difficult to standardize due to the uniqueness and complexity of the defect in individual patients. Results from the early recipients are very encouraging: the first four patients were able to eat, drink, and speak within 10 days of transplantation [29]. As new functional units such as tongue and lacrimal gland are added to the

facial allograft, these outcomes are likely to improve even more.

*2.4. Burden of Immunosuppression.* Unlike solid organ transplantation, greater scrutiny has been placed on immunosuppression-induced complications in VCA recipients. This is largely appropriate as VCA has been deemed life enhancing as opposed to a life saving intervention. On this basis, recipient selection has been very stringent thus far in VCA. The majority is physically healthy individuals suffering from severe tissue defects of the face or limbs [30]. Despite the thorough vetting of potential recipients, the observed postoperative complications have largely mirrored those described in solid organ transplantation.

Metabolic complications have been reported in 69% of hand recipients and include diabetes, hypertension, and renal dysfunction including the need for renal replacement therapy in 1, Cushing's syndrome and aseptic vascular necrosis of both hips needing replacement [6]. The majority of recipients developed infectious complications: CMV infection occurred in 10/33 hand recipients. Interestingly, severe CMV infection was noted in two of the first four face allotransplant recipients [31]. Posttransplant lymphoma and basal carcinoma of nose have been reported in hand recipients [6].

Graft loss has been reported in the hand transplant literature: 7 patients from China due to an inadequate immunosuppression; 3 patients from the West from the cessation of immunosuppression, transplant vasculopathy, and bacterial infection [6]. More concerning is the reported mortality following face allotransplantation: 2 of 17 recipients to date have died: one Chinese recipient died 2 years after the procedure from an unknown cause and the world's first recipient of simultaneous face and hand transplant died at 2 months [32].

Clearly, there is a significant price to pay for the immunosuppression currently essential for the successful VCA. While we strive to gain better understanding of the immunology of VCA, the results thus far urge us to find ways to minimize the need for immunosuppression in these recipients. It is time to consider a clinical application of tolerance data accrued from animal experiments and the clinic.

## 3. Clinical Success in Organ Transplantation Tolerance

Induction of chimerism has been shown to be a reproducible method to induce tolerance in the laboratory. Studies published in 2008 supported strategies to achieve tolerance by donor bone-marrow infusion in living donor renal transplant recipients. The limitations of the studies were success only in HLA matched pairs in one study [2] and only short lived chimerism (undetectable after 2 weeks) and engraftment syndrome in the other [3]. A recent approach using a different strategy has reported durable chimerism (5 of 8 patients at 15−30 months) and tolerance induction in mismatched living donor renal transplant recipients [4]. The technique is based on nonmyeloablative conditioning using cyclophosphamide, fludarabine, and 200 cGy of total body irradiation (TBI). The renal transplant is followed by infusion of a bioengineered mobilized cellular product enriched for hematopoietic stem cells and facilitating cells. The facilitating cells have been previously demonstrated to promote engraftment without an increase in GVHD [33]. Based on the experience in the first 4 patients, the appropriate dose of $\alpha\beta$ T-cell dose has been defined. Subsequent patients have demonstrated durable multilineage chimerism and have been successfully weaned off all immunosuppression. None of the recipients developed GVHD or donor-specific antibodies. Some of the complications that have been reported in the above tolerance induction studies include engraftment syndrome with reversible acute kidney injury [34] and the loss of renal grafts from rejection and viral sepsis induced vascular thrombosis [4, 34].

Thus this approach appears very promising as a potential way forward in solid organ transplantation. But, more importantly, it may hold important lesions to enable a wider application of VCA. The reluctance on the part of the plastic and reconstructive surgeons in embracing VCA is the fear of long-term complications that are part and parcel of conventional immunosuppression. Future refinement of the above-mentioned tolerance strategies to enable use with deceased donor transplantation, further elucidation of the mechanistic components of these studies and a longer-term followup of the "tolerant patients" could help persuade reconstructive surgeons to use VCA.

## 4. Animal Models in VCA Study

It is known that the current challenge in the widespread clinical applicability of VCA is the toxicity of high-dose postoperative immunosuppression. The use of preclinical animal models is essential in developing novel cellular immune-reduction therapies that will ultimately make VCA a safer and viable treatment option. Both small and large animal models have been employed in studying VCA immunology thus far.

*4.1. Small Animal Models.* Small rodent animals are widely preferred for VCA studies for certain reasons. Logistically, such animals are relatively inexpensive and easy to maintain and handle. Biologically, these animals have short lifespans and accelerated reproduction rates that allow for VCA-related immune activities to be observed along varied time points of the life history [35]. VCA is complicated due to the disparate antigenicities of the composite tissues. Small rodent models can be an invaluable tool to investigate these various tissue antigenicities, and rodent surgical models serve well to evaluate acute and chronic rejection events postoperatively [36]. Additionally small rodents are widely used as functional models to study nerve regeneration, a unique challenge to VCA [37].

Mice and rats are the predominant small animal models used in VCA studies. The mouse model is a more valuable tool for basic immunologic research due to the numerous genetic variations of inbred and knockout strains and the

commercially available genetic probes and antibodies [35, 36]. Mouse models are commonly used to study specific tolerance induction therapies. In contrast, the rat model has been more prevalent in functional studies. The rat hind-limb allograft model is a hallmark in evaluating the post-operative function of composite allografts and rejection events.

However, there are limitations of small animal models as it pertains to VCA. The most significant obstacles have been the technical challenges during microsurgery. Dissection and microanastamosis in small vessels in mouse models are problematic due to the fragility of the vessel walls [38, 39]. Similar microsurgical difficulties limit rat VCA-related models. Yet novel microsurgical techniques are in development to improve and expand VCA research potentials using mouse and rat models.

Despite the technical limitations of small animal models, such animals continue to be a cornerstone of VCA research. Various composite allografts have been performed in mice and rats in the past decade, including hind-limb transplants, bone-marrow transplant (BMT), a hemiface allograft, and even an allograft of the groin region has been performed [40, 41].

*4.2. Large Animal Models.* Immune-reduction protocols that have been successfully demonstrated in small rodent models are further evaluated in large VCA animal models for efficacy and safety before clinical trials in humans. Large animal models allow for immune-reduction therapies to be further examined in more complex biological systems that are more realistic and representative of the human immune system [35, 42–45]. Models that have been used related to VCA thus far include canines, swine, and nonhuman primates. In particular swine and nonhuman primates have a clinical relevance presenting MHC antigens similar to humans. Nonhuman primates are highly preferred in pre-clinical human immunologic studies due to the genetic similarity between primates and humans. Certain species of macaque primates are also favored for they are relatively small in size and demonstrate acceptable homology to cross react with most human immune molecules [35].

Tolerance of VCA can be induced in small rodent models, but tolerance protocols established in rodents are difficult to translate to preclinical large animal models and eventually clinical human trials. In contrast to the isolated, pathogen-free lab rodents, large animal models are exposed to uncontrolled environmental factors over longer lifespans, resulting in immune-reduction protocols that are challenging to stabilize. Further, unlike rodent models, the complex immune system in large animals requires significant potent doses of immunosuppression for graft survival in unrelated donor/recipient pairs [46]. Examples of successful immune-reduction protocols in rodent models that are more difficult to demonstrate in larger animals include limb allografts and face transplant models. Both limb and face transplants are more difficult to replicate in large animals due to the higher doses of post-operative immunosuppressants involved and the differing responses of large animals to immune-reduction protocols and methods established in mice [47, 48]. Consequently direct translation of animal protocols to human clinical trials still remains daunting due to the toxicities that may be induced by concentrated post-op immunosuppressant requirements [35, 42, 43]. Thus limitations in large VCA animal models are not purely technical. The use of animal models, both small and large, in VCA research has yielded significant progress and will continue to do so. Tolerance induced via mixed chimerism has demonstrated promise in minimizing, even eliminating, postoperative immunosuppressants, enabling VCA as a widespread clinical option.

## 5. Mixed Hematopoietic Chimerism and VCA Tolerance

A major factor limiting VCA is the requirement for lifelong immunosuppression and the toxicities associated with the use of these agents [9]. Virtually all expected complications associated with the use of chronic immunosuppression, including renal failure and death from infections, have occurred now in recipients of VCA [11]. Efforts to immunomodulate the VCA graft and recipient to induce donor-specific tolerance would be transformational in organ and VCA transplantation. Immunological tolerance would achieve permanent VCA survival and abrogate the need for chronic immunosuppression.

*5.1. Conditioning for Induction of Mixed Chimerism.* The establishment of donor hematopoietic chimerism in organ transplant recipients leads to donor-specific tolerance [49–52]. Chimerism refers to a state of a conditioned recipient in that the donor hematopoietic stem cell engrafts and produces multiple lineages of blood cells. A new immune system including that of the donor, therefore, is established in the recipient. The tolerance associated with chimerism is permanent, stable, and not easily broken [53, 54]. Immunosuppression is not required to prevent graft rejection once chimerism is present. Chimerism is the only approach that has been generalizable to all species tested, including humans [2–4]. There are two types of chimeras: full chimerism, where the donor hematopoietic system totally replaces the recipient system, and mixed chimerism, where the donor and recipient HSC coexist. To establish a full donor chimerism, the recipient's entire hematopoietic system is ablated by lethal conditioning and replaced by the donor system. In 1985, Ildstad et al. [53] reported that mixed allogeneic chimerism induces tolerance to donor-specific skin grafts. Mixed chimeras exhibit superior immunocompetence due to the presence of recipient antigen-presenting cells to which lymphocytes of both recipient and donor origin are restricted [55]. However, in this pioneer study, mixed chimerism was established with ablative irradiation and transplantation of a mixture of T cell-depleted host and donor BMC. The application of mixed chimerism to induce tolerance in transplantation has been limited by the side effects associated with myelotoxic conditioning. As a result, we and others developed clinically relevant reduced-intensity conditioning to establish chimerism in animal models [56–61]. This

critical paradigm shift allowed for the development of reduced-intensity immune-based conditioning approaches to establish mixed chimerism which has been successfully translated to the clinic [62]. The immunomodulation of the host-versus-graft immune response could provide a novel form of conditioning to establish chimerism and may completely eliminate the need for TBI and myelosuppressive conditioning agents. Recently, a mixed chimerism was shown to be established by a nonmyeloablative conditioning with TBI as low as 300 cGy in an allogeneic rat model [51] and 100 cGy in a mouse model [63].

*5.2. The Association between Mixed Chimerism and VCA Tolerance.* Mixed chimerism induces donor-specific tolerance to virtually all the organs or tissues tested including skin, heart and lung, kidney, intestine, pancreas, islets, and composite tissue allografts [51, 64–66]. In an earlier study, 950 cGy ablative TBI was used in a rat model as conditioning for chimerism followed by donor hind-limb transplantation [65]. Their results showed stable chimerism and reliable limb allograft survival. However, a safe and reliable method to facilitate the induction of mixed hematopoietic chimerism for VCA tolerance is needed. Rahhal et al. [51] had recently reported that the long-term acceptance of VCA could be induced by mixed chimerism established by nonmyeloablative conditioning with TBI as low as 300 cGy combined with a short course of immunosuppressive therapy (anti-$\alpha\beta$-TCR mAb, FK-506, and antilymphocyte serum). The BMT conditioning strategies based on costimulatory blockade of CD28 or CD40 ligand in combination T-cell depletion and low doses of irradiation have also reported to induce long-term acceptance of VCA in rat [64] and to prolong VCA survival in mouse [67]. The advantage of nonmyeloablative conditioning is that the recipients will survive from their autologous reconstitution of self-stem cells if the BMT fails to take. The optimal level of donor chimerism in tolerance induction for VCA was investigated using an MHC incompatible rat model and a reduced-intensity conditioning [68]. The chimerism level correlated positively with the incidence of GVHD and long-term CTA. Levels of 20–50% donor chimerism at day 28 were optimal for VCA acceptance with minimal or no GVHD in this rat model. Higher levels of donor chimerism were also found to be associated with VCA acceptance with nonmyeloablative conditioning of anti-$\alpha\beta$-TCR mAb, FK-506, and anti-lymphocyte serum and 300 cGy TBI for BMT [51]. There was a correlation between higher levels of donor chimerism at one month after BMT and graft acceptance in animals from all groups that accepted flap allografts ($38.6 \pm 2.1\%$) compared to animals that rejected their flaps ($18.9 \pm 3.6\%$).

*5.3. The Vascularized Bone-Marrow Transplant in VCA.* One unique feature that distinguishes VCA from other transplants is the presence of its own hematopoietic microenvironment and supportive stromal cells from accompanying donor bone. Bone-marrow-derived cells, especially plasmacytoid precursor dendritic cells (p-preDC) and the regulatory T cells ($T_{reg}$) they generate, maintain self-tolerance through regulatory feedback loops. They also show promise as a cell-based therapy to promote allograft acceptance. Bone marrow has long been appreciated to possess immunomodulatory properties [50, 69–72]. Therefore, the vascularized BM transplant (VBMT) model has been developed as a better source for hematopoietic cell reconstitution than transplantation of cellular BMC [73]. This model promotes long-term mixed chimerism and tolerance [74] with a decreased incidence of GVHD [75]. The sources of the vascularized bones tested have been hind limb [76, 77], sternum [78], femur [79], maxilla [80], and ilium [81].

*5.4. The Timing between Chimerism Induction and VCA.* The timing between BMT and VCA is also an important and clinically relevant issue in tolerance induction by chimerism. Historically the VCA was performed in established chimeras about 1-2 month after BMT [51, 65, 68] and termed a sequential model (Figure 1). The delay between BMT to solid organ transplantation is clinically applicable in the case of living donor organ transplantation. However, this would not be the case in VCA transplants because the VCA is always a clinical scenario of deceased donor donation setting. The clinically relevant VCA model would be that BMT and VCA are performed simultaneously or the chimerism established after recovery from the VCA. The feasibility of this simultaneous BMT and VCA model is established in rat models. Prabhune et al. reported in an ablative conditioning model that tolerance to hind-limb transplants can be established through the simultaneous transplantation of hind limb and BM in recipients [65]. The rat simultaneous BMT and VCA can also be successfully performed with reduced-intensity nonmyeloablative conditioning (Figure 1. Simultaneous model) (Xu et al., submitted to *Transplantation* 2012) which is closer to the clinical reality. Although simultaneous HSCT-induced mixed chimerism offers an opportunity for tolerance induction, there are obvious drawbacks that would prevent its clinical application. The major concern is that simultaneous BMT and VCA may increase the risk of complications from combined major operation and conditioning for BMT. Moreover, nonmyeloablative conditioning regimens usually require a period of time from days to condition the recipient, which takes five to six days. Attempts to compress the conditioning to $\leq 2$ days would result in unacceptable toxicities. As such, an approach to establish chimerism electively after recovery following VCA (Figure 1. delayed tolerance induction model) using frozen BMC is of critical importance, as the living donor transplant is not clinically feasible for VCA. To address this concern, Chen et al. explored a delayed tolerance induction approach in which BMT was performed 2 months following VCA [82]. They found that donor-specific tolerance can be successfully achieved in VCA when HSCT was performed electively after full recovery from the VCA transplant. The major concern in delayed tolerance induction is that the recipient may become sensitized to donor alloantigens as a result of a prior transplant and may be more prone to BMC rejection [83, 84]. More conditioning and a higher dose of donor BMC are required for engraftment in sensitized recipients as

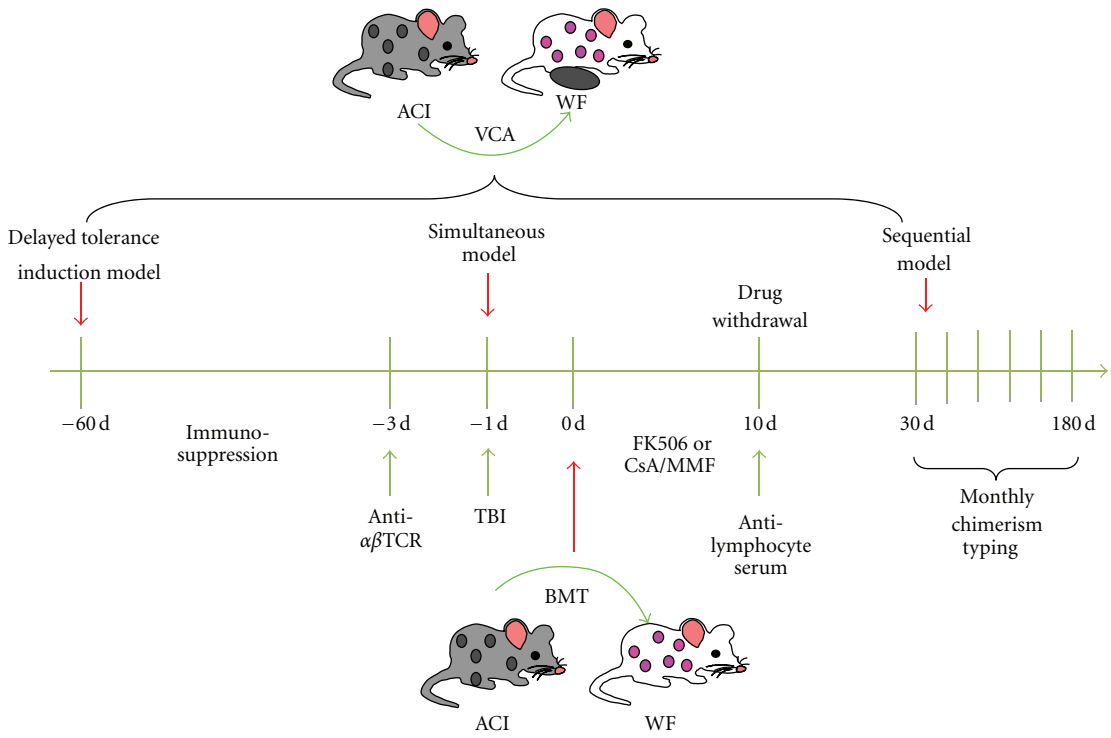


Figure 1: Schema for inducing VCA tolerance.

preformed antidonor antibodies contribute as a dominant barrier for the survival of donor cells [85–87]. However, they found that the continuous immunosuppression after VCA and before BMT prevented the generation of antidonor antibodies and effector/memory T cells [82]. This has ensured the success of subsequent donor BMT. The delayed tolerance approach is now being translated to the clinic in an FDA and IRB approved study in living donor kidney transplant recipients.

*5.5. The Mechanisms of Tolerance Induction by Mixed Chimerism.* The pluripotent hematopoietic stem cells engraft and co-exist with recipient stem cells to give rise to all hematopoietic lineages in the recipient. Mixed chimerism is a hybrid immune system. The mutual tolerance in this hybrid immune system must be systemic as suggested by stable and durable coexistence of genetically different donor and recipient hematopoietic components in mixed chimerism. The mutual tolerance in mixed chimeras should be systemic including adaptive immune tolerance (T and B cells) and innate immune tolerance. T-cell tolerance in mixed chimeras has been well studied *in vivo* and *in vitro*. The mechanism of T-cell tolerance is through central deletional mechanisms, in which the allo-activated T cells are deleted by negative selection in the thymus [59, 88]. Functional donor-specific T-cell tolerance has been detected in *in vivo* MLR assays as lymphocytes from mixed chimeras specifically did not respond to host and donor alloantigens, but are competent to respond to genetically disparate third-party alloantigen. Although donor antigens are continuously presented in mixed chimeras, the recipients do not generate antidonor antibody, and vice versa. These data indicate that B-cell tolerance is established in mixed chimeras [89, 90]. T-cell-dependent B-cell immune responses should serve as the mechanism of humoral tolerance [91–93]. As activated T cells are deleted by negative selection in mixed chimeras, there are no donor-specific antigen activated T cells in the periphery to interact with B cells as B cell-activation uniquely requires interaction with activated helper $CD4^+$ T cells. The general innate immune tolerance in mixed chimeras is evidenced in an *in vivo* cytotoxicity assay where similar cytotoxicity to donor cells (16.4% ± 8.7%) and to syngeneic cells (9.9% ± 0.8%) occurred and significant cytotoxicity to third-party cells (72.3% ± 3.4%, $P < 0.005$) was detected in mixed chimeras [63]. These results suggest donor-specific innate immune tolerance is achieved in mixed chimeras as the effectors mediating BMC rejection at the early time (<3 days) are innate immune cells.

*5.6. Preferential Localization and Persistence of Chimerism in Transplanted Donor Bone.* Mixed chimerism achieved by nonmyeloablative conditioning has been shown to induce donor-specific tolerance in fully MHC-mismatched VCA recipients. In a paper published by Rahhal et al. [51], WF recipients conditioned with 400 to 100 cGy TBI, transplanted with $100 \times 10^6$ T-cell-depleted ACI donor bone-marrow cells, and treated tacrolimus, antilymphocyte serum, and anti-$\alpha\beta$TCR showed between 1.8% and 35% donor chimerism 1 month after BMT. Donor engraftment was multilineage in these chimeric recipients 1 to 2 months after BMT. Chimeric

animals were then subjected to heterotopic osteomyocutaneous flap transplantation 4–6 weeks after BMT. Over 57% of animals conditioned with 400 cGy TBI and 33% of animals conditioned with 300 cGy TBI showed long-term VCA acceptance though peripheral blood chimerism was lost 5 months after BMT. Interestingly, when donor chimerism was analyzed in various hematopoietic compartments in long-term VCA acceptor animals, there was significantly higher donor chimerism detected in the transplanted donor bone (15.7% ± 4.5%, $P = 0.0079$), recipient bone (4.2% ± 1.0%, $P = 0.004$), spleen (3.1% ± 0.91%, $P = 0.011$), mesenteric lymph node (1.6 ± 0.47%, $P = 0.014$), and thymus (1.6% ± 0.60%, $P = 0.036$) compared to peripheral blood (0.09% ± 0.06%). The highest level of donor chimerism was detected in the transplanted donor bone. The authors hypothesized that the donor bone may either serve as a tolerizing source of donor lymphoid cells for systemic microchimerism as previously observed [94] or have no underlying effect as tolerance may have been induced at the time of donor BMT. In any event, loss of peripheral blood chimerism did not affect long-term VCA graft acceptance suggesting a role for microchimerism in peripheral blood. Under conditions of low donor chimerism, regulation of immune responses can be maintained by other mechanisms involving regulatory T cells ($T_{reg}$) [95].

## 6. Role of T Regulatory Cells in Long-Term Allograft Acceptance

$CD4^+$ $CD25^+/FoxP3^+$ $T_{reg}$ play a principal role in regulating immune responses to allogeneic antigens and are robust suppressors of T-cell activation [96]. Using a similar rat model, Bozulic et al. [97] evaluated the function of $T_{reg}$ in peripheral tolerance to VCA. WF recipients were conditioned with 400 cGy TBI, transplanted with $100 \times 10^6$ T-cell-depleted ACI donor bone-marrow cells, and treated with tacrolimus, anti-lymphocyte serum, and anti-$\alpha\beta$TCR. Recipients were monitored for engraftment and then transplanted with a heterotopic osteomyocutaneous flap. Peripheral blood donor chimerism at 1 month after BMT was approximately 30% and was multilineage for both lymphoid and myeloid cells. Sixty-seven percent of transplanted animals displayed long-term acceptance. The group demonstrated that sorted $CD8^-CD4^+/CD25^+$ $T_{reg}$ from spleens of VCA transplanted animals could significantly suppress cell proliferation when plated in a 1 : 1 ratio with either WF responders/ACI stimulator or WF responder/F344 stimulator. Interestingly, when these sorted cells were restained for $FoxP3^+$ $T_{reg}$, VCA rejector animals demonstrated higher absolute numbers of $FoxP3^+$ $T_{reg}$. Similarly, Bunnag et al. showed higher levels of FoxP3 mRNA levels in rejected human renal tissue compared to nonrejected tissue [98]. In addition, there was a 12-fold increase in the absolute number of recipient-derived $FoxP3^+$ $T_{reg}$ 6 months after-CTA which suggested a potential role for $T_{reg}$ in peripheral tolerance whereby newly induced $T_{reg}$ could potentially migrate to target tissue in high numbers as needed. Because $FoxP3^+$ cells were detected in the peripheral blood and appeared to increase over time, immunofluorescent assays were carried out to investigate the presence of $FoxP3^+$ $T_{reg}$ at the VCA graft site. Though $FoxP3^+$ $T_{reg}$ were not detected in skin samples from VCA-rejected animals, skin samples from long-term VCA acceptor animals stained positive for $CD4^+FoxP3^+$ $T_{reg}$ (Figure 2). Recently, $FoxP3^+$ $T_{reg}$ were detected in biopsies from human hand allografts [99] and in human hand transplants undergoing severe rejection [100]. To confirm that the detection of $FoxP3^+$ cells in the VCA acceptor animals was not due to the VCA transplant itself, syngeneic controls were performed where WF rats were conditioned in a similar manner but received WF bone-marrow cells and a WF CTA. No $CD4^+FoxP3^+$ cells were detected in the transplanted graft samples from these animals.

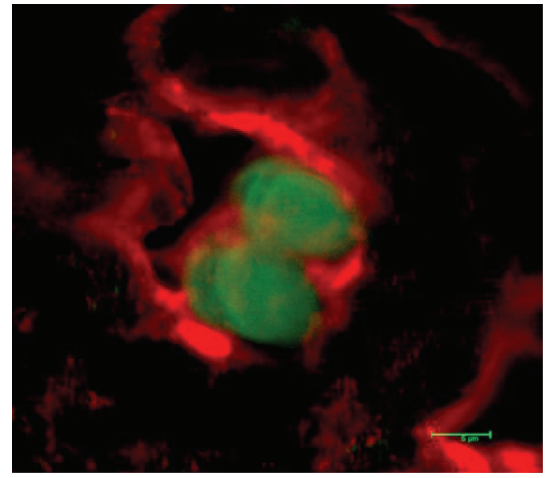

Figure 2: Skin sample from long-term WF-ACI VCA acceptor animals stained for CD4 (red) cell surface staining and FoxP3 (green) intracellular staining. The merged image shows $CD4^+/FoxP3^+$ cells.

The lack of $FoxP3^+$ $T_{reg}$ expression in the skin of CTA-rejector animals correlated with the increase in $FoxP3^+$ $T_{reg}$ expression in the spleen of rejected animals. Lu et al. showed elevated numbers of mast cells and $T_{reg}$ in tolerant skin grafts in a donor-specific transfusion/anti-CD154 model [101]. Similarly, Mathes et al. demonstrated increased numbers of $CD3^+FoxP3^+$ $T_{reg}$ in the skin and muscle of tolerant composite allografts [102]. However, $FoxP3^+$ $T_{reg}$ have also been detected in rejected allografts. Biopsies from human hand transplant recipients undergoing severe rejection demonstrated elevated $FoxP3^+$ $T_{reg}$ [100]. In addition, increased numbers of $T_{reg}$ were detected in skin biopsies from patients with acute GVHD compared to patients without GVHD [103]. Time-course studies suggest that $T_{reg}$ are recruited to the site of antigenic challenge early after transplantation to effectively prevent the infiltration of effector T cells [104]. Similarly, Chauhan et al. showed that $T_{reg}$ suppress the induction phase of immune responses in draining lymph nodes rather than the effector phase in the periphery [105]. As such, there may exist a pool of $FoxP^+$ $T_{reg}$ that home to various tissue sites as needed to induce tolerance early on after antigenic challenge and then maintain peripheral tolerance. Recently, Hoerning et

al. demonstrated that circulating $CD4^+FoxP3^+CXCR3^+$ $T_{reg}$ correlate with renal allograft function and that peripheral immunoregulation depends on $T_{reg}$ allograft homing [106]. In addition, the timing of acquired biopsies may account for when $FoxP3^+$ $T_{reg}$ are detected in tolerant or rejected tissues. Bunnag et al. showed that in human renal transplants, FoxP3 expression increased with time after-transplant. As such, late biopsies had greater FoxP3 expression than early biopsies [98]. Taken together, both time and location of infiltrating $FoxP3^+$ $T_{reg}$ may be important in tolerance induction and long-term VCA graft survival.

## 7. The Future of VCA

Significant progress has occurred in VCA in the past decade. Alexis Carrel, Peter Medawar, and Joseph Murray would be pleased to find that their pioneering work would one day make hand and face allotransplantation a reality. However, the next major advance to make VCA widely available is to minimize or avoid the toxicities of the immunosuppressive agents altogether. Based on the recent clinical success in renal transplantation, tolerance induction may be a path ahead. Future research should focus on establishing safe, simple, and durable donor-specific tolerance in HLA-mismatched recipients of VCA. Drug-free graft approaches to achieve acceptance have been termed the "holy grail" in transplantation and would represent a transformational achievement for VCA to reconstruct traumatic combat-related and civilian injuries, allowing unlimited tissue for repair.

## Conflict of Interests

S. T. Ildstad has a significant equity interest in Regenerex, LLC, a start-up biotech company based on the facilitating cell technology. All anothers have no conflict of interests to declare.

## Authors' Contribution

K. V. Ravindra and H. Xu contributed equally

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*Review Article*

# Improving the Safety of Tolerance Induction: Chimerism and Cellular Co-Treatment Strategies Applied to Vascularized Composite Allografts

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Although vascularized composite allografts (VCAs) have been performed clinically for a variety of indications, potential complications from long-term immunosuppression and graft-versus-host disease remain important barriers to widespread applications. Recently it has been demonstrated that VCAs incorporating a vascularized long bone in a rat model provide concurrent vascularized bone marrow transplantation that, itself, functions to establish hematopoietic chimerism and donor-specific tolerance following non-myeloablative conditioning of recipients. Advances such as this, which aim to improve the safety profile of tolerance induction, will help usher in an era of wider clinical VCA application for nonlife-saving reconstructions.

## 1. Introduction

It has become increasingly appreciated that vascularized composite allografts (VCAs) can, in select patients, provide the best reconstructive alternative for complex losses, such as of total larynx [1], total hand/forearm [2–4], and composite subtotal facial deficits [5], which have until now been essentially non-reconstructible. The problems that hold VCA back from completely revolutionizing reconstructive surgery are predominantly immunological [6]. VCAs contain highly immunogenic (e.g., skin) and immunocompetent (e.g., marrow and blood) tissues that, when revascularized by the recipient vessels, instigate a battle of immune systems leading to transplant rejection and/or graft-versus-host disease (GVHD) unless intricately controlled exogenously by immunosuppression. If robust tolerance between VCA and host could be achieved repeatedly and predictably without causing significant host morbidity, the gates could be opened for a reconstructive paradigm shift led by VCA. The literature addressing the concepts highlighted in this paper is immense and it is not intended that it be exhaustively reviewed. Instead, we provide an overview of the current trends in chimerism-based tolerance research and focus particularly upon recent cellular co-treatment strategies that have real potential for translation to the clinical setting.

## 2. Current Immunosuppression to Avoid VCA Rejection

VCA became a clinical reality using immunosuppression regimens imported from solid organ transplantation [6]. Transplant recipients require life-long immunosuppression, commencing with induction followed by maintenance therapy. Maintenance may be interspersed with rescue therapies

if episodes of acute rejection occur. The goals of tailoring immunosuppression according to recipient and transplant response are to prevent acute and chronic rejection, to minimize the toxicities of immunosuppressants and the rates of infection and malignancy, and to maximize patient and graft survival without GVHD [7].

Induction therapy for clinical VCA consists of anti-lymphocyte antibody or anti-T-cell therapy (using blocking or deleting agents) administered parenterally for a short course immediately posttransplant [8]. The underlying rationale for using these agents is their potent anti-T-cell immunosuppressive properties. Induction therapy is used in conjunction with maintenance agents to minimize early rejection episodes [8]. Induction agents include OKT3, anti-thymocyte globulin (ATG), daclizumab, and basiliximab [9]. Whilst induction therapies may be used in an attempt to induce a tolerogenic effect to donor alloantigen, the experimental evidence indicates that tolerance is unlikely to follow if such treatments are used alone [10, 11]. Maintenance agents used in VCA include corticosteroids (prednisone), cyclosporine, tacrolimus, azathioprine, mycophenolate mofetil (MMF), and sirolimus.

In the early 1990s cyclosporine-AZA steroid-based regimens were used in a series of clinical CTAs to reconstruct nerves, tendons, muscle, bone, joint, and laryngeal defects [6]. In 1997, tacrolimus/MMF/prednisone-based regimens developed by the Louisville group were used successfully to prevent VCA rejection, especially of the skin, while causing minimal systemic toxicity in a preclinical swine forelimb model [6]. This tacrolimus/MMF/prednisone-based combination therapy, similar to that used for solid organ transplants, was utilized thereafter for hand transplantations.

Despite rigorous immunosuppression, however, transplants are not necessarily completely spared from acute rejection episodes and, additionally, chronic rejection is likely deleterious to the long-term function of VCAs [12]. Hence, subjecting patients to life-long immunosuppression regimens that do not completely control against acute rejection episodes nor against chronic functional decline of a non-life-saving reconstructive transplantation remains ethically problematic.

## 3. Graft-Versus-Host Disease in VCA Recipients

A critical feature of VCAs that distinguish them entirely from solid organ transplants is the massive lymphoid armament of donor immunocompetent cells from marrow and lymph nodes that attempt to reject the recipient and may cause GVHD. T cells have been identified as the most important effector cellular subset in this reaction although other cell populations may also participate [13, 14].

In attempts to relieve the risks of GVHD, attention has recently been paid to the preparation of more tolerogenic cell populations, notably plasmacytoid dendritic cells (DCs) [15], hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs) [16]. Infusion of MSCs from a third-party reduced GVHD in allogeneic bone marrow transplantation (BMT) in leukemia patients [16]. MSCs have also been shown to facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in rats [17].

Additionally, removal of mature T cells from the transplanted bone marrow graft has prevented GVHD effectively in mice, rats, and humans [18]. Depletion of both $\alpha\beta$ T cells and $\gamma\delta$ T cells from the donor marrow inoculums prevented GVHD, implicating a role for either or both types of T cells as effectors in GVHD [19]. Importantly, this approach to T-cell depletion does not remove facilitating cells (FCs), nor does it compromise engraftment [18]. The phenotype of FCs is similar to that of plasmacytoid DCs, which are known to mediate antigen-specific tolerance and induce $CD4^+$ as well as $CD8^+$ regulating T cells *in vitro* [15, 20, 21].

To prevent the occurrence of GVHD in VCAs, T-cell depletion of grafts has been explored in an attempt to lower transplant-related mortality [22]. Selective techniques to prevent GVHD without causing immune deficiency and increased infection provoked by systemic T-cell depletion can be achieved by preirradiating the transferred hindlimb with a lethal dose [23] or by lymphadenectomy [24]. However, irradiation of the donor tissue may increase graft failure, and lymphadenectomy is a time-consuming procedure that has its own complications. These two methods have limited potential for clinical practice. An alternative promising approach is graft perfusion with anti-T cell receptor (TCR) monoclonal antibody (mAb) [22]. This approach immunomodulates the vascularized bone graft to reduce GVHD after VCA and concomitantly promotes long-term donor-specific tolerance in the host.

## 4. Tolerogenicity of Vascularized Bone Grafts within VCA

Ideally, VCA recipients would be treated with an effective antirejection therapy that could be tapered quickly to a maintenance dose and then stopped to reduce immunosuppression-related complications [25]. Complete withdrawal of immunosuppression would be possible if donor-specific tolerance develops and the functional recovery of the transplant would not be jeopardized by its withdrawal, hence providing solutions to both chronic rejection and immunosuppressive drug toxicity.

The induction and maintenance of tolerance to allotransplants constitutes an active process involving multiple mechanisms that work cooperatively to prevent graft rejection [26]. The creation of hematopoietic chimerism through BMT remains the most stable method for inducing transplantation tolerance [27]. Chimerism refers to a state in which two genetically different hematopoietic systems are harmoniously present and functioning in one organism [28]. Successful achievement of mixed chimerism tolerizes T cells and B cells to both donor and host tissues [29]. Whilst chimerism is associated with the induction of tolerance, our research has indicated that it depletes after several months and yet allograft tolerance is maintained [30, 31]. Therefore, cell migration and chimerism are believed an invariable early event in graft acceptance [32]. Irradiation of the recipient

promotes cell migration and engraftment of the infused donor HSCs. Conventional BMT can induce chimerism leading to tolerance, but involves the following sequence: host conditioning with irradiation, donor BMT, characterization of chimerism by flow cytometry (at 28 days), and allotransplantation [27]. This 28-day period has been considered a requirement for engraftment and repopulation of the donor bone marrow cells in the host [27]. Allotransplantation performed before successful engraftment of donor bone marrow may interfere with the establishment of tolerance [27]. To overcome this 28-day delay, operational tolerance through cyclophosphamide, anti-CD2 mAb, or thymic irradiation was successfully induced in four out of five patients with end-stage renal disease receiving bone marrow transplantation in addition to a kidney from related living donors [31]. Despite withdrawing immunosuppression, renal function remained normal for up to 5.3 years after transplantation. In addition, immediate tolerance to skin graft was observed in rodent models using BMT and costimulatory blockade with reduced myeloablative host conditioning. However, exposing an otherwise healthy patient with a non-life-threatening functional deficit to irradiation and the creation of chimerism is ethically difficult.

A unique feature of some VCAs (e.g., hand/forearm, knee) that has been exploited recently is the presence of a vascularized bone marrow transplant (VBMT) component within the incorporated long bone(s). This instantly and continuously produces bone marrow cells once transplanted and directly provides the niche for reconstitution of HSCs [30]. Bone marrow additionally contains stromal cells, including fibroblasts, adipocytes, endothelial cells, and osteoblasts, derived from MSCs that are known to influence the HSC microenvironment [33]. Stromal cells appear to be capable of supporting HSCs and progenitor cells *in vitro* and *in vivo* and a stromal microenvironment is essential for the proliferation and differentiation of hematopoietic progenitors [33].

Early engraftment and reconstitution of multiple hematopoietic lineages may allow for instant establishment of chimerism and earlier tolerance to VCAs. VBMT within VCA, and not conventional BMT, was critical to the long-term establishment of chimerism and tolerance under partially myeloablative conditioning and tacrolimus-based treatment [30]. It can be concluded therefore that the VBMT within VCA provides critical signaling and modulatory functions that initiate tolerance induction. Importantly, this study showed that it was therefore possible to overcome the 28-day delay to engraftment because the VBMT within VCA could, under the correct conditions, induce mixed chimerism and tolerance simultaneously.

## 5. Other Methods of Tolerance Induction for VCA

*5.1. T-Cell Depletion.* T cell depletion prior to VCA transplantation followed by T-cell repopulation after allotransplantation has been associated with allograft acceptance in animal models and in humans [34, 35]. Nonspecific T-cell depletion (lymphodepletion) medications such as ATG [36, 37], Orthoclone mAb OKT3 [38], and humanized Campath-1H CD52 mAb [39] are frequently used clinically for induction therapy before transplantation for prevention and treatment of acute rejection episodes. Selective T-cell inhibition using $\alpha\beta$-TCR monoclonal antibodies combined with cyclosporine has been associated with robust mixed chimerism and long-term VCA survival in animal models [40, 41].

*5.2. Costimulation Blockade.* Full T-cell function requires binding of the TCR to MHC molecules on DCs or other antigen-presenting cells (APCs), alongside profoundly influential co-stimulatory signals, examples of which include the CD154 and CD28 interactions with CD40 and B7 ligands, respectively [42, 43]. Without adequate co-stimulation, T cells may undergo apoptosis, inactivation, or anergy [42]. Selective interference with these co-stimulators is therefore an attractive way to influence the behavior of T cells that encounter specific antigens.

Costimulation blockade, with or without BMT, using anti-CD154 (anti-CD40L) mAb alone or together with CTLA-4 Ig (CTLA-4 immunoglobulin fusion protein) has been reported to prolong survival or induce tolerance to VCA in rodents and large animals [42–48]. Various mechanisms have been proposed in prolongation of solid organ allograft survival using co-stimulatory blockade, including anergy, suppression, and deletion. The most recent data suggest that deletion of peripheral alloreactive T cells has a major role in the establishment of mixed chimerism using co-stimulatory blockade. The use of co-stimulatory blocking reagents in BMT protocols can facilitate the induction of mixed chimerism while markedly reducing the potential toxicity of conditioning using total body irradiation (TBI), thymic irradiation or host T-cell depletion. CTLA-4 Ig is a biological agent consisting of the extracellular domain of CD152 fused to the Fc region of IgG1. As CTLA-4 Ig is potentially tolerogenic through costimulatory blockade it has been explored extensively in transplantation. It was subsequently deduced in mice that administration of CTLA-4 Ig resulted in the induction of indoleamine 2,3-deoxygenase (IDO) in professional APCs, like DCs [49]. IDO is induced during inflammation by IFN-$\gamma$ [50] and other proinflammatory cytokines and acts to deplete the local microenvironment of the essential amino acid, tryptophan. The resulting low levels of extracellular tryptophan act as a signal to inhibit T-cell proliferation.

*5.3. In Vitro Manipulation of Donor Dendritic Cells.* Dendritic cells (DCs) are considered the most potent class of APCs, the mature form of which express MHC class I and II molecules. They bear a variety of costimulatory signal molecules such as CD40, CD80, and CD86 that are responsible for presenting antigen to T cells for T-cell activation. The majority of DCs are functionally immature and therefore incapable of presenting donor antigen to T-cells efficiently, leading to disabled T-cell activation [51]. This has been exploited to support VCA tolerance induction by, prior to transplantation, infusing pharmacologically stabilized

Table 1: Methods of tolerance induction for VCA listed chronologically.

| Methods | Induction pathway |
|---|---|
| Chimerism | Bone marrow transplantation<br>Vascularized bone grafts |
| T cell depletion | ATG, OKT3 mAb, Campath-1H (CD52) mAb, $\alpha\beta$ TCR/$\gamma\delta$ TCR mAb |
| Costimulation blockade | CD154, CD28, and/or CD40 mAbs, B7 ligand, CTLA-4 Ig (fusion protein) |
| Donor dendritic cell | *In vitro* manipulation followed by intravenous infusion |
| Mesenchymal stem cell | *Ex vivo* expansion followed by intravenous infusion |
| Regulatory T cell | *In vivo* induction followed by intravenous infusion |

immature DCs that have been pulsed with donor antigen [52, 53]. DCs are also noted to induce tolerogenic regulatory T cells ($T_{reg}$) that in turn promote allograft tolerance [52, 53]. The administration of recipient-derived DCs prolonged VCA survival in animal models but DCs *per se* were unable to induce transplantation tolerance to VCA [54, 55].

*5.4. Mesenchymal Stem Cells.* Another intriguing recent finding that requires further research has been the ability of infused syngeneic, allogeneic, and even third-party adipose-derived MSCs to promote long-term survival of VCA between fully MHC-mismatched rats without causing GVHD [56]. Clinically, MSCs could modulate immune responses and ameliorate GVHD after hematopoietic-stem-cell transplantation [57]. How exactly MSCs modulate the immune response is not completely understood. However, some mechanisms involved in such modulation have been proposed, which include the expression of HLA-G molecules, direct interactions with DCs preventing them from differentiation and maturation, and modulation of the expression of cytokines/factors such as IL-10, TGF-$\beta$, IDO, TNF-$\alpha$ and INF-$\gamma$ [58]. Co-treatment of MSCs with BM cells before VCA transplantation with low-dose (3 Gy) irradiation conditioning significantly prolonged allograft survival without GVHD as compared to animals that did not receive MSC co-treatment [59].

*5.5. Regulatory T-Cell Therapy.* Further study into our model [60] demonstrated that the $T_{reg}/CD4^+$ ratio in the peripheral blood of VCA-accepting chimera was negatively correlated with mixed chimerism levels. This suggests a significant role for $T_{reg}$ in maintaining VCA tolerance when mixed chimerism is less robust. A body of literature reports that $T_{regs}$ have exceptional therapeutic effects on autoimmune diseases [61], organ transplantation [62, 63], and GVHD models [64, 65], but do not induce skin graft tolerance across full MHC barriers when utilized alone [66, 67]. Interestingly, combined therapy with $T_{regs}$ and allogeneic BMT has been reported to achieve durable mixed chimerism and long-term tolerance to nonvascularized skin allografts without cytoreductive conditioning in mice [68]. This approach was recently tested in rats in our laboratories [69]. A combination of $T_{regs}$ prepared from the recipient strain and VBMT treatment, with a short course conditioning of recipients with costimulation blockade and rapamycin, led to long-term multilineage hematopoietic mixed chimerism (12–18%) and long-term donor-specific tolerance to VCA (89% acceptance rate) without GVHD. Neither stable mixed chimerism nor VCA acceptance was observed in recipients without $T_{reg}$ treatment. Interestingly, $FoxP3^+$ $T_{reg}$ cells infiltrated VCA near the donor/allograft tissue junction in VCA-accepting chimera, further suggesting an importance for them in permitting long-term VCA survival [68–70]. Of note is that the $FoxP3^+T_{reg}$ cells found in the donor/allograft tissue junction were mostly of recipient origin (JY Lin and SK Liao, unpublished). Nevertheless, the question as to whether they belong to natural or induced $T_{reg}$ remains to be determined. The presence of $T_{regs}$ in the donor skin of hand allotransplantation recipients has been demonstrated as has increased FoxP3 expression during rejection episodes at a later time point after transplantation [71, 72]. These findings also support that $T_{reg}$ may play a significant role in maintaining allograft acceptance and prevent allograft rejection by downregulation of donor-reactive effectors infiltrating the donor graft. However, the clinical relevance and exact mechanism of immunomodulation by $T_{regs}$ in tolerance induction to VCA remains unclear and further investigations are warranted.

Allogeneic $T_{reg}$, third-party $T_{reg}$ [73, 74] and MSC preparations [75, 76] are just some examples of how immunosuppressive functions from diverse cellular sources have been exploited to ease tolerance induction in recent years. Encouragingly, these biologicals can be cryopreserved in liquid nitrogen and hence could be made available in large quantities when required to facilitate VCA acceptance in the future. So far, stable mixed chimerism, which has been successfully established in rodent models for tolerance induction to VCA, has not been observed in the majority of clinical VCA transplantations without recipient conditioning, thus making translation of such protocols into clinical application less likely. The major hindrance to widespread use of mixed allogeneic chimerism as a strategy to induce VCA tolerance in the clinical setting will be the requirement for recipient conditioning and the risk of GVHD. We believe that combination approaches, such as our noncytoreductive $T_{reg}$-VBMT protocol or tailored cell-based therapies alongside low-dose immunosuppression, may have improved potential for clinical application in VCA transplantation. Current widely researched methods of tolerance induction are summarized in a chronological order in Table 1.

## 6. Conclusions

Encouraging results continue to be obtained from VCA investigations in rats focusing on developing nonmyeloablative methods of establishing mixed chimerism. So far there has been a lack of evidence showing the presence of mixed chimerism or donor-specific unresponsiveness in all clinical VCA transplantation recipients (including 53 hand transplantations that inherently included VBMT and one face transplantation with bone marrow infusion), suggesting stable mixed chimerism cannot be induced without host conditioning in humans. Clinical VCA transplantations will benefit from further investigations searching for tolerance protocols involving less toxic host conditioning. Protocols that have promise incorporate co-treatment strategies, including the use of cell-based therapies involving VBMT, $T_{reg}$, and/or MSCs. Whilst progress is made in improving the safety of tolerance induction, it will become increasingly important to develop recipient monitoring measures that can accurately reflect the success or failure of tolerance induction in these animal models. Such tests should ultimately be considered an integral part of tolerizing protocols to be used clinically for VCA.

## Conflict of Interests

None of the authors has a financial interest in any of the products mentioned in this paper.

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*Review Article*

# Mechanisms and Mediators of Inflammation: Potential Models for Skin Rejection and Targeted Therapy in Vascularized Composite Allotransplantation

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Vascularized composite allotransplantation (VCA) is an effective treatment option for patients suffering from limb loss or severe disfigurement. However, postoperative courses of VCA recipients have been complicated by skin rejection, and long-term immunosuppression remains a necessity for allograft survival. To widen the scope of this quality-of-life improving procedure minimization of immunosuppression in order to limit risks and side effects is needed. In some aspects, the molecular mechanisms and dynamics of skin allograft rejection seem similar to inflammatory skin conditions. T cells are key players in skin rejection and are recruited to the skin via activation of adhesion molecules, cytokines, and chemokines. Blocking these molecules has not only shown success in the treatment of inflammatory dermatoses, but also prolonged graft survival in various models of solid organ transplantation. In addition to T cell recruitment, ectopic lymphoid structures within the allograft associated with chronic rejection in solid organ transplantation might contribute to the strong alloimmune response towards the skin. Selectively targeting the molecules involved offers exciting novel therapeutic options in the prevention and treatment of skin rejection after VCA.

## 1. Introduction

Acute skin rejection is a frequent challenge, and long-term immunosuppression is a necessity in vascularized composite allotransplantation (VCA) [1]. The toxicity profile of such a drug treatment includes metabolic side effects, opportunistic infections, malignancy, and organ damage [2–6]. This illustrates the need for immunosuppressive-sparing protocols in order to limit side effects of this quality-of-life improving procedure and widen the indications for VCA.

The infiltration of alloantigen specific T cells into the skin allograft has been identified as a central element of acute skin rejection in VCA [7, 8]. Histologically, the appearance of skin rejection shares many common features with inflammatory skin diseases and may be difficult to distinguish [9, 10], suggesting that underlying immunological mechanisms might be similar in some aspects. In inflammatory skin conditions, T-cell recruitment to the skin is orchestrated by a multitude of adhesion molecules, cytokines, and chemokines [11]. In part, this concept of inflammation and immune activation holds also true for the initiation and progression of allograft rejection in solid organ transplantation (SOT) [12]. A mechanism currently discussed to be involved in the development of chronic allograft rejection is the formation of lymphoid neogenesis and tertiary lymphoid organs (TLOs) in the transplant [13–15].

The mechanisms and dynamics of skin allograft rejection have been partially understood and remain the subject of

numerous trials aiming at a better understanding of the pathophysiology and novel and targeted drug development. We herein review the molecular events and key players of inflammation as well as new therapies with particular regard to skin inflammation and allograft rejection in SOT and discuss them in the light of acute and chronic skin allograft rejection of VCAs.

## 2. Adhesion Molecules: Anchors for Lymphocyte Recruitment to the Skin

Adhesion molecules play a crucial role in the function of immune cells. They are the central actors helping leukocytes to immediately convert from an inactive, nonsticky status to an adhesive status, though permitting adhesion to the vascular endothelium with transmigration to inflamed tissues. Further they support cell-cell interactions through various homophilic and heterophilic interactions and have the ability to transmit costimulatory signals to the interacting cells. The expression pattern of adhesion molecules is characteristic for each cell population and changes during the maturation process of a cell [16].

### 2.1. Adhesion Molecule Families

*(1) Selectins.* 3 subtypes of selectins, characterized through their N-terminal lectin domain, are defined [17, 18]: E-selectin is mainly expressed by activated endothelial cells, whereas endothelium of noninflamed tissue does not express E-selectin. Potent stimuli of E-selectin expression are IL-1 and TNF [19]. The "P" in P-selectin stands for "platelet", but P-selectin is also expressed in activated endothelial cells, where it is stored in Weibel-Palade bodies [20] and is released upon stimulation [21]. In contrast to E- and P-selectins, L-selectin is constitutively expressed on lymphocytes, neutrophils, and monocytes and is known to play a crucial role in homing of lymphocytes to secondary lymphoid tissues through binding to its counter-receptor addressin, which is expressed by high-endothelial venule cells [22, 23]. However, there is now growing evidence that all three types of selectins contribute to leukocyte extravasation in the skin with overlapping effect. E- and P-selectin seem to play the most important role in leukocyte homing into the skin [24]. This idea is supported by the failure of monoselectin antagonists and the success of pan-selectin agonists in targeting leukocyte extravasation [25, 26]. All types of selectins bind to carbohydrate ligands such as the tetrasaccharides Sialyl-Lewis-x or P-selectin glycoprotein ligand-1 (PSGL-1) [27, 28].

*(2) Integrins and the Ig Family.* Leukocytes (neutrophils, monocytes, lymphocytes, and natural killer cells) express the integrins lymphocyte function-associated antigen-1 (LFA-1) and Mac-1 (both sharing a common $\beta$2-subunit [29]), which bind intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, two members of the Ig superfamily expressed by vascular endothelial cells, and leukocytes. While ICAM-1 expression on vascular endothelium and leukocytes can be stimulated [30], ICAM-2 is constitutively expressed on the endothelium as a target for beta2 integrins [31]. Another integrin expressed on mainly lymphocytes and monocytes is very late activation antigen (VLA), which binds to vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [32]. VCAM-1 has been shown to mediate several steps in the process of leukocyte extravasation. It is not only involved in firm adhesion, but also in rolling of T cells and transmigration through the endothelium [33].

### 2.2. Functions during Inflammation

*(1) Cellular-Vascular Interactions.* an important function of adhesion molecules is the mediation of cellular-vascular interactions, enabling leukocytes in a multistep cascade to exit the blood vessel and to migrate into the inflamed tissue [34]. The selectin-sialyl-Lewis-x interaction between vascular endothelium and the leukocyte first leads to cell rolling through reversible tethers between the leukocyte and the vessel wall. The leukocyte is slowed down and thus brought into closer proximity to the endothelium. A more firm adhesion is required before the effective transmigration can occur: this arrest of the rolling leukocyte is provided through interactions between immunoglobulins and integrins. For the induction of tight adherence, costimulating ligands and chemokines enhance the avidity of integrins on leukocytes [35–37]. For transmigration junctional adhesion molecules (JAMs), which are constitutively expressed at the borders between endothelial cells, play an important role through interaction with VLA-4 or Mac-1 on the leukocyte [38].

*(2) Cell-Cell Interactions.* In addition to cellular-vascular interactions interaction between different types of immune cells and leukocytes is required for an orchestrated cellular immune response. "The immunological synapse" is an assembly of adhesion molecules, which provides relatively stable interactions between cells of the immune system thereby supporting antigen recognition and enabling bilateral stimulation of the cells. One important mode of cell-cell interaction is the binding of naïve T lymphocytes to antigen-presenting cells (APCs) such as dendritic cells (DCs) [39, 40]. The interaction between cells through adhesion molecules allows the T-cell receptor (TCR) to scan the surface of a DC for appropriate major histocompatibility complex (MHC-) displayed peptides [41–43]. Furthermore, the interaction between T-cells and B-cells [44, 45] and T-cell-mediated killing (through T cell-target cell adhesion and as well the natural killer cell-target cell interaction) relies on adhesion molecules [46]. In this context adhesion molecules are not only anchors for the cells, but also transmitters of important costimulatory signals for many immunity-related functions.

*2.3. Adhesion Molecules in Inflammatory Skin Diseases.* In inflammatory skin diseases such as psoriasis, allergic contact dermatitis, and atopic dermatitis, it has been shown that T cells play a central role in the initiation and/or perpetuation

of cutaneous inflammation [47, 48]. While the distinct entities seem to be related to environmental as well as genetic factors, they can be uniformly characterized through a subset of T lymphocytes found in inflammatory skin lesions staining positive for cutaneous lymphocyte-associated antigen (CLA) [49]. CLA is a modified carbohydrate ligand interacting with E-selectin during skin homing of these lymphocytes. However, further characterization of this distinct cell population revealed that they are a heterogenous population of CD4+ and CD8+ T cells. It has been shown that CLA-bearing T cells preferentially extravasate through the endothelium of the superficial dermal plexus [50]. Skin homing of T lymphocytes therefore seems to be a central mechanism in inflammatory skin diseases.

For psoriasis the crucial role of T cells homing to the skin has been clearly demonstrated in several in vitro and animal studies [51, 52] and this concept is further supported by the effectiveness of therapies targeting either the number/proliferation or the extravasation of T lymphocytes [53, 54]. The histological pattern of psoriasis shows a hyperproliferation and hyperkeratosis of epidermal keratinocytes as well as cellular infiltration into dermis and epidermis. Epidermal keratinocytes in psoriatic lesions have been shown to display upregulation of MHC class II antigens as well as induced expression of ICAM-1. Furthermore, the vascular endothelial cells upregulate adhesion molecules of all classes: E-selectin, ICAM-1, VCAM-1, and MHC class II antigens [55]. The understanding of the relevance of lymphocyte homing into the skin for the development of psoriasis has initiated a quest for potential treatments [56]. However, the redundancy and the many overlapping functions of adhesion molecules have made the development of effective therapeutics difficult [57]. The insufficient therapeutic potency of several substances such as monoclonal antibodies against E-selectin has revealed the difficulty of this therapeutic approach.

Nevertheless, there is a strong need for new anti-inflammatory substances for the treatment of inflammatory skin diseases and induction of long-lasting remissions. The integrin inhibitor efalizumab (Raptiva), which is a monoclonal antibody against the alpha-subunit of LFA-1, has clinically shown to alleviate skin inflammation in plaque psoriasis [58]. However, EMEA and FDA recommended withdrawal of this substance from the market because of a severe side effect: progressive multifocal leukoencephalopathy was observed in a few patients. Numerous compounds have been introduced inhibiting selectin function. Efomycine M [59], BMS-190394 [60], OJ-R9188 [61], and TCB-1269 [62] showed an effect in preclinical models of psoriasis, delayed-type hypersensitivity (DTH), and atopic dermatitis. However, only insufficient response was reported for most inhibitors in phase I/II trials. Other substances, which are still being evaluated in preclinical trials, include inhibitors of fucosyltransferase IV [63] (an enzyme which modifies carbohydrate ligands to CLA, a high-affinity ligand for E-selectin). Furthermore, alefacept, a fusion protein of LFA-3 and the Fc-portion of human IgG, has been reported to cause long-term remission in at least a subpopulation of psoriasis patients [64].

*2.4. Adhesion Molecules in Solid Organ Transplantation.* Ischemia-reperfusion injury (IRI) is an event of excessive inflammatory response that occurs after temporary absence of blood supply, such as shock, infarction, and transplantation. Key events during IRI are the generation of damage-associated molecular patterns (DAMPSs) and upregulation of inflammatory cytokines and adhesion molecules, which contributes to recruitment of leukocytes [65, 66]. In liver transplantation IRI affects the outcome and results in 2–10% early graft failures [67]. Moreover, it has been speculated that IRI may also lead to higher incidences of acute and chronic rejection. Gene expression profiling of IRI in human liver allografts has revealed an upregulation of adhesion molecules and integrins [68]. Several preclinical and clinical trials have focused on prevention of IRI in SOT, and blocking of adhesion molecules has shown promising results in many models. In the setting of liver transplantation, blocking P-selectin with a monoclonal antibody resulted in decreased incidence of IRI in mouse models [69, 70] and most recently in a clinical phase II study [71]. The leukocyte adhesion cascade in myocardial IRI remains an interesting target for therapeutic intervention. Molecules such as B$\beta$15–42 [72] and FX06 [73] have shown promise for limiting damage in myocardial IRI.

As the mechanisms of leukocyte recruitment to the allograft in the course of rejection is similar to leukocyte recruitment during inflammation [12, 74], strategies to block adhesion molecules also demonstrated effects on allograft survival in different settings. inhibition of LFA-1 prolonged graft survival in murine heart allotransplantation [75, 76]. Prolonged allograft survival was achieved in an islet transplant model in nonhuman primates [77]. The monoclonal antibody against LFA-1, efalizumab, has demonstrated efficacy in a clinical phase I/II study of renal transplantation [78]. However, an increased incidence of posttransplant lymphoproliferative disorder was observed in these patients [79]. A study published by Langer et al. [80] in 2004 showed prolonged survival of rat kidney allografts using the selectin inhibitor OJ-R9188. This effect was mainly due to a reduction of infiltrating T cells and macrophages as well as decreased intragraft expression of cytokines and chemokines.

*2.5. Adhesion Molecules in Vascularized Composite Allotransplantation-Potential Targets for Therapy.* We have recently published an analysis of more than 170 biopsies taken from five human hand and forearm transplant recipients demonstrating the upregulation of adhesion molecules during skin rejection [7]. Immunohistochemical staining of skin samples has revealed a strong correlation of LFA-1 (also found to be expressed in keratinocytes), ICAM-1, and E-selectin with the severity of rejection, while none of these markers was found to be upregulated in nonrejecting skin. Quantitative PCR analysis, however, showed no correlation between the severity of rejection and the gene expression of these molecules, which may indicate that these adhesion molecules are not solely regulated at the gene level.

In a series of experimental studies using a rat hindlimb transplant model, adhesion molecule blockers were

administered subcutaneously (SC) into the allograft after a short course of systemic immunosuppression (tacrolimus) to prevent rejection. Targeting E- and P-selectins using the small-molecule inhibitor Efomycine M resulted in long-term (150 days) allograft survival in 5 out of 6 animals [7]. Histology on day 150 showed a mild lymphocytic infiltrate in the dermis and only single vacuolized keratinocytes in the epidermis. Local intragraft administration of anti-ICAM-1 and anti-LFA-1 significantly prolonged graft survival when compared to controls. In 3 out of 4 animals long-term graft survival was achieved (paper in preparation). In another attempt to address local inhibition of adhesion molecules, the fibrin derivative B$\beta$15–42, which blocks VE-cadherin, revealed a statistically significant prolongation of hind-limb allograft survival in the rat when combined with subtherapeutic doses of tacrolimus. When local treatment with B$\beta$15–42 was then combined with an induction with IL-2 Fc and a short course of cyclosporin A, long-term allograft survival with significant reduction of CD4+ and CD8+ T cells was achieved (paper in preparation). These data indicate the potential of leukocyte migration blockers to prevent skin rejection in a rat VCA model.

## 3. Cytokines and Chemokines as Important Mediators for Cell Trafficking

Attraction of mononuclear cells to sites of inflammation does not only require membrane-bound adhesion molecules but also a close interplay of the inflammatory signal presented by a variety of soluble or membrane-borne chemoattractive factors. It is known that the specific expression pattern of chemokines and their receptors determines the type of cell that is attracted to the inflamed tissue. This pattern of chemokines is regulated by the local cytokine milieu. For example, interferon-$\gamma$ (IFN-$\gamma$) induces upregulation of chemokines, which subsequently attracts neutrophils, monocytes and T helper-1 (Th1) cells. Further, a T helper-2 (Th2-) dominated cell recruitment pattern is induced by chemokines upregulated upon exposure to IL-4 and IL-13 [81].

Chemokines can be characterized as a family of cytokines with chemotactic activity for leukocytes. To this day, approximately 60 chemokine members have been identified. They are divided into C, CC, CXC, CX3C subfamilies based on the cysteine motifs near the aminoterminal end of the molecule [82]. Several studies emphasize the importance of chemokines and their receptors in the allograft rejection process and their role in leukocyte recruitment, Th1 and Th2 cell differentiation and DC movement and maturation [83–87]. Studies on human renal biopsies delineated that the expression of Th1 chemokine receptors (CCR5 and CXCR3) and their ligands (CXCL10 (=IP10), CXCL9 (=Mig) and CCL5 (=RANTES) is associated with acute rejection [88]. Mig was increased in a lung transplant model and its inhibition decreased intragraft migration of mononuclear cells [89]. The importance of CCR5 was shown in islet allografts since targeting CCR5 resulted in significant prolongation of these grafts [90]. Increased CXCR3 expression was demonstrated in a murine skin allograft model during rejection and peptide nucleic acid (PNA) CXCR3 antisense significantly prolonged allograft survival by blockade of CXCR3+ T-cell infiltration into the allograft [91]. Li et al. [92] investigated the intragraft expression profile of 11 chemokines from all four chemokine subfamilies in a murine skin transplantation model and demonstrated that CCL5/RANTES, CCL17/TARC, and FKN were expressed at equivalent levels in iso- and allografts. The expression of eight chemokines was upregulated in allografts compared with isografts also in dependence of postoperative days. The most significantly elevated chemokine was I-TAC (CXCL11), which peaked during rejection (postoperative day 7), and when inhibited via intradermal injection of anti-I-TAC monoclonal antibody significantly prolonged skin allograft survival. Most studies in transplantation have concentrated on rather few chemokines. To analyze their roles in a meaningful manner, novel techniques including commercially available multiprobe ribonuclease protection assays, antichemokine and antichemokine-receptor monoclonal antibodies, and gene-knockout animals are now available [93, 94]. Despite these prospects, it is important to emphasize that many data from in vitro experiments demonstrated the presence of multiple ligands for one chemokine receptor and often multiple receptors for one chemokine. This may help to explain, why allograft rejection was not abrogated in any of these trials. Thus a cocktail of reagents directed to multiple recruiting chemokines may be required for efficient inhibition of T-cell infiltration into allograft. In this context we believe that this may also provide a future promising strategy in VCA.

## 4. Mediators of Inflammatory Skin Diseases: Parallels to Skin Rejection

Skin rejection in VCA presents with erythematous macules that may progress if not treated to scaly violaceous lichenoid papules covering the complete surface of the graft [8]. These alterations are not specific for rejection and may mimic inflammatory dermatoses. Kanitakis [10] emphasized the diagnostic challenges in early or mild skin rejection; differentiation from contact dermatitis, insect bites or dermatophyte infections may be difficult in these stages.

Parallels between acute skin rejection and inflammatory dermatoses (e.g., contact dermatitis, psoriasis, and atopic dermatitis) also exist on the molecular and Cellular level. Allergic contact dermatitis for example is a T-cell-mediated DTH reaction that occurs upon hapten stimulation in sensitized individuals [95]. Therefore, the differentiation by histological and macroscopic criteria can be difficult. It has been demonstrated, that T cells (CD4+ and CD8+ cells) are critical and that elements of the innate immune system (e.g., natural killer cells) may play a key role [96]. Epidermal Langerhans cells as the most competent APCs in the skin as well as keratinocytes are regulating this inflammatory process. Cytokines derived from Langerhans cells (e.g., IL-12) and from T cells (IFN-$\gamma$, IL-4, and IL-10) play a pivotal role in the induction and initiation of this skin disease [97].

Given the close interaction of chemokines in the inflammatory process and immune response, it is not surprising that a number of dermatological diseases are a result of chemokine dysregulation [98]. Strong chemokine expression in allergic and inflammatory skin diseases such as psoriasis and contact hypersensitivity (CHS) has been documented [99–102]. Specifically, CXCL8/IL-8 and the related CXCL2/Gro-$\beta$ are significantly upregulated in psoriatic skin lesions and thus responsible for the typical intraepidermal aggregation of neutrophils. CCL2/MCP-1 and CCL5 are responsible for attracting predominately monocytes and T cell subsets, and CXCR3 ligands attract Th1 cells [103]. The expression of cytokines and chemokines during the sensitization and elicitation phase of CHS has been well studied [104]. Watanabe et al. [105] has shown that TNF-$\alpha$ and IL-1$\beta$ play a main role in the sensitization phase of CHS, meanwhile the elicitation phase is predominately characterized by IFN-$\gamma$, IL-1, IL-4, and TNF-$\alpha$ expression.

## 5. Tertiary Lymphoid Organs: Do They Play a Pivotal Role in Chronic Rejection of VCAs?

The role of chronic rejection in VCAs is poorly understood so far. As reconstructive transplantation is a relatively young field, follow-up periods of VCA recipients are currently limited to 13 years. Allograft vasculopathy is the main feature in chronic rejection of solid organ allografts. Only a limited number of reports on vascular changes of graft vessels in a VCA are available at this time [106, 107]. It is hypothesized that multiple (untreated) acute rejection episodes imitate a state of chronic inflammation, which may trigger myointimal proliferation and occlusion of allograft vessels [106, 108].

TLOs are lymphoid-like structures that can be found in chronically inflamed tissues [109]. They are composed of B- and T-cell aggregates, specialized populations of DCs, well-differentiated stromal cells, and high endothelial venules (HEVs), but they are not encapsulated [110]. Many of the molecular signals and events leading to the development of secondary lymphoid organs have been shown to be as well involved in the formation of TLOs [14, 111]. Mesenchymal lymphoid tissue organizers express CXCL13, MAdCAM, ICAM, and VCAM and thereby recruit CD4+CD3− haematopoietic lymphoid tissue inducers. The expression of lymphotoxin on these inducer cells further upregulates chemoattractants and adhesion molecules via a positive feedback loop, resulting in recruitment of immune cells and formation of HEVs.

*5.1. TLOs in Chronic Allograft Rejection.* The formation of ectopic lymphoid structures is thought to enhance the efficiency of alloantigen presentation and generation of alloreactive lymphocytes and might therefore enhance the alloimmune response. This is speculated to be a mechanism in several chronic inflammatory conditions, such as rheumatoid arthritis, Sjögren's syndrome, and Hashimoto's thyroiditis [112–114]. A retrospective analysis of 350 renal allografts revealed the formation of regional inflammatory infiltrates consisting of T and B lymphocytes, plasmocytoid cells, and DCs [115]. The authors found a strong correlation between the formation of TLOs and an increased incidence of chronic rejection and graft loss. Baddoura et al. [13] reported lymphoid neogenesis in murine cardiac allografts in the course of chronic rejection. 78% of chronically rejected allografts revealed either classical TLOs with organized T- and B-cell zones and peripheral node addressin+ (PNAd+) HEVs or PNAd+ HEVs without organized lymphoid accumulations. Interestingly, the architecture of TLOs has been shown to be related to the immune activation status of the host [116]. In an attempt to address the role and function of TLOs during rejection Nasr et al. [117] reported that TLOs are able to generate effector and memory T cells. In a murine transplantation model full thickness skin grafts containing TLOs due to transgenic expression of lymphotoxin-a (RIP-LTa) were transplanted to recipients lacking all secondary lymphoid organs. These allografts were rejected, while wild-type allografts were accepted. When RIP-LTa and wild-type allografts were transplanted simultaneously both were rejected. Furthermore, Thaunat et al. [118] demonstrated the production of alloantibodies specific for donor MHC class I molecules in germinal centers of TLOs in a rat aortic interposition model, suggesting a local antibody-mediated alloimmune response. In a mouse model of autoantibody-mediated cardiac allograft vasculopathy administration of a lymphotoxin blocker, LT$\beta$R-Ig fusion protein, abolished allograft TLO formation and inhibited the effector humoral response [119]. Taken together these findings suggest that TLOs in allografts are not only a result of the chronic inflammatory stimulus, but also a site where the alloimmune response is being executed and enhanced.

This contrasts findings by Brown et al. [120], who reported the presence of TLOs in a murine kidney allograft model of tolerance to be associated with superior graft function and survival. In summary, it remains unclear at this point whether TLOs are associated with a destructive or beneficial response in organ and tissue transplantation and if they should be targeted or induced in order to promote long-term graft survival.

## 6. Conclusion

A perivascular infiltrate of mainly CD3+ T lymphocytes in the dermis marks the advent of skin rejection in VCA [9, 121]. The cellular infiltrate then further spreads into the dermis and epidermis leading to dermal-epidermal separation and necrosis if not treated successfully. Since a variety of adhesion molecules as well as cytokines and chemokines are responsible for lymphocyte trafficking towards the epidermis during acute rejection, selectively blocking leukocyte recruitment to the site of inflammation seems a promising approach to prevent and also treat skin rejection in VCA. Moreover, novel concepts targeting intragraft lymphoid neogenesis and the formation of TLOs might be considered in the treatment of chronic allograft rejection, while it remains a puzzling feature in VCA. Targeted therapy, inspired by the novel treatments for inflammatory skin diseases, could evolve to

a promising treatment option for VCA patients, lowering their burden of long-term systemic immunosuppression.

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