The unique immunobiology of the skin: implications for tolerance of vascularized composite allografts

Radhika Chadha[a], *David A. Leonard*[b,c,d], *Josef M. Kurtz*[b,e], *and Curtis L. Cetrulo Jr*[b,c]

Purpose of review
Vascularized composite allograft (VCA) transplantation restores function and form following major soft tissue and musculoskeletal injury. Lifelong immunosuppression is necessary for graft function and survival but acute skin-targeted rejection episodes remain common. We review recent advances in skin immunobiology, emphasizing findings in clinical and experimental VCAs. We also highlight advances in immunotherapy and tolerance protocols with implications for the prevention of VCA rejection, and ultimately, induction of clinically applicable strategies for VCA tolerance.

Recent findings
There is now an increasing appreciation for the role of skin-specific mechanisms, including lymphoid neogenesis, in VCA rejection. In contrast, expression of the regulatory master-switch FOXP3 was demonstrated to be significantly upregulated in the skin of tolerant VCAs in large animal models compared with normal skin and rejecting controls.

Summary
Most VCA transplant centers continue to utilize antibody-mediated induction therapy and triple agent maintenance immunosuppression. Skin remains the primary target of rejection in VCAs, and current multicenter studies hope to elucidate the mechanisms involved. Proposed standardized procedures for skin biopsies, and diligent reporting of clinical data to the international registry, will be important to maximize the strength of these studies.

Keywords
immunosuppression, skin immunobiology, tolerance, transplant, vascularized composite allograft

INTRODUCTION

Vascular composite allograft (VCA) transplantation emerged as a clinical reality over the past decade [1,2]. To date, more than 89 hands and up to 27 faces have been transplanted throughout the world since the first successful hand transplant was performed in 1998 [3]. This advancement in reconstructive surgery has revolutionized the treatment of individuals with devastating upper extremity and facial injuries, allowing for superior restoration of form and function compared with outcomes following prosthetic wear [4,5]. However, patients remain vulnerable to the risks associated with long-term immunosuppressive regimens including nephrotoxicity, metabolic disturbances and increased susceptibility to infection and malignancy [6–8]. Such adverse effects are difficult to justify in the context of VCA as the procedures, although life-enhancing, are not considered to be immediately lifesaving. Thus, as with solid organ transplantation, induction of donor-specific tolerance, defined as lack of a destructive immune response to the transplanted tissue in the absence of immunosuppression, remains an important goal for the field.

The skin now represents a primary goal for tolerance as it represents the most critical component of a VCA. Recent studies have sought to further understand the immunobiology of skin with the intent of developing strategies specifically aimed

[a]University of Oxford, Oxford, UK, [b]Transplantation Biology Research Center, [c]Division of Plastic and Reconstructive Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA, [d]Department of Plastic and Reconstructive Surgery Research, University of Manchester, Manchester, UK and [e]Department of Biology, Emmanuel College, Boston, Massachusetts, USA

Correspondence to Curtis L. Cetrulo, Jr, MD, FACS, FAAP, Head, Vascularized Composite Allotransplantation Laboratory, Transplantation Biology Research Center, MGH East Building 149-9019, 13th Street, Boston, MA 02129, USA. Tel: +1 617 643 7294; e-mail: ccetrulo@partners.org

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KEY POINTS

- Conventional immunosuppressive protocols achieve 96% hand transplant survival at 1 year but 85–90% of patients experience at least one episode of acute skin rejection during this period.
- Acute skin rejection following VCA has been correlated with expression of PNAd, a marker of lymphoid neogenesis and tertiary lymphoid organs.
- Normal human skin contains large numbers of resident T_{EM} cells including a FOXP3$^+$ T_{REG} subset.
- The presence of T_{REGS} and enhanced expression of FOXP3 mRNA has been demonstrated in the skin of tolerant VCAs in large animal models.
- Establishment of mixed chimerism is sufficient for induction of kidney transplant tolerance in clinical trials and has resulted in tolerance of all components of VCAs across minor antigen, single haplotype MHC class I and class II and full MHC barriers in large animal models but further work is required to develop clinically applicable protocols.

at preventing and treating skin rejection. This review will highlight results of such studies investigating the 'skin immune system' and discuss the impact on VCA, as well as the development of novel alternatives to current immunosuppression protocols.

RECENT ADVANCES IN SKIN IMMUNOBIOLOGY

The concept of a unique skin immune system gathered momentum when Clark *et al*. [9] demonstrated that normal human skin harbors twice the number of T cells that circulate in the blood. Not only did the T cells express the skin-homing addressins cutaneous lymphoid-associated antigen and CCR4, but 80% of them also expressed a phenotype consistent with T effector memory (T_{EM}) cells [9]. Interestingly, this finding of skin-resident T_{EM} cells challenges the conventional model whereby T_{EM} cells are recruited from the circulation to the tissues following an inflammatory stimulus [10,11]. These results may also be important in the context of work carried out more than 30 years ago that observed the presence of antigen-presenting Langerhan's cells in the epidermis [12]; recognition of alloantigen by skin-resident T_{EM} cells could be predicted to elicit a more potent response than would occur following activation of naïve T cells.

Recent in-vitro and in-vivo studies have aimed to clarify the function of Langerhan's cells in the skin. Resting Langerhan's cells appear to selectively and specifically induce the activation and proliferation of cutaneous regulatory T cells (T_{REG}) [13,14]. However, they take on an opposing role in the presence of foreign pathogen inducing proliferation of T_{EM} cells, differentiation of naïve CD4$^+$ T cells into T_H2 [15], T_H17 [16] and T_H22 phenotypes [17] and restraining activity of T_{REG}. It is clear from these studies that Langerhan's cells may play an important role in the maintenance of tolerance in skin and therefore further investigation into their specific activity is required.

Dissection of the skin-resident T-cell population revealed that 5–10% display a T_{REG} phenotype [18,19]. Cutaneous T_{REG} express high levels of CD25, L-selectin, GITR and FOXP3 as well as high levels of the skin-homing addressins cutaneous lymphoid-associated antigen, CCR4 and CCR6. In-vitro experiments demonstrated the ability of cutaneous T_{REG} to suppress effector T cells from the same skin sample in a cell contact-dependent manner in the presence of IL-15 and dermal fibroblasts (both mimic the environment of chronically inflamed skin), as well as with use of CD3 and CD28 antibodies [18]. These T_{REG} cells also proliferate *in vivo* under inflammatory conditions [20] controlling the inflammatory response both locally [21] and following migration to the draining lymph nodes [22]. Perhaps suggesting more potent regulatory properties, the CD25^{hi} expressing fraction of the cutaneous T_{REG} population are prolific producers of IL-10, transforming growth factor-β and cytotoxic T-lymphocyte antigen 4 compared with their lymph node counterparts [23].

An additional subset of T cells isolated from human blood and found to be skin-trophic includes memory T_H9 cells. These cells express tumor necrosis factor-α and granzyme B, and blocking studies have demonstrated that IL-9 was required for maximal production of interferon-γ, IL-9, IL-13 and IL-17 [24▪]. It is therefore possible that aberrant activation of this discrete subset may contribute to inflammatory processes in the skin and initiate a rejection cascade in the context of VCA.

Recent work by Lakkis and colleagues [25▪▪] reviewed the classical paradigm of leukocyte migration by investigating the effect of pertussis toxin, which irreversibly inhibits $G\alpha_i$ function and thus impedes chemokine signalling, on the migration of effector and memory T cells to vascularized heart and kidney grafts in mice. They observed that CD4 and CD8 effector and memory T cells pretreated with pertussis toxin migrated to heart allografts to the same extent as untreated T cells, implying that $G\alpha_i$-signalling was not essential for their migration to vascularized organ transplants. However, the group demonstrated that

antigen recognition by effector and memory T cells was sufficient to cause their firm adhesion and transmigration into the graft. The obvious differences between mouse models and humans would preclude the direct translation of such results, but these experiments shed light on the pathogenic mechanisms that may be active in rejection of the skin component of VCAs.

Finally, research in solid organ transplant rejection has recently addressed anew the role of the innate immune system. Acute rejection mediated by neutrophils has been suggested as a relevant mechanism of rejection in VCA, in a similar manner to that described in murine cardiac allografts [26]. Other candidates for the promotion of graft rejection include endothelial IDO and upregulation of adhesion molecules on the endothelium of graft vasculature (ICAM-1, E-selectin and LFA-1) [27]. Given the barrier function and highly vascular nature of the skin, the role of innate mechanisms in VCA rejection and tolerance should not be overlooked.

SKIN IMMUNOBIOLOGY IN VASCULAR COMPOSITE ALLOTRANSPLANTATION

The importance of accurate diagnosis and prompt, effective therapy of skin rejection episodes to prevent persisting damage to, or loss of, allografts has been recognized from the outset of the clinical VCA era. Indeed, protocols for standardization of skin biopsy technique, processing and analysis across VCA centers at different institutions have recently been proposed [28]. This recognition is being followed by an increased focus on skin-specific immunologic cell populations and mechanisms in both clinical and laboratory research. An interesting clinical observation that supports the possibility of local regulation by cutaneous T_{REG} is the observation of increasing levels of FOXP3 mRNA in skin biopsies of an upper extremity VCA 6 years posttransplant. This was detected in combination with an intragraft suppressive cytokine profile and was in contrast to a complete absence of FOXP3 mRNA expression in the contralateral leg, suggesting a potential role for T_{REG} in the long-term survival of this allograft [29]. More recently, a multicenter study has demonstrated the presence of lymphoid neogenesis and formation of tertiary lymphoid organs, phenomena strongly associated with chronic rejection and thought to be sites of T-cell activation and alloantibody production in solid organ transplants, in the skin of upper extremity transplants. These findings were first observed in a case of antibody-mediated rejection following forearm transplantation [30]. Subsequent analysis of clinical biopsy specimens, supported by nonhuman primate and rodent studies, demonstrated a correlation between expression of peripheral node addressin (PNAd) and infiltration of both T and B cells with evidence of both cellular and antibody-mediated rejection [31▪]. Further work is required to determine the significance of lymphoid neogenesis in VCA rejection and to define the mechanisms involved, but PNAd may ultimately prove useful as a biomarker of cutaneous rejection.

Recently published data from our laboratory describe the induction of immunologic tolerance of VCAs across major histocompatibility (MHC) barriers in a clinically relevant miniature swine model [32▪▪]. Success was achieved via the establishment of stable hematopoietic mixed chimerism. Recipient conditioning consisted of T-cell depletion with CD3-immunotoxin, 100 cGy total body irradiation prior to hematopoietic cell transplantation (HCT) and a 45-day course of cyclosporine A. VCAs (a primarily vascularized fasciocutaneous flap) transplanted into both stable chimeras, and those rendered chimeric simultaneous to VCA transplantation, accepted all components including skin. Resident populations of T cells, including $FOXP3^+$ cells, were detected by immunohistochemistry in the tolerized VCAs up to the experimental end point that was over 1 year following cessation of immunosuppression. Ongoing studies have demonstrated the presence of both donor and host-derived cells within these resident cell populations (Leonard, unpublished data).

Similarly, in a canine model, Mathes *et al.* [33▪▪] have demonstrated the induction of VCA tolerance by simultaneous HCT across minor histocompatibility barriers. In this study, $FOXP3^+$ cells were also identified in the skin and muscle of accepted VCAs, and using RT-PCR analysis, expression of FOXP3 mRNA demonstrated to be significantly elevated in tolerant VCAs in comparison with both nontolerant (rejecting) VCAs and normal skin. Interestingly, expression of Helios mRNA was raised in tolerant VCA muscle despite higher levels in both tolerant and rejecting grafts following analysis of skin expression.

Ideally, in order to maximize donor availability and minimize waiting times for suitable donors, a clinical tolerance protocol would be effective across all MHC barriers including between fully mismatched individuals. Although this is an ambitious target, proof of principal for the induction of VCA tolerance across a full MHC barrier has been demonstrated in a porcine model following in-utero establishment of mixed chimerism [34▪]. T cell-depleted adult bone marrow was injected intravascularly into midgestation fetuses under ultrasound

guidance and, following delivery, chimerism was detected by flow cytometry as well as donor-specific unresponsiveness demonstrated in a mixed lymphocyte reaction. Donor-matched VCAs were subsequently transplanted once chimeric piglets reached a suitable size to undergo surgery under the cover of a 12-day course of cyclosporine A to facilitate induction of tolerance of minor antigen differences between the MHC-matched bone marrow and VCA donors. Chimeric recipients accepted VCAs to their experimental end points between 150 and 260 days posttransplant, whereas nonchimeric littermates rejected by day 21. Interestingly, mild lymphocytic infiltration was detected in skin biopsies of accepted VCAs. Unfortunately, detailed characterization of these cells was not possible in this study but in the context of persisting mixed chimerism, donor-specific unresponsiveness *in vitro* and the absence of gross signs of rejection or progressive histological change, it is conceivable that these cells represent the $FOXP3^+$ T_{REGS} demonstrated in single haplotype MHC class I and class II-disparate porcine [32▪▪] and MHC-matched canine [33▪▪] models. Clearly, this approach lacks direct clinical applicability but, nonetheless, is an important proof of concept and should offer encouragement for induction of mixed chimerism and tolerance across full MHC barriers beyond the fetal stage.

Establishment of mixed chimerism across MHC barriers is now considered a reasonable proposition in which matched donors are not available. However, despite significant advances in nonmyeloablative conditioning and HCT protocols, graft-versus-host disease (GvHD) remains a potential complication in this setting. Even so, it is noteworthy that GvHD has not been observed in any of the mixed chimerism-mediated tolerance protocols for renal allografts to date [35,36]. Even so, continued work to mitigate the risk of GvHD following HCT is warranted, as this would quite clearly be an unacceptable side-effect of any tolerance protocol. Considering the broadly similar pathogenesis of VCA rejection and cutaneous GvHD, it can be expected that studies leading to improved understanding of the mechanisms determining VCA skin rejection and acceptance will also yield insights applicable to the avoidance and management of GvHD.

CURRENT PROGRESS IN IMMUNOSUPPRESSION

Most immunosuppressive protocols employed for VCA are extrapolated from regimens utilized in solid organ transplantation. Protocols often commence with an antibody-based induction therapy, most commonly antithymocyte globulin, but in some cases anti-interleukin 2 receptor (daclizumab, basiliximab) [4,37,38], anti-CD3 (OKT-3) [39] and anti-CD52 (campath-1H, alemtuzumab) [40] monoclonal antibodies have been used. Maintenance immunosuppression is most frequently achieved with conventional triple-therapy regimens consisting of a calcineurin inhibitor (tacrolimus), an antiproliferative agent (mycophenolate mofetil) and corticosteroids that are typically weaned to low dose or even withdrawn [3,41]. Some centers convert from tacrolimus to sirolimus (a mammalian target of rapamycin inhibitor) in an attempt to reduce side-effects such as nephrotoxicity, chronic vasculopathy and impaired glucose tolerance [42].

These protocols are successful at controlling acute episodes of rejection and have been reported to produce a 96% hand transplant survival rate at 1 year [43]. However, despite this impressive 1-year survival data, acute rejection is common with approximately 85% of patients experiencing one or more episodes during their first year posttransplant [3]. The reasons for this disparity between incidence of rejection and graft loss to rejection are not yet entirely clear but are likely due to the intrinsic immunogenicity of skin versus ease of monitoring and access for diagnostic biopsies without invasive surgery, and potentially, the benefits of prompt initiation of antirejection therapy. Even with the efficacy with which rejection episodes can be treated, these remain a source of morbidity for VCA recipients in combination with the burden of maintenance immunosuppression-related side-effects. Therefore, efforts to improve these protocols or avoid the need for long-term therapy are welcomed.

In this regard, the immunomodulatory protocol introduced by the Pittsburgh/Johns Hopkins group for upper extremity transplantation represents a possible method to reduce immunosuppression. Following induction with lymphocyte depletion with alemtuzumab, patients received an infusion of unmodified donor bone marrow 14 days posttransplant. Immunosuppression was then maintained with tacrolimus monotherapy [44]. At 1 year, most patients on this protocol were on reduced immunosuppression, but all had experienced reduced glomerular filtration rate. At the time of publication, it had been reported that glomerular filtration rates were returning to baseline and that some patients were on monotherapy. Although these initial results are encouraging, extended follow-up reports are necessary to validate the long-term safety of this approach. The working hypothesis behind this protocol is that by creating 'space' in the host, via alemtuzumab induction prior

to donor bone marrow infusion, donor and recipient-derived clones are better able to accommodate each other thereby increasing the potential for acceptance of the graft. However, evidence for such a mechanism is lacking as mixed chimerism was not detected following bone marrow transplantation, and donor-specific hyporesponsiveness was not reliably demonstrated.

In contrast, cotransplantation of donor bone marrow following nonmyeloablative conditioning has been utilized in renal transplantation for induction of transplant tolerance at a number of centers. Protocols have differed in the details of conditioning treatments used and in the nature of chimerism established, with both transient mixed chimerism and stable, predominantly donor, chimerism both permitting patients to be weaned from all maintenance immunosuppression [35,36,45,46]. The successful, deliberate withdrawal of patients from immunosuppression following induction of kidney transplant tolerance is a landmark achievement built on relentless investigation of the mechanisms of tolerance and rejection, and dedicated commitment to clinical translation of research findings. Although it is apparent that VCAs may pose a greater challenge to tolerance induction, and certainly some additional logistical constraints imposed by transplantation from deceased donors must be faced, it can be hoped that with similar dedication to translational research the recent progress in preclinical VCA studies will result in direct benefit to patients in due course.

Use of the mixed chimerism approach for induction of tolerance of VCAs transplanted from deceased, unrelated donors will require a protocol that reliably achieves mixed chimerism with a negligible incidence of GvHD. Recent developments in the field of HCT offer encouragement in this regard, particularly the use of posttransplant cyclophosphamide to selectively deplete alloreactive cells following transplantation of bone marrow across HLA barriers [47].

With regard to the logistics of tolerance induction, successful kidney transplant protocols have benefited from the availability of living donors and the resulting ability to perform transplantation on an elective basis with pretransplant conditioning of the recipient. Deceased donor transplantation, be it of VCAs or solid organs, precludes such preconditioning. To address this issue, the concept of delayed tolerance induction has been proposed. Under this paradigm, the patient undergoes surgical transplantation under conventional immunosuppression at which time donor bone marrow is procured and cryopreserved. After a period of months, during which the patient recovers from surgery and the associated inflammatory milieu abates, the patient then undergoes conditioning and donor bone marrow transplantation [48]. This approach has proven successful for renal allografts in nonhuman primate studies in which the depletion of memory T cells using a novel anti-CD8 monoclonal antibody was found to be a key component [49]. Extensions of this work have successfully induced tolerance of heart–kidney cotransplants and lung allografts (J. Madsen and J. Allen, personal communication), and studies currently under way are investigating the efficacy of this approach for upper extremity transplantation.

CONCLUSION

The last two decades have witnessed impressive progress in the field of vascularized composite allotransplantation. It is now a viable reconstructive and restorative option for patients with significant disfigurement and soft tissue loss. Steady progress is being made at the laboratory level into our understanding of the immunobiology of VCAs and the development of novel approaches to immune management of VCA recipients. Clinically, the incidence of acute rejection in the first year posttransplantation remains high with graft survival and successful functional outcomes being dependent on careful compliance with lifelong immunosuppression. Unfortunately, the risks and side-effects of such immunosuppression protocols represent a significant limitation on the uptake of these undoubtedly transformative, life-enhancing procedures.

The induction of donor-specific tolerance would obviate the need for long-term immunosuppression and would hold the potential to prevent chronic rejection, which although still poorly defined in VCA, is the focus of increasing scrutiny as follow-up durations extend [50]. However, the immunogenicity of the skin component of VCAs continues to pose the primary obstacle to the development of a clinically applicable tolerance protocol. Thus far, mixed chimerism is the established front-runner in this search having alone proven successful both in large animal models of VCA transplantation and at every stage of the translational research cycle in kidney transplantation.

The mechanisms responsible for the particular susceptibility of the skin component of VCAs to acute rejection, and the difficulty of inducing tolerance of this tissue, have not yet been fully elucidated. Evidence at a systemic level for mechanisms resulting in such tissue-specific effects has been lacking. In contrast, our appreciation of the scale and complexity of the cutaneous immune network is growing steadily. It is our belief that a more comprehensive understanding of the

skin-specific mechanisms operational in both VCA rejection and tolerance will yield important insights leading to development of novel therapeutic agents, and ultimately, development of a successful clinical protocol for the induction of VCA tolerance.

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Conflicts of interest

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