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REVIEW

Immunobiology in VCA

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SUMMARY

Transplantation of vascularized composite tissue is a relatively new field that is an amalgamation of experience in solid organ transplantation and reconstructive plastic and orthopedic surgery. What is novel about the immunobiology of VCA is the addition of tissues with unique immunologic characteristics such as skin and vascularized bone, and the nature of VCA grafts, with direct exposure to the environment, and external forces of trauma. VCAs are distinguished from solid organ transplants by the requirement of rigorous physical therapy for optimal outcomes and the fact that these procedures are not lifesaving in most cases. In this review, we will discuss the immunobiology of these systems and how the interplay can result in pathology unique to VCA as well as provide potential targets for therapy.

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Introduction

Vascularized Composite Allotransplantation (VCA) is a unique field, and almost every VCA graft is unique within the field. Unlike kidney or heart transplantation, where the graft is fairly homologous, with similar tissue and vessels transplanted, almost every VCA recipient has a different tissue defect that must be reconstructed. Every graft has a different amount of skin, muscle, and bone. This review focuses on transplantation of the upper extremity and the face, where there is a composite of skin/bone/muscle/tendon and nerves as well as vessels and lymphatics. Additionally, these types of transplants are unique in that they interact directly with the physical environment of the recipient. Hand and face recipients often undergo subsequent invasive procedures to improve graft function and cosmesis. VCA allografts have the advantage of direct observation of the graft, which may be partially responsible for the very high rates of diagnosing acute rejection (100% in the first year at this center). However, the field is at a disadvantage in that there are no assays for graft rejection similar to those used in solid organ transplantation for monitoring purposes (e.g., creatinine levels in kidney transplants). Furthermore, hand transplant function is not influenced by rejection until it is quite severe or results in edema that physically limits hand function. Most hand transplant recipients enjoy good function and use their hands in activities of daily living (Fig. 1).

At this time, there have been more than 130 hand and face VCA transplants worldwide [1]. There are other types of VCA which are included in this field, such as uterine or larynx transplantation; these are less 'composite' in that they lack skin or bone. In addition to their unique aspects, hand and face transplant recipi-

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ents must deal with all of the same challenges that any solid organ allograft recipient does. Acute rejection, humoral rejection, and chronic rejection, as well as complications from immunosuppressive drugs, all influence outcomes in VCA. This review focuses on the interplay of the tissues and immune cells within the VCA. It is these unique interactions that can either result in a 'Perfect Storm' of aggressive vasculopathy or lead to graft accommodation with routine long-term dual-drug immunosuppression maintenance.

Tissue components of composite allografts

Skin

Arguably, the most unique feature of VCA grafts is the skin. In recent years, it has become apparent that the skin may be as much a part of the immune system as the lymph nodes and spleen. The skin is composed of two main layers, the epidermis and the dermis. The epidermis is avascular but contains immune cells such as dendritic epidermal T cells (DETC). This is a recently described population of γδT cells which populate murine skin [2] and, to a lesser extent, human skin [3]. This population may be of particular interest in VCA recipients as they have been shown to be one of the first immune cells to migrate from the peripheral blood to the skin in damaged or irritated human skin [3] and play a significant role in the initiation and maintenance of psoriatic inflammation [4]. The dermis lies beneath the epidermis and is the site where skin-resident immune cells as well as blood and lymphatic vessels are located. Additionally, the dermis holds hair follicles, sweat glands, and nerve endings. These structures have implications in VCA as follicles and sweat glands are reduced in patients by what appears to be chronic rejection. The ability to sweat is reduced or absent in many hand transplant grafts due to reduced sympathetic innervation. This lack of sweating has implications in activities of daily living, such as turning pages or picking up small objects.

Figure 1 Patient 8, transplanted in 2012, uses his transplanted hand to prepare tomatoes for dinner.

The immunobiology of the skin as it relates to VCA has recently been reviewed by Chadha *et al.* [5]. Surprisingly, normal skin under resting conditions contains nearly twice the total amount of T lymphocytes than are found in the peripheral blood [6]. Importantly, the findings of Clark *et al.* [7.] suggest that 98% of the cutaneous lymphocyte antigen (CLA)+ effector memory T cells actually reside in the skin. The T-cell receptor Vβ repertoire is diverse, and the cells are functionally skewed toward a Th1 phenotype. About 5-10% of the T cells are regulatory T cells (Treg), and these skin Tregs tend to produce higher amounts of cytokines than those found in the peripheral blood [8]. This suggests that the Tregs are poised to react when large amounts of effector T cells are stimulated by the skin antigen-presenting cells (APC) and cytokines secreted by innate immune populations. The epidermis is home to the Langerhans cell, which along with dendritic cells, captures antigen and takes it to the lymph nodes for presentation to naïve T cells. However, presentation of antigen to memory T cells by dermal dendritic cells or other APCs such as macrophages occurs locally within the skin [8]. It is unclear how much direct antigen recognition occurs between the memory T cells and antigen expressed by endothelial cells and fibroblasts. This model of the skin as a repository of antigen experienced T cells that accumulate through life may also help to explain the high levels of acute rejection in VCA recipients. Under normal circumstances, upon activation within the skin, the resident memory T cells will effectively call in other lymphocytes and innate immune cells which will then work to clear offending stimuli [7]. In an allograft setting, the arriving 'cavalry' of immune cells are exposed to the new antigens from the donor in a setting ripe for effector differentiation. In addition, the trafficking of T memory cells between the skin and

draining lymph nodes and back has been proposed as an explanation for the development of tertiary lymphoid organs (TLO) during episodes of rejection in VCA grafts [9]. These TLO are thought to be local sites of T-cell activation and alloantibody production. These *de novo* lymphoid tissues resemble lymph nodes and are made up of clusters of T and B lymphocytes, high-endothelial venules (HEV), antigen-presenting and follicular dendritic cells, and occasional germinal centers [9]. A multicenter study demonstrated an upregulation of peripheral node addressin (PNAd) in skin biopsies during rejection and with increasing time post-transplant [9]. Alternatively, other groups have suggested that TLO should not be identified by PNAd expression alone, but rather by their morphology. Highly organized tertiary lymphoid tissue that arises *de novo* during chronic inflammation has been referred to as lymphoid neogenesis. Lymphoid neogenesis has been detected in chronically rejected grafts in kidney, heart, and lung transplantation. A complete recapitulation of this program seems crucial for the development of a fully functional tertiary lymphoid tissue harboring germinal center reactions in which recipient's naive B cells are converted into memory B cells and anti-HLA antibodies-producing plasma cells. Intragraft TLOs display the same microarchitecture as 'professional' secondary lymphoid organs. The function of TLO can be evidenced by expression of AID and Ig class switch in intragraft TLOs and by the evidence of local intragraft secretion of alloantibodies [10,11]. Whether the skin TLOs observed by Hautz *et al.* play a role in VCA would be substantiated by similar functional studies and examination of these structures in patients who are undergoing chronic rejection.

Intuitively, one might expect that the cells which are infiltrating the allograft, especially during episodes of rejection, are primarily of recipient origin. However, a study published recently by Lian *et al.* [12] demonstrates this may not be the case in VCA allografts. The finding by Clark *et al.* [7.] that normal skin contains twice as many T effector cells as in the peripheral blood suggests that the donor skin should be home to many donor T cells, even after the transplant. In a study of 113 biopsies from face transplant recipients, during active rejection, the majority of the lymphocytes spatially associated with areas of injury were of *donor* rather than recipient origin [12,13]. Additionally, most of these donor lymphocytes are CD8+, and of a T resident memory (T_{RM}) phenotype. The authors hypothesize that this response of the resident donor memory T cells in the allograft skin is against recipient T cells and APCs that are migrating into the graft. In this scenario, it may actually be an influx of recipient FoxP3+ T regulatory cells that express skin homing receptors (CLA+) that control this donor 'graft versus host' type response which is unique to VCA patients [14]. This hypothesis is further supported by the fact that virtually all human peripheral blood CD4 + Treg also express the chemokine receptor CCR4, and 80% express the CLA [15]. Based on this observation, it could be argued that this 'GVH' donor T-cell response would migrate to the recipient skin and literally cause Graft versus Host Disease (GVHD). However, to date, there have not been clinical reports of GVHD in humans, although GVHD has been reported in dog [16] and pig [17] VCA models that also received stem cell transplants.

The novel findings by this group, as well as the description of unique anatomical features seen during rejection, suggest that the current Banff criteria for histologic grading of skin rejection should be revisited, especially for face transplantation. Currently, grades of rejection are based on histologic detection of presence and location of infiltrating mononuclear cells. There is no assessment of the dynamic nature of an infiltrating cell, whether it is pro-inflammatory or anti-inflammatory and recipient or donor derived.

In addition to the unique immunologic features of skin that affect VCA outcome, the skin type and area of the graft can also impact the rejection process. Hautz *et al.* [18] compared three distinct areas of skin in a rat hindlimb allograft model. Skin from the thigh, dorsum, and planta pedis on post-op day 5 with active rejection ongoing was compared with respect to cellular infiltrate, cytokine expression, and histopathologic appearance. Among the differences in these skin types are thickness and amount of hair. The thigh skin is thick with hair, the dorsum of the foot is thin with hair, and the planta pedis is thick and hairless. The cell infiltrate was distributed differently, with diffuse distribution in the dermal layer for the thigh skin, less infiltration in the dorsum of the foot, and primarily perivascular infiltration in the planta pedis [18]. There was not a large difference in the ratio of CD4 + to CD8 + T cells in the infiltrate between the three areas, but a difference was noted in cytokine expression. The hair bearing skin areas had higher levels of MCP-1, IL-4, and GRO-KC, compared to the palm type skin [18]. Clinical VCA grafts of the hand, face, and abdominal grafts also have different skin types, and area-specific rejection processes are likely occurring in humans as well.

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Recently, the AST VCA Advisory committee polled a number of VCA groups around the world and submitted a list of proposed changes and areas of investigation for a Banff Working group on VCA. This proposal was discussed at the 2015 Banff meeting in Vancouver and will be addressed at an upcoming Working group meeting. Questions include whether the grades should be expanded to deal with 'in between' grades of rejection. Many centers continue to report histologic grades of 0-I, I-II, and II-III from review of VCA skin biopsies. Other criteria that should be considered in scoring criteria are loss of capillaries and importance of evaluating small vessel vasculopathy. In addition, there are the distinct differences between infiltrates and histology of skin from hand vs. face transplants, and the fact that current criteria do not note that the level of involvement in the graft (i.e., focal vs. diffuse). Practically, the histologic score is used interchangeable as 'Grade of Rejection'. An actual 'grade of rejection' vs. 'grade of histology' would reflect clinical parameters such as level of involvement, edema, and induration of the skin and graft. Other questions focus on whether a clinical–pathological 'point' system be considered for grading/predicting rejection and how vascular targeting/vasculopathy fit into the grading of rejection schema. These and many other areas of investigation were suggested. As with all Banff criteria, how we evaluate rejection in VCA transplants will continue to change and evolve as our experience and clinical observations change.

Bone

Another unique property of hand and face VCA grafts is the presence of vascularized bone. Like all properties of VCA transplants, this varies a great deal from graft to graft and may also be significantly affected by the age of the donor. With respect to the amount and quality of bone marrow and skin, a hand transplanted at the wrist from a 60-year-old donor is a far different graft when compared to a total face and mandible transplanted from an 18-year-old donor. Nonetheless, the presence of vascularized bone appears to present a more tolerogenic environment than either VCA grafts without bone, or bone marrow cell infusions [19]. In a study of nonhuman primates, Barth *et al.* [20] demonstrated that the presence of facial vascularized bone marrow (VBM) prolongs graft survival compared to VCA grafts without bone. Additionally, animals without VBM were more likely to develop IgM and IgG alloantibody. In these studies, withdrawal of immunosuppression demonstrated that the presence of vascularized bone in an immunosuppressed recipient did not induce tolerance in of itself, but did promote stable acceptance of the graft under moderate immunosuppression. The advantage that vascularized bone offers has been demonstrated by other groups as well [21]. Ramierz *et al.* studied the effect of the presence or absence of vascularized bone as well as the ratio of vascularized bone to VCA mass on graft survival and induction of donor-specific tolerance. Interestingly, tolerance was induced in this rat model with a regimen of transient immunosuppression and recipient adipose-derived stem cells (ADSC) [22]. The authors compared graft survival in animals receiving a full-thickness abdominal wall vs. an osteomyocutaneous hindlimb flap of a similar mass. These two groups were compared to animals who received both grafts with a mass approximately twice as large, but with the same amount of vascularized bone. Immunosuppression alone (ALS and short-term CsA) prolonged survival in all three groups, but prolongation was longer in the group with the hindlimb flap only. However, with the addition of the tolerogenic ADSC, three of the eight animals in the hindlimb flap-only group developed donor-specific tolerance, compared to none of the animals in the abdominal flap-only group, or the group which received both grafts [21]. The increased survival in the hindlimb flap-only group was also reflected in higher levels of donor chimerism found in the peripheral blood by flow cytometry. These studies suggest that while vascularized bone promotes graft survival and development of tolerance, the advantage can be overcome by high graft to VBM mass ratios. In a rhesus macaque model of vascularized fibula with a donor skin pedicle, Mundinger *et al.* [23] showed that the donor marrow was completely replaced by host cells at six months post-transplant. This could be a type of reverse chimerism, but the study was complicated by a high rate of chronic rejection and some technical issues. Despite these issues, the bony unions remained intact, suggesting this type of transplant may provide a good alternative for long bone reconstruction when autologous donor bone is not available.

Vascularized bone marrow appears to offer the benefit of prolonged survival, as well as increased capacity for the induction of donor-specific tolerance, at least in animal models. It must be noted that to date there have been no reports of long-term chimerism in clinical VCA recipients who received vascularized bone without additional stem cell transplantation. Analysis of two patients at our center failed to find even micro-chimerism [24]. However, there are early indications that graft survival in clinical VCA recipients may be superior to solid

organ transplantation. Graft survival rates and the incidence of chronic rejection in compliant patients at 5 and 10 years look promising. However, the numbers are still very small.

It has been estimated that one-quarter of the cells in the human body are blood cells [25,26]. The hematopoietic niche in vascularized bone is made of a group of cells which form a microenvironment that can establish, maintain, or reactivate hematopoietic stem cells [26]. Hematopoietic stem cells (HSC) can be found in most regions of the long bones. The trabeculated regions of the metaphysis are preferred areas that transplanted HSC home to, compared to the endplates (epiphysis, or the diaphysis (the shaft of the long bones) [26]. Additionally, there are two types of niches thought to occur, an endosteal niche and perivascular niche. The endosteal niche is thought to contain physiological characteristics such as hypoxia and increased calcium ions which help dictate HSC proliferation. The perivascular niche is thought to support HSC quiescence, in part from direction by a mesenchymal stem cell in close proximity to the vessels in bone marrow [26]. However, these relationships are a matter of debate. Using a mouse model of un-ablated recipients without activating or damaging the marrow, Ellis *et al.* found that transplanted cells homed to an endosteal niche in close association to blood vessels and that these blood vessels express a unique repertoire of adhesion molecules such as hyaluron (HA). The expression of these adhesion molecules on the blood vessels near the endosteal niche may play a role in how the HSC home and are regulated [27].

As with most VCA programs, our hand transplant recipients receive an annual bone density scan to monitor for osteoporosis. This is primarily to monitor for this complication associated with the use of systemic prednisone immunosuppression. Our program also monitors and maintains vitamin D levels. Over the past 17 years, we have seen changes in bone density in the hips and spine. One recipient has undergone two hip replacements [28]. However, we have not seen evidence of bone loss or thinning in the allograft to date. Our third recipient has presented evidence of chronic rejection of the skin, but review of graft X-rays over time does not indicate bony changes in the hand. The changes in bone density appear to be restricted to the native bone and are a complication of immunosuppression.

Another aspect of bone and the how vascularized bone associated with VCA may affect the development of tolerance and the stem cell niche is the effect of biomechanics on the bone and the niche [29]. Mechanotransduction is now being proposed as an additional means of how cells interact with the physical environment via mechanical forces, resulting in changes to protein organization and even gene expression [29]. This relatively new field may help the understanding of how trauma appears to affect the incidence of rejection and vasculopathy in the field of VCA. Anecdotally our center has noticed an association of rejection and trauma to the grafts, and this has been presented by other centers as well.

Vessels

The first of the nine OPTN criteria for defining a VCA is that the graft is vascularized and requires blood flow by surgical connection of blood vessels to function after transplantation (1 42 CFR §121.2 (2014). Although initially it appeared that the field of VCA might be spared from some of the intermediate post-transplant pathology experienced by solid organ transplants, unfortunately that does not seem to be the case. As in cardiac transplantation, hand transplant recipients have experienced an aggressive vasculopathy that has resulted in graft failure [28]. More recently, Kanitakis *et al.* [30] reported graft vasculopathy of vessels within the skin of a hand recipient, as well as deeper vessels in the graft. Conversely, the same group has reported evidence of chronic rejection of the skin in a face transplant recipient with macules on a background of hypopigmentation and telangiectasias, resulting in a poikilodermatous (sun damage like) aspect. Skin biopsies showed epidermal atrophy, basal cell vacuolization, and diffuse dermal sclerosis in the absence of significant dermal cell infiltration [31]. The dermal capillaries showed thickened walls and narrowed lumina, but unlike the previously reported VCA patients [28], the large vessels did not show significant alterations [31].

The immunobiology of vasculopathy has been studied in great detail in cardiac transplantation [32,33]. The term is sometimes used interchangeably with chronic rejection, but evidence suggests vasculopathy is more complex than an incompletely controlled rejection response, although suboptimal immunosuppression has been shown to induce vasculopathy in animal models [34]. However, vasculopathy can progress in the absence of an ongoing allogeneic response, if supplied with cytokines from nonalloimmune processes [35]. Mitchell describes vasculopathy as a variation on stereotypic healing of vessels. When injured, vessels are capable of finite responses with respect to repair based on the proliferation of smooth muscle cells and extracellular matrix to buttress the damaged wall [32]. The insults

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that damage the wall in the first place can be due to ischemia, mechanical trauma, hypertension, hypercholesterolemia, drug toxicity, as well as alloimmunity. Of note, while veins can show evidence of vasculopathy, the literature and our own center's experience have demonstrated that most of the graft threatening stenosis of vasculopathy is limited to the arteries. The same is true for atherosclerosis [32]. In addition, some centers report that infection, specifically Cytomegalovirus (CMV) infection, is associated with a higher incidence of vasculopathy in cardiac transplant recipients [36]. While there are conflicting reports [37] and immunosuppression regimens employed may also play a role, it appears that infection may also be a triggering factor for vasculopathy. This may be important in VCA as CMV reactivation/infection has been commonly reported and in some cases was difficult to manage [38,39]. However, in the majority of cases, including VCA patients at our center, the CMV viremia responded well to treatment and was cleared.

In the Louisville VCA program, we have seen two types of vasculopathy, an aggressive diffuse confluent vasculopathy that appears relatively early in the post-transplant course, and a minimal focal vasculopathy that is slow to progress and can been seen in most hand recipients [28]. While suboptimal immunosuppression cannot be ruled out as a contributing factor of the aggressive vasculopathy, subjects at our center with multiple episodes of rejection over many years have not developed the aggressive form, suggesting that rejection alone is not sufficient to induce the pathology.

Allograft vasculopathy differs from atherosclerosis in that it is diffuse, seems to involve the entire arterial tree, but is restricted to the allograft, is rapid, immune mediated and does not result in complex plaque lesions containing thrombi [32,40]. Time and additional clinical experience are needed, but in general, the pathogenesis in VCA will likely mimic that of cardiac allograft vasculopathy with a initiating injury, likely at the time of transplant from donor brain death, graft recovery, and ischemia/reperfusion, and is exacerbated by immune-mediated endothelial cell and vascular wall injury. Initially, the innate immune response of neutrophils and macrophages gives way to a T-cell-driven alloimmunity and then remodeling of vessel in response to this injury. This remodeling involves the recruitment of smooth muscle cells and can occur in either a positive (stenotic) or negative (aneurysm) remodeling. Negative remodeling is mediated by smooth muscle cell loss due to apoptosis and increased degradation of the media extracellular matrix. This process results in dilation of the vessel and, in extreme cases, aneurysm. Conversely, and what has been seen in hand transplantation, is inward remodeling, with medial scarring and reduced matrix turnover. Inflammation and the resulting adventitial scarring in the early post-transplant period, and intimal hyperplasia in later stages, results in luminal stenosis [41].

Similar to the emerging story regarding the presence of donor T cells in the infiltrate skin rejection, studies have demonstrated that the majority of the smooth muscle cells recruited into the stenotic lesions of allograft vasculopathy are of host origin, rather than donor [42]. Surprisingly, the progenitors of the host-derived cellular components of the thickened intima are of bone marrow origin [43,44]. This suggests the thickening of the intima is an inflammation-derived recruitment of smooth muscle cell (SMC) progenitors, rather than merely a proliferation of smooth muscle cells already present in the donor intima. However, our understanding of the cellular makeup of these lesions is evolving. Studies in atherosclerosis suggest the cells that make up intimal lesions may be more complex than just smooth muscle cells. In a recent review, Tabas *et al.* [45] cited several studies [46–48] that suggest SMCs and macrophages in atherosclerotic lesions have been misidentified. This statement is based on the fact that SMC marker-positive cells in lesions can be derived from multiple cell types other than SMC. Additionally, the SMC can lose marker expression, and cells of nonhematopoietic origin can express macrophage markers [45]. Wong *et al.* [41] have proposed that the loss of luminal volume in graft vasculopathy is a balance between three processes: (i) deep vessel and adventitial injury leading to shrinkage, especially of the smaller vessels; (ii) intimal injury leading to SMC recruitment and intimal thickening; and (iii) compensatory remodeling triggered by hemodynamic factors. A case could also be made in hand and face transplantation for vessel remodeling being triggered by trauma, vibration, or thermal injury to the graft.

As the vasculopathy observed in VCA recipients strongly resembles cardiac allograft vasculopathy, intuitively one would expect the risk factors for Cardiac Allograft Vasculopathy (CAV) would also be important in vasculopathy in VCA recipients. In a recent study of late failing heart allografts, Loupy *et al.* [49] compared the pathology of CAV with antibody mediated rejection, specifically looking for antibody-mediated damage in endomyocardial biopsies. The authors found that 19 of 40 explanted hearts showed evidence of antibody-

mediated rejection, and biopsies showed evidence of subclinical antibody-mediated rejection years before allograft loss. And of course the enigma of CAV is that 21 of the 40 explanted hearts did not show evidence of ABMR. Few VCA patients to date have demonstrated evidence of vasculopathy, despite presentation of *de novo* Donor Specific Antibody (DSA) production in VCA recipients. In the case of aggressive vasculopathy that resulted in graft loss at 9 months, DSA was not detected, and the explanted tissue did not show evidence of antibody deposition [28]. However, DSA was detected 3 days after amputation and cessation of immunosuppression. Whether this was a subclinical DSA or an immune response in the absence of immunosuppression is unclear.

An additional characteristic of VCA grafts, especially those with significant areas of skin, may be an issue of lymphatic drainage following transplantation. During conventional replant surgery, lymphatic surgical connections are rarely made, as lymphatics connect spontaneously, and the surgery is already very long and complex. However, edema is a common finding following VCA transplantation and may reflect a combination of rejection, possible venous outflow issues, and inadequate lymphatic drainage. Cavadas *et al.* [50] did a scintigraphic study of three upper extremity hand recipients and found evidence of moderate lymphedema in two of the subjects. Interestingly, both of the affected patients were bilateral recipients, and one side was affected with normal contralateral lymphatic function. The block of lymphatic flow was not complete, and no cutaneous back flow was seen in the affected limbs. These abnormal flow patterns may also impact the immune response to the allograft. The lymphatic vessels play a key role in trafficking of B and T cells to the lymph nodes [51,52]. Stasis of lymphatic fluid has been shown to result in increased fibrosis, adipogenesis, and inflammation [53]. This increased inflammation may result in subsequent graft rejection. Studies of lymphatic draining using indocyanine green (ICG), imaging in a nonhuman primate model of face transplantation demonstrated that reduction of facial swelling post-transplant correlated with superficial donor–recipient lymphatic channel reconstitution [54]. However, the normal rapid flow of dye from the superficial to the deep lymphatic system through multiple small channels did not develop post-transplantation [54]. Interestingly, rejection episodes did not seem to alter the lymphatic drainage patterns. The authors concluded that VCA graft edema might be overcome by performing lymphaticovenous and or lymphaticolymphatic anastomosis during the transplant procedure [54]. Our center is currently implementing protocols using subcutaneous ICG injection as a method to monitor lymphatic drainage and pilot studies have also suggested a dominance of superficial vs. deep drainage in transplant recipients vs. a normal control.

Muscle

An additional component of most VCA allografts is muscle tissue. Transplantation of the face and hand and especially of the upper extremity brings a significant amount of muscle tissue with the transplant. The largest experience with muscle allotransplantation is cardiac transplantation, and a wealth of literature and reviews are available. Additionally, there is a large literature on fulminant myocarditis, most of which is attributed to viral infection [55]. This syndrome, which often results in the need for a heart transplant, involves both direct virally mediated myocyte injury and immune-mediated tissue injury. The resulting myocyte necrosis may result in cardiac dysfunction and sudden death [55]. In cardiac transplantation, endomyocardial biopsies routinely show cellular infiltration into the cardiac muscle with myocyte necrosis. However, this type of acute rejection responds to treatment and is not a primary cause of graft loss [56]. In heart transplants, significant cellular infiltrate and acute rejection in the first year post-transplant is not associated with a decrease in short-term survival. On the other hand, more acute rejection in the first year after heart transplantation is associated with decreased survival due to cardiac allograft vasculopathy [56].

In VCA allografts, the muscle tissue seems relatively spared with respect to acute rejection as compared to the skin. There are multiple animal models that have examined the muscle components of VCA, including a model of transplanting the cremaster muscle only to examine microcirculation [57]. Kuo *et al.* [58] followed a heterotopic porcine VCA graft as it rejected in the absence of immunosuppression and found that all tissues eventually had significant mononuclear cell infiltrate, but that muscle had less than skin, and more than bone or cartilage. In a detailed comparison of all tissues present in face and hand transplants, Petruzzo found that deeper muscle biopsies showed a sparse interstitial lymphocytic infiltrate in patients 1, 2, and 4, and a mild interstitial lymphocytic infiltrate between muscle fibers in patient 3 [59]. When the same tissues were compared by MRI analysis, muscles were the only tissue that showed a variable degree of hypotrophy, particularly of intrinsic muscles, accompanied by fatty

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degeneration. However, the changes are more likely associated with denervation and changes in muscle activation pattern rather than rejection. When the tissue from the graft that was amputated at 9 months due to ischemia secondary to vasculopathy at our center was examined, we also found relative sparing of the skin, muscle nerves, and tendons compared to the severe vasculopathy of the donor vessels [28]. Histologic evaluation of the muscle of the graft explant showed some changes including atrophy and muscle fibrosis. However, it is also possible that the changes in the muscle was due to the moderate-to-severe ischemia of the graft that developed over a 2-week period [28]. While rejection of the muscle tissue can and does occur, no evidence in either clinical or experimental models has been presented where there is significant rejection of the muscle tissue in the absence of skin rejection.

Nerve

While hearts, kidneys, and livers are transplanted as denervated organs, VCA grafts require regeneration of nerves for optional functional outcome. Intrinsic muscle function and sensory input require reinnervation and that nerve regeneration can be affected by rejection. Moore *et al.* [60] reviewed nerve allotransplantation with respect to transplant of isolated nerves versus the transplant of nerves within VCA allografts. Nerve allograft regeneration appeared to be dependent on host schwann cell (SC) migration into the nerve allograft [61]. Schwann cells are a target of allogeneic rejection [62] and are preserved by immunosuppression [63]. The theory is that host SC migration would be inhibited on the distal end of the nerve within a VCA graft. Therefore, if the regeneration was dependent on donor SC, any rejection and loss of the donor SC would inhibit or prevent nerve regeneration [60]. However, more recent studies in a rat hindlimb model suggest that a single episode of rejection that is treated does not affect regeneration [64]. Yan *et al.* also investigated the effect of late rejection on nerve regeneration. While untreated late rejection did result in loss of function, the authors did not test whether the animal would recover from late treated rejection. Our clinical results in hand transplant recipients support the findings in this report and suggest that nerve regeneration continues throughout the first decade post-transplant and continues to occur even after treated rejection episodes. As expected, the speed and completeness of nerve regeneration in an adequately immunosuppressed recipient appears to have more to do with how proximal the nerve repairs are than the presence or absence of clinical or subclinical rejection episodes.

In a fascinating review, Larregina *et al.* discussed a report by Riol-Blanco *et al.* [65] which demonstrated that nociceptive sensory neurons drive IL-23-mediated psoriasiform skin inflammation. This finding demonstrates that peripheral nerves within the skin can regulate cutaneous immune responses. Larregina *et al.* [66] noted that neuropeptides released in peripheral tissues modulate both innate and adaptive immune responses. The sensory function of surface tissues such as the skin depends on innervation provided by sensory Aδ and C nerve fibers. It is these fibers that express nociceptors that detect the presence of chemical and mechanical stimuli and differences in temperature and pressure [67,68]. After these receptors are activated, the brain perceives sensations of itch or pain, and by antidromic reflex pro-inflammatory neuropeptides are secreted in the affected tissues. This occurs especially in type I and type 2 hypersensitivity reactions [66]. Graft rejection is mediated primarily by type 1 hypersensitivity. This is an important new area of investigation. Data from our own center as well as others have suggested that surgery, burns, and physical trauma can trigger acute rejection episodes [69,70]. The authors suggest that depletion of sensory nerve fibers may be a new treatment alternative to control rejection [66]. While that would not be an appropriate treatment for hand or face VCA recipients, these studies have significant implications in understanding how trauma and environmental influences can trigger rejection in VCA recipients.

Conclusion

In summary, the immunobiology of VCA parallels much of the experience to date in solid organ transplantation, with the caveat of the unique environment of the skin and its under-appreciated role as a lymphoid organ. The expansion of skin-resident Treg and T effectors as well as the ability to develop TLO within skin is an example of the forces in VCA allografts that can drive the immunologic state from quiescence to rejection and back (Fig. 2). The presence of more than one type of tissue with differing susceptibility to rejection, as well as to ischemia reperfusion injury (muscle is quickly damaged by ischemia compared to skin, tendons), additionally complicates the immunobiology of this type of transplant. The high incidence of acute cellular graft rejection (nearly 100% in most centers) during the first year could be attributed to several factors:

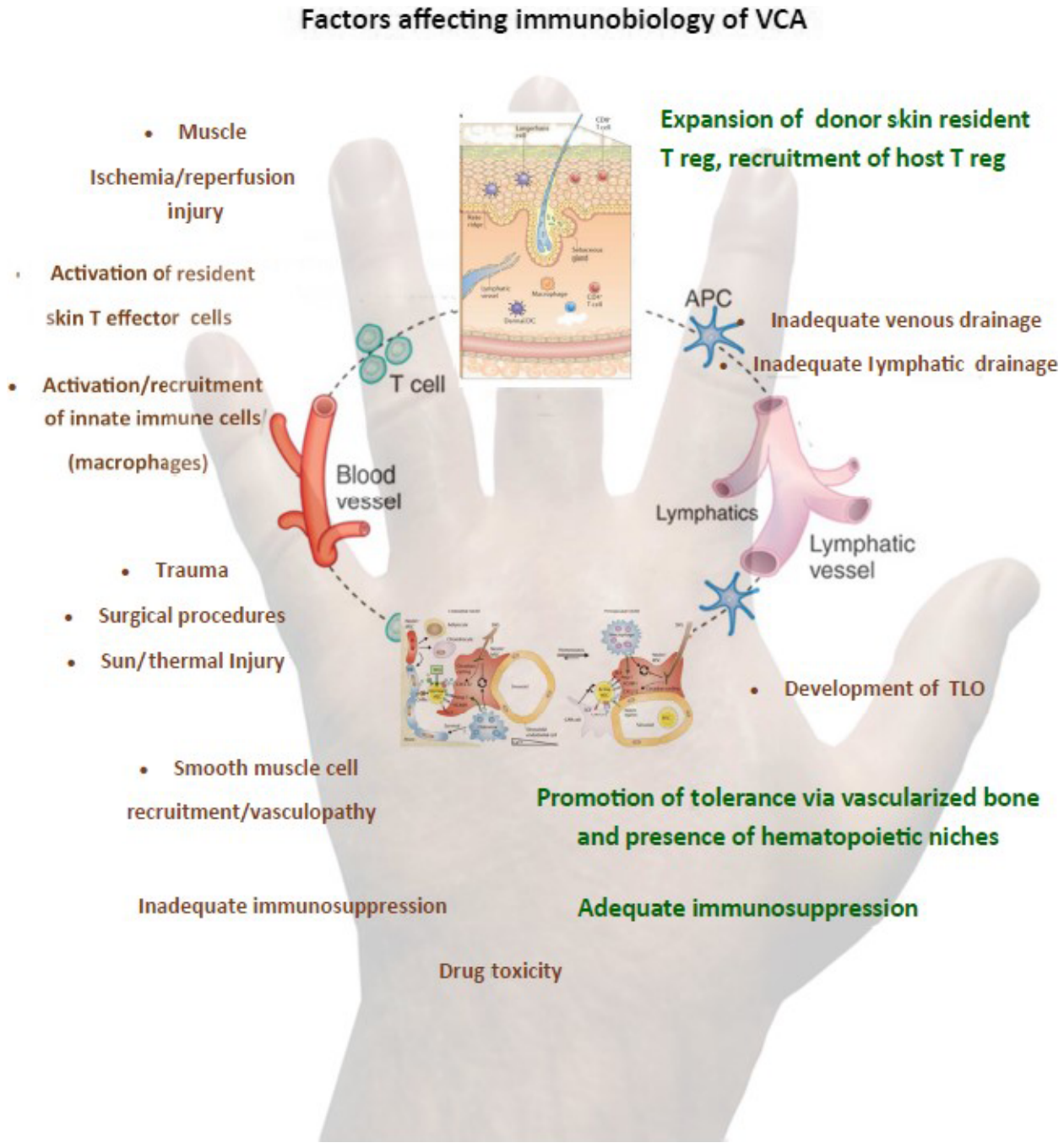

Figure 2 Overview of factors affecting the immunobiology of VCA.

(i) the ability of transplant physicians and the patient to directly observe the graft, and ease of taking frequent skin biopsies, (ii) the presence of resident T effector cells and the role of the skin as a lymphoid-like organ, and (iii) the influence of external trauma during activities of daily living along with frequent surgical damage to the graft via biopsy procedures and subsequent operations to improve function and cosmesis after the transplant. These challenges and characteristics also make VCA transplants an excellent model to study transplantation immunology and lessons learned will advance all types of transplant as well as conventional reconstructive surgery. Finally, a better understanding of the transplant immunology of VCA and novel procedures using cell therapies such as adipose-derived stromal vascular fraction cells and mesenchymal stem cells may allow the

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more widespread application of VCA to the thousands of patients who could benefit.

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