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REVIEW

Vascularized composite allograft-specific characteristics of immune responses

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SUMMARY

Vascularized composite allograft (VCA) transplantation, or reconstructive transplantation, has revolutionized the treatment of complex tissue and functional defects. Despite arriving during an age in which the immunology of solid organ transplant rejection has been investigated in much detail, these transplants have offered new perspectives from which to explore the immunobiology of transplantation. VCAs have a number of unique molecular, cellular, and architectural features which alter the character and intensity of the rejection response. While much is yet to be clarified, an understanding of these distinct mechanisms affords new possibilities for the control of immune responses in an effort to improve outcomes after VCA transplantation.

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Introduction

Vascularized composite allograft (VCA) transplantation has revolutionized the treatment of the most challenging tissue defects. Over 100 patients have now received VCA transplants comprising entire functional units such as hands, faces, lower limbs, and abdominal walls. While 'replacing like with like', VCA transplantation is associated with major immunological challenges due to the allogeneic nature of the transplanted tissue. Importantly, the rate of acute rejection of VCAs in the first year is over 80% in comparison with approximately 10% for renal allografts [1–3]. Hence, while the principles of rejection that apply to traditional organ transplants [4] are also applicable to VCA transplants, there are a few special considerations, which may explain this disparity in rejection rates. These considerations are related to the presence of multiple tissue types within each VCA and in particular the presence of vascularized skin and bone. Much has been written regarding the clinical features of VCA rejection and the challenges in histopathological diagnosis. This review will focus principally on the cellular and molecular immunobiological mechanisms that are unique to VCAs and that may explain the clinical features that have been observed.

The early response to a VCA

The immune system exists to protect and clear the host from foreign material, be it infectious microorganisms or mutated cells. VCAs are subject to the same response, in this context termed an alloresponse as it is against foreign cells from an allogeneic individual. In general, the alloresponse results from the interplay between innate (largely nonspecific) and adaptive (lar-

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gely specific) immune responses. However, a large body of evidence is emerging to indicate that these two mechanisms are not distinct and that a number of cells 'bridge the gap' [5]. Examples include innate lymphoid cells, which although belonging to the lymphoid lineage do not respond in an antigen-specific manner [6]; and natural killer (NK) T cells, which although share properties of both T cells and NK cells, respond to glycolipids presented in the major histocompatibility complex (MHC) I-like molecule CD1d through their semi-invariant T-cell receptor (TCR) rather than a conventional TCR. While it is well established that innate immune activation promotes the adaptive response, the emerging concept is of a reverse model in which the adaptive immune system senses specific antigens to then activate innate immunity to augment alloresponses further.

The innate immune response is mediated largely through macrophages, dendritic cells (DCs), neutrophils, NK cells, and the complement cascade. These cells and molecules provide a preformed system of immunity that responds to 'danger'. The appreciation that the context in which an antigen is encountered determines whether an immune response is activated has been elegantly described as the 'danger hypothesis' [7]. Danger signals such as the ischemia–reperfusion injury (IRI), surgical trauma, and brain death in the donor promote the activation of innate immune responses. Invariant pattern recognition receptors on innate leukocytes recognize damage-associated molecular pattern molecules (DAMPs) on the allograft that have been altered by the inflammatory processes following transplantation. These activated cells produce chemokines and preformed P-selectin, which recruit leukocytes to the transplant site. Recruited macrophages produce pro-inflammatory cytokines such as interleukin (IL)-1 and IL-6, which potentiate the response against the transplant and help to recruit and activate antigen-presenting cells (APCs). At the same time, APCs migrate out of the transplant into the host where they may be involved in direct allorecognition [8–10]. In theory, the innate response will therefore be activated even in the absence of genetic disparity between the transplant donor and recipient as long as the danger signals of IRI and surgical stress are present. However, there is evidence from VCA transplantation models that there is greater cellular trafficking into allografts than isografts [11].

The influx of cells and migration of APCs to and from VCAs differs to that of conventional secondarily vascularized skin grafts [11]. The logical explanation for this is that VCAs are primarily vascularized therefore providing a route of access for these recipient leukocytes. However, there is also evidence that Class II MHC expression only appears on the endothelium of VCAs and not skin grafts, which may have an impact on the access of leukocytes to the allograft [12]. Moreover, the migration of recipient APCs into the transplant is more prominent in VCAs than conventional skin grafts [11]. These observations may explain disparities in the synchronicity of rejection when using a distant vascularized or nonvascularized skin transplant as a 'sentinel' for rejection of a VCA. In a rat study, nonvascularized skin sentinel grafts preceded the rejection of a hindlimb VCA by approximately 1 day [13]. Similarly, in a clinical program of hand transplantation, signs of rejection at a distant nonvascularized skin graft preceded rejection of a hand transplant by 1 week [14]. However, the majority of clinical VCA programs have used vascularized sentinel skin transplants, which appear to demonstrate signs of rejection synchronously with the VCA. [15,16].

All VCAs are subjected to periods of both cold and warm ischemia, followed by rapid reperfusion after revascularization. Ischemia times have long been known to be associated with poorer outcomes after solid organ transplantation (SOT), predisposing to both acute and chronic rejection [17–20]. Methods to minimize ischemia time are therefore critical to ensuring the detrimental alloresponse is kept to a minimum, as well as to keeping the transplant viable – a critical concern in muscle and bone-containing VCAs. Moreover, a prolonged ischemia time in autologous free flaps is associated with a no-reflow phenomenon in which neutrophils and activated endothelium produce pro-adhesive molecules and cytokines that result in intravascular stasis within the microvasculature [21]. The reperfusion event itself may also be associated with an augmentation of the alloresponse through IRI-related mechanisms. Indeed, free radical formation and oxidative stress have an adverse effect of SOT outcomes through the activation of immune responses, and methods for the targeting of oxidative stress may improve outcomes after transplantation [22]. One method that has recently been advocated in abdominal wall transplantation is remote revascularization on the forearm as a temporary measure while the visceral transplants take place [23]. Such a method may also provide an element of remote ischemia preconditioning, which has been shown to suppress pro-inflammatory gene expression [24].

Skin and the acute cellular response

A discussion of the immune mechanisms of rejection of VCAs is not complete without a focused discussion regarding the importance of skin. Figure 1 is a sum-

mary of some of the aspects of skin immunology that may be relevant to transplant rejection. Some argue that skin presents the greatest immunological challenge due to its immunogenicity [25]. Indeed, there are studies that show that skin sits at the top of the hierarchy in terms of 'antigenicity' [26]. However, there is evidence that skin does not produce as potent an immune response as once thought, suggesting that antigenicity is not in fact the causative factor. Specifically, skin transplants do not induce a greater T-cell response than other SOT experimentally [27]. However, skin is usually the first and almost always the only tissue to reject in VCAs [28,29], and controlling rejection of the skin component in VCAs is more challenging than the other components [30,31]. An argument may therefore be made for the hypothesis that skin is not more antigenic than other tissues; rather it is more susceptible to rejection.

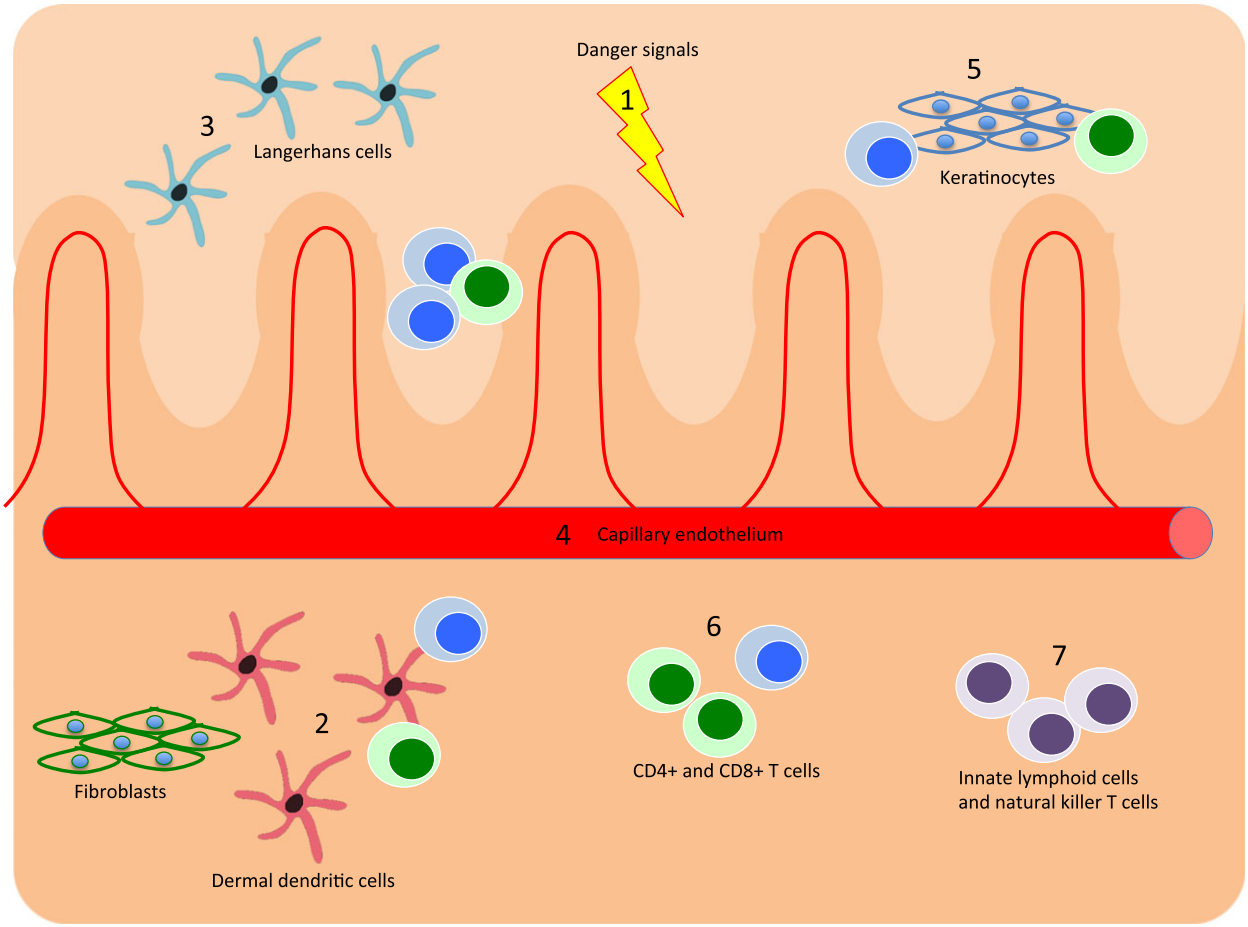

Figure 1 Skin contains a self-sufficient immune system capable of activating the host immune system. This microenvironment contains a number of elements, which increase its susceptibility to rejection. (1) Danger signals, which may come in the form of trauma, ischemia, irritants, and UV radiation, activate resident innate cells through Toll-like receptors and other pattern recognition receptors. The ability of the external environment to activate skin leukocytes must not be underestimated and is unique to barrier tissues. (2) Activated innate cells such as fibroblasts produce inflammatory cytokines such as IFN-α, IFN-γ, TNF, and IL-6 to activate professional antigen-presenting cells (e.g. dermal dendritic cells) in order to present antigen to T cells. Activated dendritic cells produce chemokines to assist in the recruitment of T cells from the peripheral blood. (3) Langerhans cells may produce inflammatory cytokines to facilitate dermal dendritic cell activation or present antigen themselves. (4) Activated capillary endothelium expresses human leukocyte antigen (HLA) Class I and II and may recruit resting memory cells. Endothelial cells also express lymphocyte adhesion molecules to assist with the transendothelial migration of circulating lymphocytes. The width of dermal capillaries is less than that of a single T cell, forcing cells to come into contact with HLA molecules on the endothelium. (5) Keratinocytes stimulated with pro-inflammatory cytokines such as IFN-γ may also present antigen to T cells via HLA Class I and II. Keratinocytes may also express unique skin-specific antigens. (6) T cells undergo clonal expansion and mature into effector and memory cells which migrate throughout the dermis and in more severe cases into the epidermis to cause destruction through multiple effector mechanisms.
(7) Bridging the innate and adaptive immune systems are innate lymphoid cells and natural killer T cells, which are capable of not only activating resident dendritic cells but of being activated and subsequently responding to certain foreign antigens through recognition via invariant and semi-invariant T-cell receptors.

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There are many parallels between the mechanisms that underlie inflammatory skin conditions and skin rejection [32]. The principal players in this respect are the endothelial cells (ECs) of the dermis and the APCs and keratinocytes of the skin. In terms of the keratinocytes, a unique feature in comparison with the other components of the VCA and indeed SOTs is their ability to express human leukocyte antigen (HLA) Class II (and subsequently activate T cells) when stimulated by inflammation [33–35]. This feature has led to postulation that only the skin component of a VCA may induce a humoral immune response [36]. Furthermore, in comparison with SOT, the microvasculature of the skin is a powerful environment for activation of the immune response [37]. Not only do ECs express HLA Class II, but also the costimulatory molecules and receptors required for T-cell recruitment and activation [38,39]. ECs are therefore powerful activators of T cells, with evidence that they are more capable of costimulation than even professional APCs [39–41]. The ability of ECs to express HLA Class II both constitutively and on stimulation with interferon-γ appears to be unique to human ECs, as mouse ECs does not express MHC Class II even on stimulation [42] – explaining why skin allografts from MHC Class II knockout mice are rejected at a similar rate to wild-type skin [43–45]. Dermal capillaries are narrower than the width of a single T cell, thus also physically forcing cells that pass through the microvasculature to come into contact with molecules on ECs. Crucially, these ECs are able to activate resting memory T cells in contrast to other tissue cells such as fibroblasts and epithelial cells, which, although express MHC molecules, are not able to activate resting memory T cells [46,47]. There is evidence that skin is able to recruit a large number of T cells despite only a small percentage of these being truly alloreactive [48]. This again is related to the function of ECs, which are also able to produce inflammatory mediators after keratinocyte damage (which may occur after ischemia). During skin rejection, lymphocyte adhesion molecules are upregulated and correlate with the severity of rejection [49]. Here, the importance of lymphocyte adhesion to ECs is highlighted by the ability to extend rat hindlimb allograft survival by blocking of E- and P-selectin. A number of leukocyte-activating cytokines and chemokines are released or expressed by ECs, including VCAM-1, ICAM-1, and E-selectin (which may all act to promote leukocyte adhesion), as well as nitric oxide, prostacyclin, and bradykinin (which promote vasodilation and increased cellular recruitment) [50,51]. While these T cells may not be directly alloreactive, they nevertheless contribute to the pro-inflammatory state through the production of damaging cytokines and nonspecific effector activity.

In the hierarchy of susceptibility to rejection, all 'barrier' tissues hold ranks at the top – skin, gut, and lung [52]. It is this immunological barrier that may also be responsible for skin's susceptibility to rejection. Elements here include the extracellular scaffold matrix that contains a high density of leukocyte adhesion molecules such as E-selectin, the ligand for cutaneous lymphocyte-associated antigen [53]. Indeed, skin harbors twice the number of T cells in the peripheral blood, the majority of which express memory T-cell phenotypic markers [53]. In addition, skin contains a high density of APCs including specialized Langerhans cells, which may assist in the activation of recruited lymphocytes [54], although there is some evidence that Langerhans cells also have a dual role in immunoregulation [55].

Many have proposed the presence of skin-specific antigens that are distinct from MHC antigens to explain the observation that the epidermis is the only tissue that is rejected in miniature swine chimeric models of tolerance [31,56–62]. In addition to these models in swine, in a rat model of skin transplantation, an increase in skin allograft survival was observed in rats that received injections of epidermal cells together with bone marrow cells from donors, compared with those that only received the bone marrow cells [63]. Potential candidates for skin-specific antigens include Skn-1, Skn-2, and Epa-1, which are present in the epidermis; however, their involvement is not clear. While Skn antigens have been identified in the mouse epidermis, they have not been shown to be sufficient to promote skin rejection in chimeric models and they are therefore unlikely to be true transplantation antigens, although, in the context of a full MHC-mismatched transplant, it is possible that they may augment the alloresponse [58,64,65]. Epa-1 may have a human homolog, and although there is evidence that skin that expresses Epa-1 rejects faster than skin that does not, there is also evidence that Epa-1 is expressed on tissues that are not as susceptible to rejection as skin, such as the heart [66].

Skin-containing VCAs may have a unique mechanism for innate immune activation due to the activity of skin alarmins, which are DAMPs that are generated endogenously by keratinocytes and resident skin leukocytes following cell death, trauma, IRI, allergic insults, or ultraviolet radiation [67,68]. Alarmins act as chemoattractants for host leukocytes and are also able to activate host APCs. Examples of these molecules include high-

mobility group protein B1, heat-shock proteins, S100 proteins, IL-1a, IL-25, IL-33, and uric acid [69–78]. IL-33 in particular has a role in the pathogenesis of other skin inflammatory disorders including psoriasis and atopic dermatitis [79]. The secretion of these inflammatory mediators through a range of nonspecific insults, and their ability to activate an immune response subsequently, may explain the observation that damaged skin (through heat or trauma, for example) may progress to a rejection episode [80]. Indeed, the differentiation between rejection and other inflammatory conditions of the skin has always proven a challenge, which may be related to the employment of similar immune mechanisms in both pathologies [28]. However, in a recent study, a number of molecular markers have been shown to help discriminate between skin rejection and allergic inflammation in a murine VCA transplantation model [81].

VCA susceptibility to rejection

At the advent of the field of VCA transplantation, a great deal of apprehension existed regarding the likely outcomes in light of both the clinical and immunological historical data regarding the susceptibility of skin to rejection. Indeed, the earliest VCA transplants did not survive before the introduction of modern immunosuppression [82,83]. However, the initial fears were unfounded and VCAs appeared to require similar immunosuppression as traditional SOTs, particularly when combined with immunosuppression reduction strategies [2,84–87]. Despite the high rate of acute rejection, none of the VCAs in the modern era have been lost due to acute rejection in patients compliant with immunosuppression, as episodes have been well controlled with boluses of immunosuppression with or without changes to maintenance immunosuppression [2]. Compliance is key in preventing episodes of acute rejection, as evidenced by data from the IRHCT registry [2], and also perhaps the improved outcomes after conversion of patients to belatacept, which is dosed monthly [88].

One of the earliest studies demonstrated that a whole limb allograft in a rat elicited less of an immune response than its individual components [26]. A number of immunobiological reasons may explain this counterintuitive observation. There is evidence that allografts of a large volume are less prone to rejection than allografts of a small volume [89,90]. Moreover, larger tissues appear to have an advantage in terms of their propensity to develop operational tolerance [91–93]. This may be related to the development of an 'antigenic sink', where leukocytes are trapped and unable to effect damage, or the increased likelihood of the development of mixed chimerism [94]. It is therefore possible that the relatively large volume of nonskin tissues transplanted concomitantly in a VCA provides relative protection, although there is currently no clear evidence for this in clinical VCA transplantation. Another explanation may be related to the fact that the majority of preclinical studies have focused on skin grafts, whereas VCAs contain primarily vascularized skin. There is evidence that primarily vascularized skin has a survival advantage over conventional (secondarily vascularized) skin allografts, which may be related to a reduced IRI after the rapid revascularization [13]. Nevertheless, within a VCA, skin remains the most susceptible tissue to rejection even when rapidly revascularized [95]. In a miniature swine model of transplantation of either conventional skin grafts or primarily vascularized skin, both types of allograft are rejected even though the musculoskeletal component of the primarily vascularized skin is accepted, leading to the concept of 'split tolerance' to skin [31,57].

Another proposed mechanism is related to the presence of bone marrow within the transplant, which enables the development of a tolerogenic chimeric state. Moreover, bone marrow aside, there is evidence that mixed chimerism is more likely to develop in primarily vascularized skin when compared to conventional skin grafts, which may partially explain some of the differences in the rejection characteristics between the two types of transplant [94]. The induction of mixed chimerism has been used both experimentally and with a good deal of success clinically in SOT for the promotion of tolerance. There has therefore been an interest in whether chimerism develops in VCA transplantation as a result of the transplantation of bone marrow as a 'passenger' within the VCA. A number of animal models of VCA transplantation have demonstrated that a T-cell-depleting antibody together with immunosuppression may result in long-term allograft survival, although mixed chimerism is not always detectable [96–102]. Importantly for these models, the presence of bone marrow within the donor allograft is necessary for achieving transplant survival [103,104]. However, clinically, there has not been any convincing data demonstrating the spontaneous development of mixed chimerism in VCA recipients [105,106]. This may be related to the very small quantities of bone marrow that are transplanted as part of a VCA, or the requirement for the recipient thymus to be fully functional in order for mixed chimerism to develop [107].

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The late alloresponse to VCAs

It is important to be clear regarding the results of clinical VCA transplantation. While graft survival rates have been encouraging and immunosuppressive drug use at a lower level than expected, acute rejection rates remain high and in the order of approximately 85% in the first year [2,3]. It is therefore important to temper the enthusiasm within the field – particularly as these are rejection rates that would be unacceptable in the field of most traditional SOTs. More importantly, acute rejection and delayed graft function in SOT is known to be predictive of future chronic allograft dysfunction [108,109]. While there was initially very little attention paid to chronic rejection of VCAs, reports are now emerging within the field. Although only a small number of VCA transplants have been performed and the follow-up period remains short, chronic rejection has been reported by a number of centers [87,110,111]. In a series from Lyon, a face transplant recipient who suffered a number of acute rejection episodes developed chronic skin rejection, evidenced by fibrosis [87,112]. In this patient, immunosuppression was reduced due to immunosuppression-related complications, triggering the episodes of acute rejection [113]. The largest series has been reported by Louisville, where a number of hand transplants have developed evidence of chronic rejection, with one also being lost due to intimal hyperplasia [114,115]. In this patient, the intimal hyperplasia was limited to the donor vessels, indicating an alloimmune process. In the sixth patient in the Louisville series, evidence of chronic rejection developed after two episodes of severe acute rejection and a turbulent clinical course. Similarly, in a case of knee chronic rejection reported from Germany, the patient also had episodes of acute cellular rejection early post-transplantation [111,116]. While it could be argued that the acute rejection episodes are predictive of future chronic rejection, in a series of five hand transplants from Lyon with follow-up periods of between 4 and 13 years, there has been no evidence of chronic rejection even in a patient who suffered 6 episodes of acute rejection [117]. Moreover, in a number of Louisville patients who suffered multiple acute rejection episodes, significant chronic vasculopathy has not been observed.

Graft vasculopathy leading to transplant ischemia can be a feature of chronic allograft dysfunction although the mechanisms that are responsible for its development remain unclear. Some have argued that the vascular tree may be the primary target of chronic allograft rejection in VCA transplantation [114]. Antibody-mediated damage is thought to be important, although it is not necessary, and indeed chronic allograft dysfunction may also be a function of the off-target effects of immunosuppressive drugs. Some have argued that the development of graft vasculopathy in the context of VCA transplantation represents underimmunosuppression [110]. In a recent report, a patient who was experiencing multiple episodes of acute rejection developed alloantibody production, which was controlled with conversion to belatacept [88]. In a study of upper limb transplantation in which immunosuppression reduction was trialed with the use of donor bone marrow infusion, there was a transient development of serum donor-specific antibodies (DSA) [118]. Antibody-mediated rejection is not very well described in VCA transplantation, although intragraft complement component 4d (C4d) deposition has been observed. Antibody is known to 'complement fix', that is to activate the complement cascade. Consequences of the proteolytic cascade that results from complement activation include the production of molecules that assist in the chemoattraction of leukocytes to inflammatory sites, the osponization of cells, the facilitation of antigen presentation and T-cell activation, and the formation of the membrane attack complex, which induces target cell lysis. However, C4d deposition has been observed in a high number of cases of VCA transplantation where there has been no correlation with poor graft function or other histological markers of rejection [49,119–121]. C4d deposition is therefore of limited value in the diagnosis of chronic allograft dysfunction. The presence of DSA in SOT has long been known to be associated with poor long-term outcomes and predictive of chronic dysfunction [122]. In VCA transplantation, DSA has been detected in only a few VCA recipients, and even then it has not correlated with C4d deposition [49,119]. Nevertheless, a recent report has highlighted a case whereby the presence of DSA was associated with B-cell associated rejection in a patient with a double forearm transplant, in which rituximab therapy successfully reversed the rejection event [123]. In a case from Louisville, DSA was detected after removal of the upper limb VCA [114], which may indicate that before amputation the antibody could have been bound to the allograft, although it is also possible that this is related to cessation of immunosuppression 4 days prior to DSA detection. Very few experimental studies have investigated DSA in the context of VCA transplantation, although models are now emerging. For example, in a model of rat hindlimb transplantation, the presence of preformed DSA has been shown to correlate with accelerated rejection [124].

Lymphoid neogenesis is a phenomenon that is strongly associated with chronic allograft dysfunction in SOT. Recently, there have been reports of this developing in VCA transplants and concerningly following antibody-mediated rejection [123,125]. This observation has been supported by data from nonhuman primate and rodent models in which peripheral node addressin expression, a marker of lymphoid neogenesis, was present and correlated with antibody-mediated rejection [125].

Conclusion

Vascularized composite allograft transplantation shares many features with the immune processes that result in SOT destruction. However, there are also a number of distinctive immunobiological features in VCA transplantation that result in a unique immune response post-transplantation. These features include the characteristics that make skin an important immunological barrier, such as a high density of resident leukocytes and special microarchitectural and immune-activating features pertaining to the microvasculature. VCA transplants also contain multiple tissues and are often large in size, thus impacting the immune response on multiple levels. For example, a specific immune response develops against skin and its adnexal structures, while the immune process that develops with the transplantation of bone marrow may differ. Examining these unique features reveals a number of potential targets for immunotherapy. These include modification to the donor to prevent or reduce the IRI, reduce the production of skin alarmins, protect the potentially beneficial transplanted bone marrow, and ensure that the transplant is not damaged by other traumatic or endogenous immunopathologies. Potential techniques that may be explored in the recipient include methods to prevent the early activation of innate immune responses post-transplantation, abrogation of the activation and recruitment of lymphocytes within the microvasculature of the skin, as well as control of lymphoid neogenesis. The ability to view and histopathologically assess VCAs or their sentinel flaps is a particular advantage in their monitoring as it provides the opportunity for early intervention in the event of rejection. In addition, VCAs are a uniquely exposed to allow access for topical immunotherapeutics. These features coupled with skin's exceptional susceptibility to rejection make VCA transplants clear candidates for the evaluation of novel transplant immunotherapeutics. While a great deal remains to be understood, including the appreciation of what constitutes chronic allograft dysfunction and the mechanisms responsible for its development in VCA transplants, the field has made great strides both clinically and experimentally over the past two decades.

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Conflicts of interest

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