REVIEW ARTICLE

Composite tissue allotransplantation: opportunities and challenges

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Vascularized composite allotransplants (VCAs) have unique properties because of diverse tissue components transplanted en mass as a single unit. In addition to surgery, this type of transplant also faces enormous immunological challenges that demand a detailed analysis of all aspects of alloimmune responses, organ preservation, and injury, as well as the immunogenicity of various tissues within the VCA grafts to further improve graft and patient outcomes. Moreover, the side effects of long-term immunosuppression for VCA patients need to be carefully balanced with the potential benefit of a non-life-saving procedure. In this review article, we provide a comprehensive update on limb and face transplantation, with a specific emphasis on the alloimmune responses to VCA, established and novel immunosuppressive treatments, and patient outcomes.

Cellular & Molecular Immunology (2019) 16:343–349; https://doi.org/10.1038/s41423-019-0215-3

INTRODUCTION

Vascularized composite allotransplants (VCAs) are grafts that are composed of multiple different tissues but are transplanted together as a single unit. A typical example is a hand graft, which consists of muscles, skin, bone, vessels, and nerves. VCAs have gained substantial clinical attention in recent years, especially for patients with injuries that are beyond repair by routine plastic surgery. Unlike solid organ transplants (e.g., liver and heart transplants), VCAs are considered a life-enhancing, rather than a life-saving, procedure, so they face a unique set of concerns and challenges in the field. To date, more than 100 upper extremities, approximately 40 faces, and, more recently, successful genito-uterine transplants have been performed worldwide.[1,2] However, there are many issues in the field that deserve immediate attention. Single-center experiences in limb and face transplants are usually small, and the analysis of immune responses remains limited. Moreover, there are individual tissue components in hand and face transplants with strikingly different features and functions. Thus, alloimmune responses against VCA grafts, either acute or chronic, may have very different presentations, especially responses against individual tissue components (e.g., skin tissue versus the bone in the same graft). Additionally, the adaptation of numerous nerves in the VCA grafts and their functional reconstitution from transplant recipients may have an additional impact on graft outcomes. It appears necessary to standardize the analysis of immunologic features by integrating the functionality of different tissue components in VCA grafts. Importantly, a better understanding of the immune responses in VCA may help utilize immunosuppressive drugs in a more tailored fashion.

IMMUNOLOGICAL CHALLENGES OF THE SKIN

Skin is a major component of all VCA grafts, and unlike other tissue components, the skin is proven to be extremely immunogenic. In fact, acute rejections occur in ~80% of all face and upper limb transplants during the first year. Notably, the incidence of acute rejections in renal allografts is considerably lower, approximately 10% among kidney transplant patients.[3–5] In both clinical and experimental models of VCA, a split tolerance phenomenon has been reported, in which rejection of the skin, but not other components such as muscle or bone, was observed.[6] In contrast, neither experimental nor clinical experience has been able to confirm the rejection of muscle tissue in the absence of skin rejection,[7] highlighting the specific and high immunogenic properties of the skin tissue in VCA grafts.[8–10]

It has been shown that a significantly higher number of T cells reside in the skin than in the peripheral circulation.[11] In addition, there is a greater representation of T cells with an effector memory phenotype in the skin.[11–13] Skin-resident memory T cells bear a diverse T-cell receptor repertoire and have a characteristic Th1 phenotype.[11] During inflammation, memory T cells can recruit other key players of innate and adaptive immunity. Skin-resident T cells have the capacity to assume immune responses independent of chemotactic activity.[13] Moreover, dermal dendritic cells (DCs) and other antigen-presenting cells (APCs) in the skin can present antigens to skin resident memory T cells directly,[14,15] resulting in their activation and effector differentiation.[16] This observation provides a novel view to the existing dogma that memory T cells need to be recruited from the circulation to the site of inflammation upon inflammatory stimuli.[17] Characteristically, an abundance of CD8+ memory T cells in the skin of VCA have been detected.[5,18] Previous studies have considered resident

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Received: 18 January 2019 Revised: 4 February 2019 Accepted: 5 February 2019
Published online: 6 March 2019

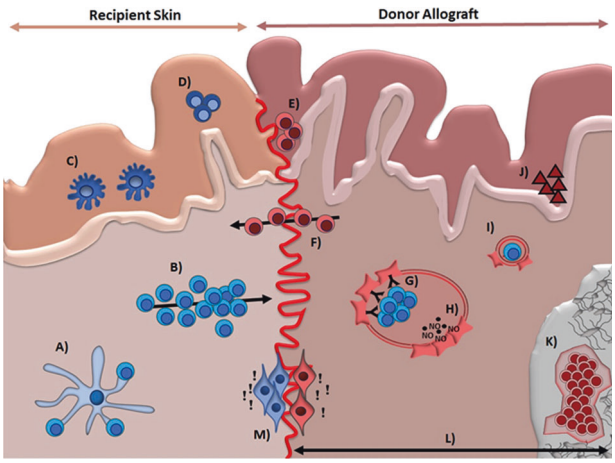

Fig. 1 Unique immunological properties of the skin affecting the rejection process. **a** Skin-resident antigen-presenting cells can directly activate tissue resident memory T cells (TRM) without the need for lymph node homing. **b** Recipient allogeneic T cells migrating to the transplanted allograft are the main driver of graft rejection in vascularized composite allotransplant (VCA). **c**) Dendritic epidermal T cells are recruited early from the blood upon tissue damage. **d** Memory Th9 T cells are skin trophic and produce copious amounts of tumor necrosis factor-alpha and granzyme B. **e** Injury-induced inflammation occurs in VCA grafts. TRM invasion at the border between allograft and recipient tissues is shown along with high levels of interleukin-1b, interferon-gamma, transforming growth factor-beta, and CCL2-5. **f** TRM, especially CD8+ T cells, are abundant in the skin. Donor-derived passenger TRM can migrate to surrounding recipient tissue, causing graft versus host disease that may, in turn, contribute to the rejection process. **g** Endothelial cells can activate lymphocytes through the upregulation of human leukocyte antigen-II and adhesion molecules. **h** Endothelial cells secrete vasoactive molecules, including NO, bradykinin, and prostacyclin, affecting the inflammatory process. **i** The width of capillaries within the skin is narrower than the diameter of T cells, leading to contact with molecules upregulated by endothelial cells. **j** Skin-specific antigens such as Epa-1 and Skn-1 and 2 contribute to the augmented immunogenicity of the skin. **k** Bone marrow in VCA grafts may ameliorate/modulate rejections. **l** Size and mass of VCA grafts may interfere with alloimmune responses. **m** Skin alarmins, released by keratinocytes upon cell death, have chemoattractive abilities. Thus, both adaptive and innate immune cells are recruited to the site of tissue damage

memory T cells the cause of graft versus host disease (GVHD), mainly due to their activation by infiltrating recipient APCs. Notably, GVHD has been detected in dog[19] and pig[20] models of VCA in combination stem cell transplants.

It should be noted that recipient T cells play a major role in skin allograft rejection. They are activated directly upon recognition of the allogeneic major histocompatibility complex (MHC) antigens presented by donor-derived APCs. Of additional relevance is the indirect allorecognition of processed donor antigens presented by the recipient's APCs.[21,22] In terms of T effector cells, memory Th9 T cells might play a special role in skin rejection, as those cells have been characterized as skin trophic with the capacity to produce tumor necrosis factor-alpha (TNF-α) and granzyme B.[17,23] Furthermore, a subset of specialized dendritic epidermal T cells that reside sparsely in human skin represents the first subset of immune cells recruited from the blood into the skin upon immune activation.[7,24]

Properties of the skin itself seem to play a role in VCAs, thus contributing to increased rejection rates. Interestingly, the microvasculature within the skin has the capacity to induce immune responses.[25,26] Endothelial cells of the skin have unique properties to recruit and activate immune cells through the upregulation of MHC class II molecules, inflammatory mediators, adhesion molecules, and vasoactive and costimulatory molecules.[27–32] Notably, the width of capillaries within the skin is narrower than the diameter of T cells, leading to endothelial cell-T cell contact.[25] This mechanism may cause proinflammatory responses after T-cell infiltration, even in the absence of allorecognition.[25,33] Moreover, specific areas of the skin may differ in their capacity to mount immune responses. In an experimental model, it was shown that the infiltration of immune cells and cytokine expression were dependent on allograft properties, with hair-bearing areas showing augmented expression of interleukin (IL)-4, GRO-KC, and monocyte chemoattractant protein-1 in a multicytokine assay.[34]

The proportion of bone marrow as part of VCA grafts seems to ameliorate rejection processes, with experimental models showing an enhanced acceptance of hemifacial allografts containing vascularized bone marrow.[35] In models of VCA flaps,[36] however, increasing graft size in the absence of bone marrow has been linked to enhanced rejection. Skin-specific antigens, which are prevalent in the epidermis, may also play a role. Epa-1, a minor histocompatibility antigen, has been identified as a target of cytotoxic T cells[37] that causes ulcerative skin lesions and nonspecific tissue damage when injected into Epa-1-positive mice.[38]

One interesting observation is that direct exposure of hand or face transplants to the environment may provoke rejections. Exposure to cold temperatures during winter months in one patient was linked to reoccurring, histologically confirmed rejections.[39] Low humidity and cold temperatures may impair skin barrier function while increasing immune cell chemotaxis.[40] A seasonable increase in circulating lymphocytes and immunoglobulins may be of additional relevance.[41] (Fig. 1)

VCAS: A COMPLEX INTERPLAY BETWEEN INNATE AND ADAPTIVE IMMUNITY

As in solid organ transplants, innate and adaptive immune responses are initiated during transplantation. In an ex vivo model of sterile skin injury, a sevenfold increase in inflammatory cytokines has been observed, followed by augmented recruitment of DCs and T cells, thus initiating the proliferation of CD4+ and CD8+ T cells.[42] The skin hosts a large number of plasmacytoid DCs that have the capacity to promote IL-17A and IL-22 production, mainly through increased IL-6 secretion upon skin injury.[43,44] Moreover, skin-specific DCs may present apoptotic cell-associated antigens that promote CD8+ T-cell cytotoxicity.[45,46] In studies that dissected the role of DCs in injury-induced adaptive immune responses, it was shown that skin grafts from K5mOVA mice transgenic for OVA in the skin displayed accelerated rejection, with augmented activation of CD8+ T cells in draining lymph nodes.[47] Notably, CD8+ T-cell responses in draining lymph nodes were significantly diminished in a CD8+ DC knockout mouse, supporting a key role for CD8+ DCs in the rejection process.[48] Moreover, keratinocytes sorted using laser capture microdissection from human skin displayed an upregulation of CCL20 and IL-23A.[49] CCL20 has chemotactic effects for CCR6-positive T cells[50] and immature DCs, recruiting them from the periphery to sites of inflammation.[51] Elevated levels of CCL20 have also been detected in the skin of psoriasis patients.[52] IL-23A secreted by DCs and macrophages residing in the skin activates Th17 cells that play a critical role during allograft rejection.[53–55] Comparing syngeneic and allogeneic VCAs,[56] CD8+ T cells infiltrated allogeneic groin flaps and accumulated close to the recipient-graft border where tissue damage was most prominent. Both syngeneic and allogeneic skin grafts revealed distinct damage-related immune responses, as shown by the upregulation of IL-1b, interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), and IL-10, while the expression of TNF-α, IL-18, and several other cytokines has been linked to the response to allogeneic skin grafts.[56]

Ischemia reperfusion injury, an inevitable part of the transplant process, causes the release of damage-associated molecular patterns (DAMPs), with the subsequent activation of toll-like

receptors fueling allograft rejection.[57,58] Skin alarmins, endogenous DAMPs that include IL-1a, IL-33, and several heat shock proteins, are released by keratinocytes and leukocytes subsequent to ischemia reperfusion injury and have chemoattractive abilities to recruit and activate leukocytes of the innate immune system.[59–61] Insufficient lymphatic drainage after VCA is likely to further promote rejection processes, as impaired lymphatic drainage may activate DC and T-cell trafficking.[7,62] Interestingly, when analyzing subcutaneous fat in a mouse model of reduced lymphatic flow, increased inflammation and fibrosis[63] have been observed.

As in solid organ transplants, prolonged ischemic times have been shown to negatively impact VCA outcomes. In a mouse orthotopic hindlimb transplant model, for example, prolonged ischemic times have been associated with diminished graft survival.[64] Notably, tolerable ischemic times have not been established for VCA grafts. Utilizing standard preservation methods, cold ischemic times up to 6 h have been cited as an upper threshold in VCAs.[65,66] Ex vivo perfusion systems, including a hyperbaric, normothermic perfusion system, have recently been explored for VCAs and showed delayed acute rejection processes of VCA.[67]

CHRONIC REJECTION: A SIGNIFICANT CHALLENGE IN VCA RECIPIENTS

Chronic rejection was thought to be a rare event in VCAs. Notably, observation times after VCAs are considerably shorter than those after solid organ transplants, and more recently, chronic rejections have also been reported in face and hand transplantation.[68–71]

In some patients, antibody-mediated processes[72] and the formation of tertiary lymphoid organs[73] in skin biopsies have been demonstrated. More recently, epidermal thinning and sclerosis have been linked to subclinical inflammation and rejection.[74] At the molecular level, proteins of the AP-1 family are also considered to play a role in promoting chronic rejection through collagen accumulation, a process that has been observed for chronic rejection in both solid organ transplants[75] and VCAs.[69] Clinical histological grading for chronic rejection in VCA remains preliminary and does not include vessel vasculopathy, loss of capillaries, or rejection of the oral mucosa.[65,76] An integrative approach involving histological findings and the underlying mechanisms may be helpful in characterizing the conditions.[74] Recurrent and insufficiently treated episodes of acute rejection may also contribute to chronic rejection in both solid organ transplants[77,78] and VCAs.[79] This aspect appears relevant, since nearly 50% of all VCA patients undergo multiple rejection episodes.[4] Moreover, the frequency of infections also seems to play a role in chronic VCA rejection.[80,81]

The premature aging appearance of grafts combined with telangiectasia and mottled leukoderma at suture lines, as well as a reduction of hair follicles, sweat glands, and nerve endings, has also been observed clinically in VCA recipients.[71,74] Pathological mechanisms may include sclerotic induration, epidermal thinning, hyperkeratosis, and vessel wall alterations; these changes are often associated with fibrosis or luminal occlusion and collagen type 1 deposition.[71,74] It is currently unclear whether the premature aging appearance of grafts represents an aspect of chronic rejection or an entirely different pathophysiology.

IMMUNOSUPPRESSION: BALANCING RISKS AND BENEFITS

As a life-enhancing rather than a life-saving procedure, intense immunosuppression in VCA patients represents a delicate balance. Compliance in taking immunosuppressive drugs is as important in VCA as it is for solid organ transplants.[82,83] Most VCA centers utilize immunosuppressive protocols for VCA patients based on experience with conventional immunosuppression used in solid organ transplant patients.[10] Approaches include an induction treatment with a polyclonal antithymocyte antibody to deplete T cells and a maintenance triple immunosuppression protocol with tacrolimus, mycophenolate mofetil (MMF), and steroids.[5,10] VCA patients often experience high rates of acute rejections, and rescue therapies include pulsed steroids.[5] The side effects of immunosuppression in VCA patients are comparable to those in solid organ transplant patients. The increased risks for malignancies[84] include Epstein-Barr virus (EBV)-related post-transplant B-cell lymphomas in facial transplant patients[85] and squamous cell, lung, colon, and Non-Hodgkin Lymphoma cancers,[86,87] in addition to opportunistic infections, such as cytomegalovirus (CMV), EBV, herpes simplex virus, and *Pneumocystis jirovecii*.[81] CMV infections occur most frequently by months 2–6, with viremia arising in nearly every seropositive donor into a seronegative recipient constellation.[88] In some cases, CMV infection has been linked to acute graft rejections.[80]

Bacterial infections have been frequently observed in VCA recipients.[89,90] Since the environmentally exposed donor skin is transferred with VCAs, its potentially pathogenic flora consisting of Gram-negative organisms, *Staphylococcus aureus*, fungi, and anaerobes may play a role. The mucosal tissue of facial VCAs exposes the recipient to donor-derived pathogens, including streptococci, *Candida* species, and anaerobes.[91] Thus, nasal cultures from VCA recipients displaying methicillin-sensitive *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Pseudomonas pneumoniae* have been reported.[88] Fungal infections at surgical sites have been shown once recipients continue with daily activities exposing them to the environmental flora.[91] However, the incidence of invasive candida infections has been low.[92] Opportunistic infections specifically associated with face transplantation include superinfected sialocele and parotitis due to remaining donor salivary gland tissue, which can be successfully treated with botulinum toxin injections.[93] Clearly, VCAs as non-life-saving procedures require balanced and effective immunosuppression.

Several groups have tested minimization protocols after VCA. Relying on the benefits of steroid-free immunosuppression in solid organ transplants, dual immunosuppression with tacrolimus and mycophenolate after alemtuzumab induction has been tested. This approach, however, has been associated with frequent acute rejection episodes.[94] In another clinical series, dual immunosuppression with tacrolimus and MMF was successful when tacrolimus trough levels had been maintained at >5 ng/ml. Nephrotoxic side effects have been more prominent in VCA recipients subsequent to steroid withdrawal.[95,96] Moreover, steroid-free maintenance immunosuppression in bilateral arm transplant recipients had been linked to intimal hyperplasia, suggesting that underimmunosuppression may contribute to the development of vasculopathy.[94,97]

Costimulatory blockade may be a promising addition to maintenance immunosuppression, with potential effects on donor-specific antibodies[98] while sparing nephrotoxic side effects.[99] While beneficial in some VCA recipients, others developed acute rejections under maintenance immunosuppression with belatacept and tacrolimus monotherapy.[100] CD57+ memory T cells lacking CD28 made up a significantly higher proportion in rejecting recipients, suggesting that screening patients for this T-cell subpopulation may be helpful prior to belatacept treatment.[100]

Topical application of immunosuppression drugs with reduced systemic side effects has been applied successfully in face and upper limb transplantation.[101] Lower-grade rejections (Banff grades 1–2) have been successfully treated with topical tacrolimus and clobetasol.[102] Interestingly, preclinical studies have shown superior effects of topical compared to systemic immunosuppression in some cases.[101] A dichotomous response upon topical tacrolimus treatment has been observed in rats receiving hindlimb transplants; half of the animals rejected the graft after 70 days,

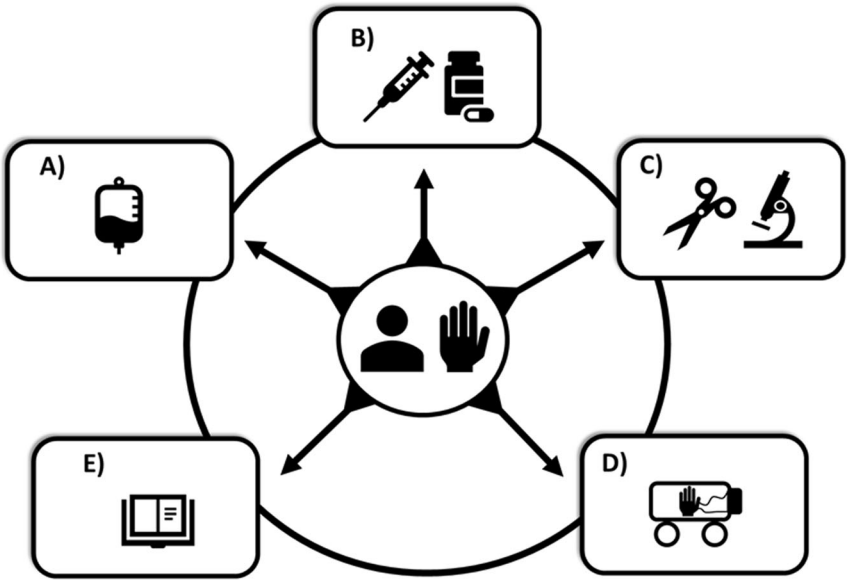

Fig. 2 Novel approaches in improving vascularized composite allotransplant outcomes. **a** Tolerance protocols involving regulatory T cells or mesenchymal stromal cells. **b** Immunosuppression minimization protocols, including topical intragraft application of immunosuppressive drugs (e.g., tacrolimus/mycophenolate mofetil). **c** Minimally invasive microsurgical techniques. **d** Novel preservation approaches involving ex vivo hypo- or norm preservation. **e** Refined rejection criteria and guidelines to assess outcomes

similar to untreated controls, while the other half did not show any signs of graft rejection 200 days post transplantation and had significantly lower pathological injury once assessed.[101] Topical high-dose application has not been linked to augmented systemic side effects,[101] encouraging VCA-specific immunosuppression.

Most recently, a formulation for topical MMF based on the ester prodrug mycophenolic acid was developed, currently allowing the simultaneous topical application of MMF, Tac, and steroids.[103] Acute rejections have been treated in most cases with steroid bolus administration; topical immunosuppression and an augmentation of maintenance immunosuppression have also been successful;[104] and rare steroid-resistant rejections require polyclonal antibodies (anti-thymocyte globulin).

TOLERANCE INDUCTION PROTOCOLS: OPPORTUNITIES IN VCA

As VCAs are not life-saving transplants and current immunosuppression protocols are lifelong and often produce debilitating side effects in transplant patients, the advantage of immune tolerance in VCA patients is obvious. There are several ongoing tolerance-inducing trials that may benefit VCA patients. Regulatory T cells (Tregs) may represent an opportunity for VCAs.[105,106] Through the perforin-dependent lysis of effector T cells, the secretion of immunosuppressive cytokines, including IL-10, IL-35, and TGF-β, and the deprivation of IL-2 through the self-expression of high-affinity IL-2 receptors, Tregs may ameliorate alloimmune responses by inhibiting T effector cells.[107] Tregs specific for donor antigens generated through priming with DCs derived from donor skin[108] represent promising candidates for alloantigen-specific immunosuppression.

Augmenting autologous Tregs in vivo may be an alternative approach. Experimentally, injections of the IL-2/anti-IL-2 complex increased murine Treg numbers 10-fold,[109] leading to prolonged orthotopic forelimb allograft survival, especially when combined with rapamycin.[110]

Injecting hIL-2/Fc fusion protein, a long-lasting form of IL-2, not only augmented the number of Tregs but also improved suppressive capacities on effector T cells specific for donor antigens in coculture experiments. Notably, responsiveness toward third-party antigens remained intact, indicating a functional immune response.[111] Subsequently, measuring FoxP3, GymB, IFN-γ, and Prf1 allowed a prediction of rejection episodes under hIL-2/Fc, ALS, and CsA treatment, enabling the reduction of immunosuppression.[111]

Injections of donor-derived allogeneic mesenchymal stromal cells after irradiation therapy causing a state of chimerism in recipients have been shown to improve allograft survival of a pig hindlimb.[112] In a pig hemifacial model, prolonged allograft survival upon repetitive high-dose application of bone marrow-derived mesenchymal stem cells (MSCs) has been reported.[113] Immunosuppression has been reduced to tacrolimus monotherapy in a hand transplant model infusing donor bone marrow after lymphoid depletion.[114] MSC infusion may also impact neural regeneration, with improved clinical and electrophysiological outcomes.[115–117] Drug interferences between MSCs and immunosuppressants are also relevant,[118] as rapamycin and tacrolimus antagonize some of the immunomodulatory effects of MSCs.[119]

The induction of chimerism in transplant recipients based on T-cell depletion and full body irradiation combined with hematopoietic cell transplantation has shown promising results in a VCA animal model.[120] GVHD induced by donor bone marrow infusion, although a theoretical complication,[17] has thus far not been reported.[121,122]

CONCLUSIONS

VCAs represent a unique procedure that has unexpected clinical needs; VCAs are life-enhancing for those with irreparable injuries but also come with enormous challenges. Although the current immunosuppression protocols in VCAs are effective in suppressing acute rejection, they produce significant side effects in transplant patients, and the drug-induced toxicity profiles are comparable to those in solid organ transplants. In some patients, however, minimization of immunosuppression protocols has been successfully applied, but the long-term outcomes of those patients remain unclear and require careful follow-ups.

As in other transplants, chronic graft vasculopathy also occurs in VCA patients, and the mechanisms remain to be defined. Another intriguing aspect of VCA patients is the observation of the accelerated aging of VCAs. This phenomenon may reflect aspects

of chronic vasculopathy or a yet defined process of "true" accelerated aging. Modern approaches for immune tolerance have been tested in experimental and clinical approaches. While some VCAs may have unique immunological properties with the concomitant transplantation of bone structures, where bone marrow cells may have protolerant features, the field overall is hampered by small numbers of transplants and a lack of clinical consortia (Fig. 2). Clearly, VCAs offer great opportunities in further dissecting the alloimmune responses that not only may facilitate improved treatments of transplant patients but may also help understand aspects of dermatological diseases.

ACKNOWLEDGEMENTS

Dr. Stefan G. Tullius currently serves as an Einstein-BIH visiting fellow. This study was supported by the Einstein-BIH Visiting Fellow Program (to S.G.T., M.M. and I.M.S.) and the Biomedical Education Program (BMEP) of the German Academic Exchange Service (DAAD to J.I.).

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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