Towards Skin Tolerance in Vascularised Composite Allografts

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ABSTRACT

Despite more than twenty years of clinical experience since the first successful human hand transplant in 1998, the mechanisms underlying acute rejection in such vascularised composite allografts (VCAs) remain poorly understood. To further compound the problem, the reporting of patient data, treatment protocols, and definition of VCA rejection remains in constant flux. On the other hand, chronic rejection, which was previously thought to not affect VCAs, has now been accepted and recognised as both a reality and likely eventuality for VCA patients, with no realistic exit plan beyond re-transplantation currently.

Between 2015 and 2017, I served as a post-doctoral research fellow at the VCA Laboratory of the Center for Transplantation Sciences, at the Massachusetts General Hospital (MGH), a teaching affiliate of Harvard Medical School in Boston, Massachusetts, USA to work on the development of immunologic tolerance protocols to VCA in both swine and non-human primate (NHP) pre-clinical VCA models. During this time, I was also a member of the clinical VCA service at MGH where I participated in the conduction of clinical VCA trials. I have maintained research collaborations with the same group since.

Data from the International Registry of Hand and Composite Tissue Transplantation (IRHCTT) have shown that more than 85% of VCA patients develop at least one episode of acute VCA rejection within the first year, compared to approximately 10% of kidney transplant patients despite the use of highly similar clinical immunosuppression regimens. These differing observations led me to hypothesise that acute rejection might develop within the dermis, due to the vicinity of the subdermal plexus and abundance of skin immune cells, before presenting clinically on the surface of VCA skin. Therefore, this thesis is based on the enclosed publications which span the gamut of clinical, basic science and translational VCA research. By targeting VCA dermis, the primary objective is successful avertion of acute rejection, with the secondary objective of allowing mixed chimerism to develop following tolerance induction through delayed donor bone marrow transplantation (DBMT) in a NHP VCA model, based on clinical trials in renal transplantation patients at MGH that successfully achieved immunosuppression withdrawal.

The first two papers (#1 and #2) in this thesis provide the clinical backdrop to the most pressing problems in VCA at present – acute and chronic rejection – for which we as a field still do not have definitive solutions to. Naturally, topical immunosuppression rapidly emerged as an attractive means of addressing acute rejection in VCA given the exteriorised skin component as compared to the typical, intra-abdominal location of solid organ transplant (SOT) allografts. It was theorised that such an approach would not only

allow earlier identification and treatment of acute rejection, which presumably would reduce the risk of progression to chronic rejection, but also potentially allow a reduction in the overall immunosuppressive load. In turn, the risk of systemic complications to the VCA recipient would also be lowered.

The above approach was explored in NHPs in the next two papers, initially in allogenic skin grafts (#3) (i.e. neovascularised) followed by VCAs, which are primarily vascularised (#4). Concurrently, ongoing research from the VCA Laboratory at MGH using a swine model suggested that rather than transient mixed chimerism (which was sufficient for renal transplantation), VCAs would require stable mixed chimerism instead. This could potentially be achieved through the DTIP approach which was successful in NHP lung transplantation at MGH. As well, analysis of swine VCA skin correlated remarkably well with MGH's clinical hand transplant patient following transplantation (#5).

Therefore, the DTIP approach was trialed in a NHP VCA model next (#6) where chronological analysis of VCA skin both in the short- and long-term reflected our clinical observations (in Papers #1 and #2) but ultimately, tolerance was not achieved due to the persistence of acute rejection. With knowledge of results from #3 and #4, the topical approach was re-engineered for local immunosuppression delivery at the subcutaneous level through a collaboration with engineering colleagues from Rutgers University in New Jersey, USA. Subsequently, acute rejection was successfully mitigated when FK506-loaded discs were implanted subcutaneously during the VCA procedure, and mixed chimerism could be induced through DTIP using the same NHP VCA model. Further work is required however, due to the persistent, premature development of post-transplant lymphoproliferative disorder, which may be an experimental and/or NHP species-specific issue.

Overall, this submission of seven papers has introduced the barriers in achieving tolerance to VCAs clinically, and described the laboratory efforts undertaken to reproduce, within ethical limits and animal welfare considerations, these immunological challenges in a robust, pre-clinical NHP VCA model. Most strikingly, the clinical observations and anticipated problems were accurately replicated in the NHP VCA studies. Potential mechanistic insight of acute rejection via VCA dermis was derived, and targeted through further studies to achieve successful proof-of-concept for the mitigation of acute rejection and ultimately, development of mixed chimerism.

CONTENTS

LIST OF RELEVANT PUBLICATIONS

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DECLARATION

I hereby declare that I have personally undertook the research projects, performed the relevant experiments, interpreted the results, drafted, edited and submitted the accompanying peer-reviewed published papers arising from my post-doctoral research fellowship (2015-2017) and ongoing research collaborations since with the Vascularized Composite Allotransplantation Laboratory of the Center for Transplantation Sciences at the Massachusetts General Hospital, a teaching affiliate of Harvard Medical School in Boston, Massachusetts, USA.

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DEFINITIONS/ABBREVIATIONS

ATG	Anti-Thymocyte Globulin
ATGAM	Equine ATG
DBMT	Donor Bone Marrow Transplantation
DCD	Donors after Cardiac Death
DSA	Donor Specific Antibodies
DTIP	Delayed Tolerance Induction Protocol
FK506	Tacrolimus
GV	Graft Vasculopathy
HLA	Human Leukocyte Antigen
mAb	Monoclonal Antibodies
MGH	Massachusetts General Hospital
MHC	Major Histocompatibility Complex
MMF	Mycophenolate Mofetil
NHP	Non-Human Primate
PRA	Panel Reactive Antibodies
PTLD	Post-Transplant Lymphoproliferative Disorder
SCS	Static Cold Storage
SNMP	Sub-Normothermic Machine Perfusion
SOT	Solid Organ Transplantation
SZNF	Sub-Zero Non-Freezing
UK	United Kingdom
USA	United States of America
VCA	Vascularised Composite Allograft

EXPLANATORY ESSAY

1. INTRODUCTION

Vascularised composite allografts (VCAs) represent the latest frontier in the fields of plastic surgery and transplantation. Upper and lower extremity, craniofacial, abdominal wall, and even genitourinary VCAs have all been performed with highly promising functional outcomes, aesthetic restoration and psychosocial rehabilitation following devastating soft tissue loss from trauma, amputation, oncological resection and even congenital absences that are not amenable to conventional, autologous reconstructive options. While the type of, and indications for such reconstructive transplantation through VCA using plastic surgical techniques such as microsurgery of the vessels and nerves, and osteosynthesis of the upper limb and craniomaxillofacial skeleton continue to evolve, as with any transplant, maintenance immunosuppression becomes obligatory for the rest of the VCA patient's life (Weissenbacher *et al.*, 2018).

Unsurprisingly, there are a myriad of associated risks and complications from such life-long immunosuppression including opportunistic infections, metabolic derangements, reno-vascular disturbances and even neoplastic processes. Moreover, despite the use of such powerful immunosuppression, VCA, like solid organ transplantation (SOT), are at an even higher risk of graft loss, likely due to the skin component which is highly antigenic (Petruzzo *et al.*, 2010, 2017; Petruzzo and Dubernard, 2011).

At Massachusetts General Hospital (MGH), pioneering clinical trials on immune tolerance through the mixed chimerism strategy via donor bone marrow transplantation (DBMT) have enabled complete immunosuppression withdrawal in renal transplant patients (Kawai *et al.*, 2013) and this approach has since been replicated with varying degrees of success at Northwestern and Stanford (Elias, Cosimi and Kawai, 2015) in the USA. Building on from such experiences, the induction of mixed chimerism was attempted, albeit with modifications, in a select number of clinical face and hand VCA patients in France (Dubernard *et al.*, 2007; Petruzzo *et al.*, 2015; Kanitakis *et al.*, 2016) and the USA (Schneeberger *et al.*, 2013) respectively. However, not only was there no clear evidence for the successful development of chimerism, some of these patients who were initially maintained on single immunosuppression with tacrolimus (FK506) have since required reinstatement of standard triple immunosuppression (FK506, mycophenolate mofetil (MMF), methylprednisolone) to maintain viability of their VCAs. Most tellingly, chronic rejection, graft loss and eventually, patient deaths have also occurred (Morelon *et al.*, 2017).

The VCA Laboratory at MGH has thus been working on developing mixed chimerism protocols in pre-clinical, large animal (swine and non-human primate (NHP)) VCA models. Concurrently, the laboratory is invested in studying the immunogenetics and immunobiology of transplanted skin because it is no longer a means of clinical assessment but a clinical goal (i.e. maintain rejection-free) at the same time for VCA patients. Based on previous NHP SOT tolerance studies at MGH (Koyama *et al.*, 2007; Nadazdin *et al.*, 2011; Y Yamada *et al.*, 2012), we know that without mitigation of acute rejection, the induction of mixed chimerism will not be successful, immunosuppression withdrawal becomes impossible, with ensuing chronic rejection and eventual, irreversible allograft loss. A similar fate would therefore await VCA.

More importantly, there remains much disconnect between clinical and histological rejection in VCA, which further confounds diagnosis and treatment (Cendales *et al.*, 2008; Schneider *et al.*, 2016). Therefore, this thesis is composed of my investigations into the possible mechanisms of VCA rejection from both clinical and laboratory NHP studies, as well as the development of therapeutic strategies in a robust, pre-clinical NHP VCA model for the induction of mixed chimerism as a means to achieve immune tolerance.

2. RELATIONSHIP BETWEEN STUDIES

While the various publications are presented separately, such a distinction is largely artificial as these studies were all essentially conducted concurrently. It should also be noted that for a mixed chimerism protocol developed in NHPs to be put forward into a clinical trial in VCA, it must be robust enough to withstand the most stringent of immunological challenges i.e. across a full major histocompatibility complex (MHC) mismatch barrier, to simulate unrelated donor-recipient transplant pairings.

The thesis submission begins with a review of clinical VCA cases performed in survivors of major burns (Paper 1), who are believed to present the strongest immunological barriers given the number and extent of prior treatments. Such procedures include allogeneic skin grafting, blood product transfusions and dialysis etc all of which are considered highly sensitising. The relevant immunological factors identified include the extent of human leukocyte antigen (HLA) matching, levels of pre-transplant panel reactive antibodies (PRA) and donor-specific antibodies (DSA). Clinical outcomes such as the number of acute rejection episodes, development of chronic rejection and overall graft survival were analysed although no obvious relationship could be observed between these various factors, likely due to the paucity of clinical cases. These observations however, portend the challenges in successfully mitigating acute VCA

rejection. Using a similar approach, another review of all clinical VCA cases with a reported diagnosis of graft vasculopathy (GV) was conducted (Paper 2). While various definitions of chronic rejection in VCA have been proffered, the study was conducted on known cases of GV in VCA patients because in SOT, GV is inevitably associated with eventual graft loss. Different possible outcomes of GV were discussed based on the patient's biomarker status for C4d and donor specific antibodies (DSA) and an estimated time to development of GV was provided at approximately 6 years.

Together, the first two papers provide the clinical backdrop to the most pressing problems in VCA at present – acute and chronic rejection – for which we as a field still do not have definitive solutions to. Naturally, topical immunosuppression rapidly emerged as an attractive means of addressing acute rejection in VCA given the exteriorised skin component as compared to the typical, intra-abdominal or intra-thoracic location of SOT allografts. It was theorised that such an approach would not only allow earlier identification and treatment of acute rejection, which presumably would reduce the risk of progression to chronic rejection, but also potentially allow a reduction in the overall immunosuppressive load. In turn, the risk of systemic complications to the VCA recipient would also be lowered.

In collaboration with our engineering colleague at Rutgers University, topical immunosuppression (cyclosporine vs FK506) in the form of a daily gel dressing was devised. This was then trialed in NHPs that had surgically-created full thickness wounds treated first with xenogeneic, followed by allogeneic skin grafts (to simulate full thickness burns and treatment within ethical limitations for animal experimentation) (Paper 3), as a prelude to future VCA studies which necessitate more intensive research labour and logistics. While the systemic absorption of immunosuppression was not observed and supported such a topical approach, paradoxically, pre-treatment of the wound bed prior to skin grafting in the hopes of prolonging graft survival actually led to earlier graft loss. Further analysis revealed that there was a profound reduction in inflammation with topical FK506 (but not cyclosporine), which suggested potential impairment of neovascularisation and thus, successful take of the skin grafts.

As such, we sought to investigate whether topical FK506 administration would be able to mitigate inflammation and thus, acute rejection in *primarily vascularised* VCAs in NHPs (Paper 4). However, similar to the clinical VCA experience from select centres around the world (Diaz-Siso *et al.*, 2015; Chen *et al.*, 2020) that have investigated topical immunosuppression, our current formulation of topical FK506 failed to demonstrate efficacy. In fact, once systemic levels of immunosuppression were lowered beyond the target maintenance thresholds, VCA rejection ensued despite continued application of topical FK506. Pharmacokinetic studies in a human cadaveric upper limb demonstrated that in fact, the topical FK506

formulation was only delivered into the *epidermis*, which most likely accounts for the lack of systemic absorption observed. Therefore, if rejection still occurred despite epidermal penetration, the *dermis* would likely be the site of development of VCA rejection and warrant further investigation of such a therapeutic approach.

At this juncture, related experimental studies in the swine VCA model from our laboratory at MGH are reviewed to provide further insight to our ongoing work in NHPs (Paper 5). Results from swine studies suggest that unlike kidney transplantation, the induction of *stable* rather than *transient* mixed chimerism, would likely be necessary for successful immune tolerance to VCA. Recent NHP work in lung transplantation at MGH have also shown that by separating the SOT and DBMT processes (i.e. the delayed tolerance induction protocol (DTIP)), not only was acute rejection averted but *stable* mixed chimerism and thus immune tolerance was achieved successfully (Tonsho *et al.*, 2015). Mechanistic insight was provided through analysis of swine skin whereby there was progressive infiltration of recipient-type T cells that replaced donor-type T cells. Remarkably, the same observations were recorded in MGH's clinical hand transplant patient (Leonard *et al.*, 2021).

Given the above observations, the DTIP was trialed in our next NHP VCA experiment with further analysis of VCA skin leukocyte populations (Paper 6). Unfortunately, while successful in mitigating acute rejection in SOT, this was not the case for VCA, as alluded to previously (Papers 1 and 4). While our laboratory's swine studies had hinted at a possible role for the extent of MHC matching in rejection outcomes, this was not the case in NHPs and both haploidentical and fully mismatched recipients developed rejection chronologically within essentially the same time frame. *In vitro* analysis of NHP VCA skin then revealed that the infiltration and turnover from donor (i.e. VCA) to recipient (i.e. host) type skin leukocytes was rapid and complete within the dermis by approximately two weeks following transplantation. Most interestingly, this phenomenon was associated with sub-clinical rejection, which was diagnosed on surveillance biopsies, despite adequate maintenance levels of systemic immunosuppression. Subsequent tolerance induction through the DTIP then failed to develop mixed chimerism. Immunohistochemistry analysis of VCA samples from NHP subjects also revealed varying degrees of chronic rejection in the long-term, with the identification of GV and concomitant C4d staining, similar to clinical observations as described previously (Paper 2).

Finally, it was decided that the topical FK506 formulation required redesigning by our engineering colleagues at Rutgers University to specifically target VCA dermis (Paper 7). Through a series of experiments, FK506 delivery to the dermis was eventually optimised in the form of a subcutaneous

implant. While systemic absorption was unavoidable given the proximity of the subdermal vascular plexus within subcutaneous fat, this iteration of the FK506 implant achieved a few firsts in the field. Not only was acute VCA rejection successfully averted in NHPs prior to DBMT in the DTIP, thereby achieving the primary objective of this submission, it was also possible to maintain the VCAs rejection-free (both clinically and on histology) without the need for additional systemic immunosuppression (unlike in Paper 4). Most encouragingly, mixed chimerism was developed successfully by targeting VCA dermis, which was the secondary objective of this submission. Unfortunately however, the NHPs developed post-transplant lymphoproliferative disorder (PTLD) following DBMT and had to be terminated from the study due to animal welfare and ethical concerns. Nevertheless, successful proof-of-concept of this approach has been demonstrated and further studies will be required to optimise the DTIP and mitigate the development of PTLD, such as through the use of rituximab, prior to clinical translation in the form of tolerance trials in VCA.

3. PERSPECTIVE WITH CURRENT STATE OF KNOWLEDGE

Clinical VCA

In the ensuing years since the completion of my research fellowship, there have been several interesting clinical developments in the field of VCA. First, building on the success of the world's first paediatric bilateral hand VCA (Gálvez *et al.*, 2016; Gurnaney *et al.*, 2016), the next youngest patient in the world to have received a VCA was only 21 when she underwent facial transplantation at the Cleveland Clinic in the USA (Knackstedt *et al.*, 2020). Of note however, she was not already on immunosuppression unlike the former, which once again, raises the perennial debate between the risks of life-long immunosuppression and improvement in quality of life in VCA (Petruzzo *et al.*, 2010, 2017; Petruzzo and Dubernard, 2011). Second, while the majority of VCA cases have been performed in Caucasian patients in Western countries, the first facial VCA in a Black patient was only reported in 2019 in the USA (Kauke *et al.*, 2021). Concurrently, there have also been reports of hand VCAs performed in India between patients of different sex and hence, skin tone; interestingly, these transplanted allografts eventually took on the skin colour of the recipient (Mt *et al.*, 2021). More recently, combined hand and face VCA have been revisited and successfully performed (Berman *et al.*, 2021) whereas this was not the case previously (Carty *et al.*, 2013).

My research by comparison, has not been designed specifically to investigate these future, new potential variables in age, ethnicity, sex and "antigenic load" on the induction of mixed chimerism and thus immune tolerance in the transplant recipient as the range of clinical indications for VCA continues to

expand. The NHPs utilised in my studies were also all male and of adult-equivalent ages based on the success of historical transplant studies (in SOT) at MGH and certainly, the influence (or not) of the Y chromosome on mixed chimerism and immune tolerance is another variable to take into consideration for future clinical trials on immune tolerance in VCA. This may, in turn, require further investigation especially with the potential interest in performing penile VCAs for transgender women who identify as men (Selvaggi *et al.*, 2018) where a theoretical, superior outcome in erogenous and erectile function compared to the current autologous option of a phalloplasty with a prosthetic pump (Blecher, Christopher and Ralph, 2019) might be achieved based on observations and experience with improved peripheral nerve regeneration in VCA with FK506 (Glaus, Johnson and Mackinnon, 2011).

Most encouragingly however, the observation that the skin tone of a VCA eventually took on that of the recipients' lends credence and indirect evidence that our NHP findings of the near-complete turnover of skin leukocytes from donor- to recipient-derived is most likely accurate and valid, rather than the currently proffered explanation of sex hormone influence (Kanitakis, 2021), which would be hard to prove. The concept of "antigenic load" and its implications in VCA immunology will be expanded upon in the next section. Briefly, this idea developed from the initial failures of simultaneous hand and face transplantation in two patients – one died due to infective complications (Lantieri *et al.*, 2011) and the other required removal of the hand VCAs (Carty *et al.*, 2013).

On a separate note, the recent development of uterine transplantation (Brännström *et al.*, 2015; Brännström, Belfort and Ayoubi, 2021), while technically considered a type of VCA, represents an entirely different consideration to the work in this submission. For one, it does not contain a skin element, which raises many challenges with regard to clinical monitoring. Additionally, the transplanted uterus is not intended to remain for the rest of the patient's life, unlike that of a hand or face VCA, and is typically removed (i.e. hysterectomy) following successful IVF, conception and delivery of a baby, the intended goal. Other rarer forms of VCA include penile transplantation (as discussed above), as well as abdominal wall transplants (as part of multi-visceral SOT) in which VCA skin serves both as a clinical goal (in providing adequate wound coverage as part of the VCA) as well as a form of monitoring of the immune status of the underlying SOT (Barnes *et al.*, 2016; Gerlach *et al.*, 2016; Giele *et al.*, 2016).

Finally, the importance of robust, psychological screening prior to proceeding with VCA cannot be overemphasized nor underestimated – the first successful human hand transplant patient had his VCA removed just a year later after defaulting on immunosuppression; similarly, one of China's face transplant patients could not afford his medications and resorted to herbal supplements instead (Petruzzo and

Dubernard, 2011). While it is well-accepted that SOTs are life-saving and VCAs life-enhancing, a key psychological difference is the chronicity of the underlying illness in the former, and typically a preceding, traumatic event in the latter within a relatively short timeframe (Smith and Cendales, 2019). Therefore, potential SOT patients have usually reached a stage where transplantation may remain the only option in order to survive. In contrast, potential VCA patients are typically otherwise healthy, so their response to transplantation may be unpredictable without prolonged, antecedent experience of chronic illness. Indeed, the lack of standardised psychological assessments of potential VCA patients has been recognised since the 2014 Chauvet workshop (Jowsey-Gregoire *et al.*, 2016), and persists even up to 2017 at the International Society of VCA meeting where the only mention of such was "involve conducting a comprehensive battery of tests and evaluations" (Rose *et al.*, 2019). In turn, this can influence the decision or not, for reimbursement of such procedures. To illustrate, while most VCA programmes depend on funding through clinical trials (Caplan *et al.*, 2019), the UK national hand transplant programme at Leeds, in comparison, has been commissioned by the National Health Service since 2016 to provide upper extremity transplants (Burdon *et al.*, 2020). It is also one of the few publicly-funded VCA programmes in the world. This is likely due, in part, to the existence of a strong psychology team.

VCA Immunology

In the current submission, the DTIP in NHP VCA studies was based on the recent success of MGH laboratory studies which separated the SOT (lung) procedure and DBMT (Tonsho *et al.*, 2015). The underlying concept is the belief that separation of the SOT and recipient conditioning processes, both of which are highly pro-inflammatory, have previously been shown to negate the reproducibility of successful mixed chimerism and thus, tolerance induction when transplantation and DBMT were performed contemporaneously i.e. "day 0" (Yohei Yamada *et al.*, 2012). Logistically, such a "day 0" approach in VCA would be nigh impossible due to the attendant recipient conditioning required starting from at least two days prior, as well as the logistics required in real life such as the need for travelling from a different city or country even (Ben-Amotz *et al.*, 2018). With a DTIP (i.e. "delayed") approach, NHP SOT studies have shown that an optimal delay period was four months (Y Yamada *et al.*, 2012) but our clinical and NHP VCA studies in this submission suggest otherwise, probably due in part to the antigenicity of VCA skin. Additionally, it should be highlighted that the success of the DTIP in NHP lung transplantation studies involved the use of anti-CD8 (for memory T cell depletion) and anti-CD154 (for costimulatory blockade) monoclonal antibodies (mAbs) that are not currently clinically available (Tonsho *et al.*, 2015). Further NHP lung transplantation studies from MGH have since attempted to replace the above mAbs with the clinical reagents – thymoglobulin and belatacept – but unfortunately, not only was

this unable to replicate mixed chimerism, but led to a high incidence of PTLD (2 of 5 NHPs) (Sommer *et al.*, 2021).

In fact, the previous belief that T cell depletion with anti-thymocyte globulin (ATG) alone would be sufficient on induction has been shown to be unlikely through the data in this submission, which suggest that further targeting of B cell pathways would likely be required. Taking this into the context of the challenges of the DTIP where acute rejection seems inevitable despite clinically adequate trough levels of systemic immunosuppression, as well as "antigenic load", it can be appreciated that additional B cell depletion in future NHP studies would likely be the most strategic approach to achieve rejection-free survival during the delay period in order to provide the highest likelihood of success of subsequent DBMT and mixed chimerism induction. Data from MGH NHP SOT studies that incorporated rituximab suggest that it may be poorly tolerated however, with severe systemic side-effects (unpublished results) although this was not the case in human renal allograft recipients (Hotta *et al.*, 2018). Most encouragingly, New York University have reported, in three VCA patients with increasing levels of "antigenic load," that induction with *both* T and B cell depletion using ATG and rituximab respectively on "day 0" achieved clinical rejection-free survival of their VCAs for at least 9 to 24 months (Gelb *et al.*, 2018). Meanwhile, institutions worldwide continue to investigate various approaches to achieve the same, from infusing adipose-derived stem cells (Stivers *et al.*, 2017) to immune cells amongst others (Anggelia *et al.*, 2022), but with no clear winner in sight still.

Regulatory T cells, in particular, have gained much interest and traction in recent years, following successful phase 1/2A clinical trials in kidney transplantation (Sawitzki *et al.*, 2020), whereby the UK was a major participant. Essentially, regulatory T cells are naturally occurring and serve to prevent both autorecognition and immune system overdrive. Indeed, after obtaining and expanding *ex vivo* from kidney transplant recipients, the infusion of regulatory T cells back into these patients allowed weaning of standard immunosuppression and maintenance on monotherapy; acute rejection rates remained similar however. This has now progressed to a phase 2B clinical trial at Oxford, with the aim of achieving successful maintenance on single-drug immunosuppression by six months after kidney transplantation (Brook *et al.*, 2022). Therefore, results from such trials are closely monitored for translation and application to clinical VCA. Indeed, a recent murine study has shown that *ex vivo* expanded regulatory T cells could be infused to mitigate against VCA rejection, and that such regulatory T cells may also have a vital role in the induction and maintenance of immune tolerance (Anggelia *et al.*, 2021).

As described earlier, VCA skin serves a dual purpose – both as a potential means of monitoring for rejection, as well as a clinical goal to achieve functional and aesthetic improvements for the VCA patient. Much effort has been channeled towards understanding of the molecular and biochemical pathways involved as evidenced by studies from various groups (Kollar *et al.*, 2019; Win *et al.*, 2021). However, it is now known that the sheer number of skin-resident leukocytes far outnumber that circulating in the peripheral blood (Clark *et al.*, 2006). As well, the complex nature of the different leukocyte populations (Ng *et al.*, 2015) suggest that it will be very unlikely for a particular signalling pathway i.e. a rate limiting step, to be identified and an effective therapeutic target developed. For now, we have shown that at least, a "blanket approach" with local FK506 delivery in the form of subcutaneous implants, is sufficient to mitigate against acute VCA rejection, with the potential to even obviate the need for regular dosing (see Paper 7), thereby ushering in the possibility of removing patient compliance with life-long immunosuppression out of the equation altogether through, presumably, the re-implantation of such local subcutaneous FK506 implants on a regular basis clinically (e.g. during scheduled surveillance VCA skin biopsies).

Related Technological Advances

During my research fellowship, it became readily apparent that while the DTIP presented many positives, it was still fraught with many potential challenges that would ultimately negate the likelihood of success with subsequent DBMT and mixed chimerism. For instance, in VCA transplants, the matching of gender, skin colour, and in particular, allograft size between potential donors and recipients are additional constraints with implications on clinical outcomes. These additional requirements further complicate the logistics involved in VCA such as coordination of travel from the point of donor identification to the recipient being prepared for surgery. In turn, the donor pool becomes limited both in geography and quantity that leads to a downstream reduction in the number of VCAs that can be performed. As such, enabling extended preservation and/or banking of VCAs is needed to provide sufficient time to address these concerns, which would then become an enabling technology for the procedure to be more widely available clinically. Therefore, in parallel, a separate line of research was conducted to facilitate, as close as possible, a "day 0" protocol. By leveraging on our MGH colleagues' expertise in preservation of rat liver grafts, the clinical standard of static cold storage (SCS) at 4^0C was modified into a sub-normothermic machine perfusion (SNMP) protocol (at 21^0C), which extended liver graft viability to 4 days after a period of "supercooling" (i.e. sub-zero non-freezing (SZNF) storage). Further scale-up and modification of SNMP for VCA using a pig hindlimb model was thus developed and tested preliminarily with successful maintenance of allograft viability for at least 3 hours following DCD (donor after cardiac death) during my time in the laboratory (Lellouch *et al.*, 2016). Since then, my colleagues have further

extended the viability of such VCAs, albeit in a rodent hindlimb model, for up to 6 hours with SNMP, with successful re-transplantation and VCA survival at up to 30 days (Burlage *et al.*, 2021). To the best of my understanding, further scale-up and modification in a swine model are in progress. More recent developments include the use of HEMO2life, a unique oxygen carrier that has been purported to address the risks of ischaemic-reperfusion damage in VCA following procurement and prior to transplantation through its ability to deliver forty times more oxygen per haemoglobin molecule (Lupon *et al.*, 2021). In fact, HEMO2life was utilised in the world's first facial re-transplantation (Lantieri *et al.*, 2020) and more recently, in bilateral upper limb transplantation in India (Thomas, 2021) although its actual impact on immunological outcomes are hard to determine conclusively.

Building on the issue of lead time to surgery, clinical VCAs can now be performed with some element of pre-operative computer planning i.e. computer aided design (CAD) or manufacturing (CAM). The adoption of such technology from clinical plastic surgery has enabled reduction of the total operative time required both in hand (Hummelink *et al.*, 2021) and face transplantation (Kantar *et al.*, 2019; Manninen *et al.*, 2021). In turn, this may potentially have some beneficial effect on the degree of surgical inflammation and presumably, the likelihood of development of acute rejection in the early post-operative period as the extent of ischaemia-reperfusion injury may be mitigated (Caterson *et al.*, 2013; Khalifian *et al.*, 2013). Interestingly, such CAD/CAM technology has even been extended to restoration of the donor following VCA procurement (Cammarata *et al.*, 2019) and is certainly an important, but often overlooked area of research as VCAs, as with any form of transplantation, remains a gift of life. These developments are certainly much more clinically appropriate and were therefore, not the focus during my research. Nevertheless, I have maintained a keen interest in their developments as I continue to thread the path between both clinical and research realms in VCA.

4. SUMMARY

The number of cases of VCA that have been performed appears to have dwindled in recent years. The "arms race" seen during the earlier years in the field, where it was arguably hijacked by some for fame and fortune, are now less rampant with only established centres remaining active it seems. However, the key to the longevity of the field lies with further understanding and development of VCA immunology, not surgical prowess. Unfortunately, and at least in my opinion, the field remains poorly understood and much research funding has been channeled towards projects with poor or at best, remote clinical relevance as I have seen in manuscripts that I have been invited to review over the last few years. Nevertheless, I have maintained a keen interest in VCA, and all these experiences have served me well as

I continue with my higher surgical training in Plastic Surgery. In particular, I am more motivated than ever to push the boundaries further with what is possible with autologous reconstructive options and prosthetics, as I know very well that a VCA may not actually be the answer at this point in time. For a start, adoption of the local FK506 subcutaneous implant as described in this thesis submission in clinical trials to investigate peripheral nerve regeneration following traumatic nerve injuries would establish not only its pharmacological safety but, actual utility prior to application in VCA patients, as a means to mitigate against the development of acute rejection and patient compliance with life-long immunosuppression, and potentially, allow withdrawal of parenteral administration altogether. In turn, the well-known sequelae of systemic immunosuppression, the current and largest rate limiting step in VCA, may then well and truly be minimised. That I think, will be a milestone in the field of VCA, stemming from the research conducted in this submission.

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