ELABORATION OF BIOENGINEERED VASCULARIZED COMPOSITE TISSUES

by
Alexandre Gaston Mickael LELLOUCH

Title Page

THESE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE GRENOBLE ALPES

Spécialité **: BIS - Biotechnologie, instrumentation, signal et imagerie pour la biologie, la médecine et l'environnement**

Arrêté ministériel du 25 mai 2016

Préparé au sein du **Commissariat d'énergie atomique et alternatif (CEA)**

Et de **l'école doctorale Ingénierie pour la santé la Cognition et l'Environnement (ED ISCE)**

ELABORATION OF BIOENGINEERED VASCULARIZED COMPOSITE TISSUES

Présentée par :

Alexandre Gaston Mickael LELLOUCH

Thèse dirigée par **Professor Walid Rachidi** et codirigée par le **Professeur Laurent Lantieri**

Thèse soutenue publiquement le 10 mars 2022 devant le jury composé de :

Examinateurs :

M, Jean-Philippe Giot Professeur Université Grenoble Alpes
M, Jérôme Lamartine Professeur Université Claude Bernard / Lyon

Rapporteurs :

Mme, Muriel Vayssade Université de Compiègne
M, Nicolas Fortunel Directeur de Recherche CEA, Chef de Laboratoire

TABLE OF CONTENTS

LAY ABSTRACT

Reconstructive surgery is a surgical specialty that takes care of the loss of substances occurring after an accident (burn, trauma) or malformities. Currently the gold standard for medical management is surgery using the patient's own tissue (autologous surgery). In recent years, many technologies have emerged to recreate new tissues in the laboratory. Currently, the only existing biomaterials on the market are dermal substitutes to optimize the results of autologous skin grafts (increasing skin thickness). The objective of this PhD is to be able to test different strategies that aim to replace missing tissues in patients (skin, muscle, tendon, bone). This thesis is also a reflection on the developments of these technologies and their clinical applicability. The creation of new composite tissues must call on different areas of expertise that intersect and must intertwine to be able to find a lasting and clinically applicable solution.

RESUME DE THESE VULGARISE EN FRANCAIS : (1000 caractères)

La chirurgie reconstructrice est une spécialité chirurgicale prenant en charge les pertes de substances survenant au décours d'un accident (brûlure, traumatisme) ou d'origine malformative. Actuellement le gold standard pour la prise en charge médicale est la chirurgie utilisant les propres tissus du patient (chirurgie autologue). Au cours de ces dernières années de nombreuses technologies ont émergé pour recréer des nouveaux tissus en laboratoire. Actuellement, les seuls biomatériaux existants sur le marché sont des substituts dermiques permettant d'optimiser les résultats des greffes de peau autologues (augmentation de l'épaisseur cutanée). L'objectif de cette thèse de science est de pouvoir tester différentes stratégies qui ont pour but de remplacer des tissus manquants aux patients (peau, muscle, tendon, os). Cette thèse est aussi une réflexion sur les développements de ces technologies et de leur applicabilité clinique. La création de nouveaux tissus composites doit faire appel à différent domaines d'expertises qui s'entrecroisent et doivent s'intriquer pour pouvoir trouver une solution pérenne et applicable cliniquement.

TECHNICAL ABSTRACT

Reconstructive surgery is a surgical specialty that takes care of the loss of substances occurring after an accident (burn, trauma) or malformities. Currently the gold standard for such defect is the autologous surgery. The limiting factors are the limited number of donor sites, morbidity, and the sequelae of these skin grafts such as scars. In addition, the complex defects affecting functional anatomical areas like the face or hands cannot be anatomically restored using autologous surgery.
In recent years many technologies have emerged to recreate new tissues. Currently, the only existing biomaterials on the market are dermal substitutes to optimize the results of autologous skin grafts (increasing skin thickness). The next step is the manufacture of a dermo-epidermal substitute in vitro. The objective of this PhD is to be able to test different strategies that aim to replace missing tissues in patients (skin, muscle, tendon, bone). This thesis is also a reflection on the developments of these technologies and their clinical applicability.
Part A will consist of testing tolerance induction protocols by mixed chimerism using bone marrow transplantation in large animals. Part B will deal with the issues of Vascularized Composite Allotransplantation in Humans. Part C will present a study on perfusion machines with the aim of preserving tissues ex vivo. Part D will address the topic of tissue engineering testing two approaches: decellularization / recellularization of porcine surgical models and in vitro creation using biomaterials.
In conclusion, the creation of new composite tissues must use different areas of expertise that intersect and must be intertwined to be able to find a lasting and clinically applicable solution.

RESUME DE THESE EN FRANCAIS

La chirurgie reconstructrice est une spécialité chirurgicale prenant en charge les pertes de substances survenant au décours d'un accident (brûlure, traumatisme) ou d'origine malformative. Actuellement le gold standard pour la prise en charge médicale est la chirurgie autologue. Les facteurs limitants sont le nombre limité de site donneurs, la morbidité induite, et les séquelles de ces prises de greffes (ajout de cicatrices inesthétiques). De plus, les pertes de substances complexes affectant des zones anatomiques fonctionnelles comme la face ou les mains ne peuvent pas être restaurer anatomiquement à l'aide de la chirurgie autologue.
Au cours de ces dernières années de nombreuses technologies ont émergé pour recréer des nouveaux tissus. Actuellement, les seuls biomatériaux existants sur le marché sont des substituts dermiques permettant d'optimiser les résultats des greffes de peau autologues (augmentation de l'épaisseur cutanée). La prochaine étape est la fabrication de véritable substitut dermo-epidermique in vitro. L'objectif de cette thèse de science est de pouvoir tester différentes stratégies qui ont pour but de remplacer des tissus manquants aux patients (peau, muscle, tendon, os). Cette thèse est aussi une réflexion sur les développements de ces technologies et de leur applicabilité clinique. La partie A consistera a testé des protocoles d'induction de tolérance par chimérisme mixte utilisant la greffe de moelle osseuse chez le gros animal. La partie B traitera des problématiques de l'allotransplantation de tissus composites chez l'Homme. La partie C présentera une étude sur les machines de perfusions dans le but de préserver les tissus ex vivo. La partie D abordera la thématique de l'ingénierie tissulaire testant deux approches : la

décellularisation/ recellularisation de modèles chirurgicaux porcins et la création in vitro à l'aide de biomatériaux.
En conclusion, la création de nouveaux tissus composites doit faire appel à différents domaines d'expertise qui s'entrecroisent et doivent s'intriquer pour pouvoir trouver une solution pérenne et applicable cliniquement.

CHAPTER 01

General Introduction and Aims of this Thesis

Alexandre G. Lellouch

Composite tissue loss caused by accident (burn, trauma) is a major clinical problem which necessitates reconstructive surgery to restore function, limit scarring and achieve optimal cosmetic outcome, all of which influence the quality of life of injured patients. Standard reconstructive techniques include autologous, pedicled or vascularized tissue flaps to replace moderate to severe composite tissue defects[1,2]. Limitations of these methods include donor-site morbidity and difficulty in shaping the graft to restore complex three-dimensional anatomy. Transplantation is defined according to the World Health Organization (WHO) as the transfer of human tissues or organs from a donor to a recipient with the aim of restoring essential functions where no alternative of comparable effectiveness exists. It became a standard of care procedure since the first successful kidney transplant performed in human in 1954[3] by Dr Murray's team. Over the past 20 years, Vascularized Composite Allografts (VCAs) have revolutionized the treatment of complex soft tissue absence by providing an anatomically exact tissue unit enabling like-for-like restoration. In recent years, there has been rapid growth in the application of VCA with face transplantation (among others)[4]. In total more than 200 VCA have been performed worldwide. Unfortunately, the first long term outcome (> 10 years) reported by our team showed many complications due to the lifelong immunosuppression (diabetes, infection, neoplasia, kidney insufficiency)[5]. The immunosuppressive regimen is documented to be more harmful in children, rendering VCAs a risky treatment choice[6]. We recently reported the first face retransplantation occurring after chronic rejection[7] witnessing the long term risk of such procedure. The persistent hurdle in VCA remain the risk of immune rejection (acute and chronic). Research is focused on preventing the risk of rejection[8] (**Figure 1**). In this thesis we will present different strategies to mitigate the immune response in VCA.

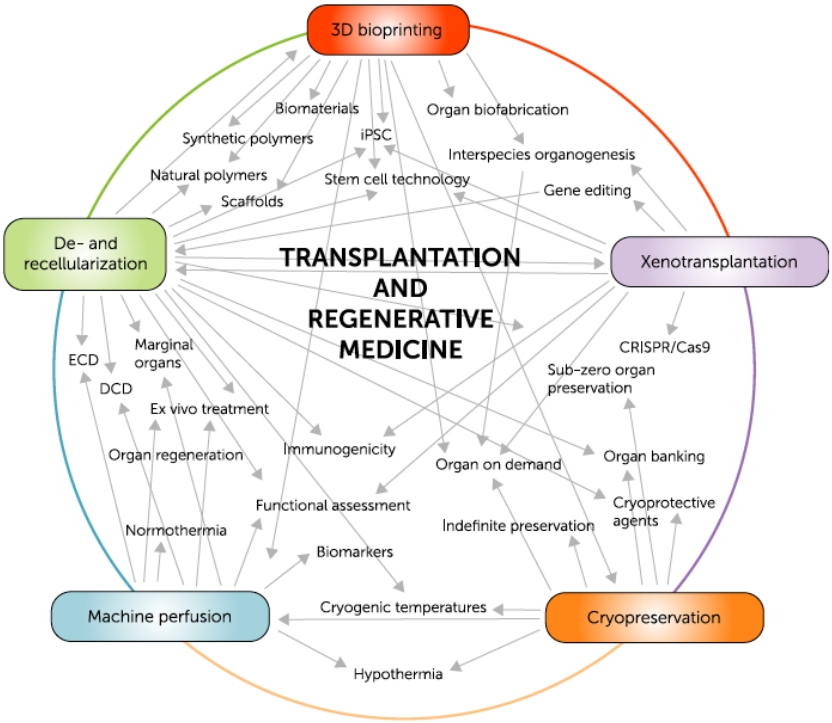


Figure 1: Interactions of different novel technologies in regenerative medicine and organ transplantation Messner et al. 2019

PART A: INDUCTION TOLERANCE PROTOCOL IN VASCULARIZED COMPOSITE ALLOGRAFT: ANIMAL STUDIES

One potential solution to the dilemma of immunosuppression-related risks and considerations in VCA is the induction of transplantation tolerance, defined as long-term, immunosuppression-free allograft acceptance with neither clinical nor histologic evidence of rejection, thereby eliminating the need for immunosuppression altogether, through mixed hematopoietic chimerism (**Figure 2**). The Chapter 2[9] is a review of the current tolerant protocols used in solid organ transplantation and VCA in large animals and human. Tolerance may also prevent the development of chronic rejection, which is a significant cause of late solid organ transplantation (SOT) allograft loss and has been reported in a face transplant recipient at 8 years post-VCA[10]. This finding is of particular relevance to the pediatric population in view of the longer lifespan ahead in comparison to adult VCA recipients. Clinically, tolerance of transplanted kidneys has been achieved in haploidentical donor-recipient pairs through donor bone marrow transplantation (DBMT), which achieved mixed donor-recipient lymphohematopoietic chimerism and immunologic tolerance of the renal allograft[11]. Stable mixed chimerism was not required for tolerance induction in this case – indeed, despite transient mixed chimerism, tolerance of the transplanted kidneys was achieved. In contrast, we have shown that VCA[12], like more antigenic SOT (e.g. lungs[13]) will require stable mixed chimerism to achieve tolerance, especially to the epidermal component[14], which will be of utmost importance in a transplanted hand or face.

Overall, it is believed that the thymus[15] is intricately linked to the success of mixed chimerism following allogeneic hematopoietic stem cells transplantation (HSCT) by contributing both directly and indirectly towards "central" and "peripheral" mechanisms of tolerance. Briefly, this involves intra-thymic clonal deletion of donor-reactive host T cells whilst simultaneously, allowing transplanted allogeneic donor cells to take up residence within the recipient's thymus wherein they become recognized as "self," proliferate and exit into the circulation to act peripherally via a number of possible mechanisms including deletion, anergy and regulation[15]. In Chapter 3, we induced tolerance in Class I mismatch swine using for the first time Bone marrow transplantation (BMT), Belatacept® and Tociluzimab®. In Chapter 4[16], we scaled-up to Non-Human Primate (NHP) but it was unsuccessful due to the acute rejection preventing the tolerance mechanism. Therefore, topical immunosuppression 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 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. As such, we sought to investigate whether topical FK506 administration would be able to mitigate inflammation and thus, acute rejection in primarily vascularized VCAs in NHPs (Chapter 5[17]).

However, like the clinical VCA experience from select centres around the world 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. Results from swine studies suggest that unlike kidney transplantation, the induction of stable [18] rather than transient[19] 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[13]. 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[20]. Given the above observations, the DTIP was trailed in our next NHP VCA experiment with further analysis of VCA skin leukocyte populations (Chapter 4).

Unfortunately, while successful in mitigating acute rejection in SOT, this was not the case for VCA. While our laboratory's swine studies had hinted at a possible role for the extent of MHC matching in rejection outcomes[21], 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. Finally, it was decided that the topical FK506 formulation required redesigning by our engineering colleagues at Rutgers University to specifically target VCA dermis (Chapter 6[22]).

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 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, but it was also possible to maintain the VCAs rejection-free (both clinically and on histology) without the need for additional systemic immunosuppression. Most encouragingly, mixed chimerism was developed successfully by targeting VCA dermis. Unfortunately, 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 the use of rituximab, prior to clinical translation in the form of tolerance trials in VCA.

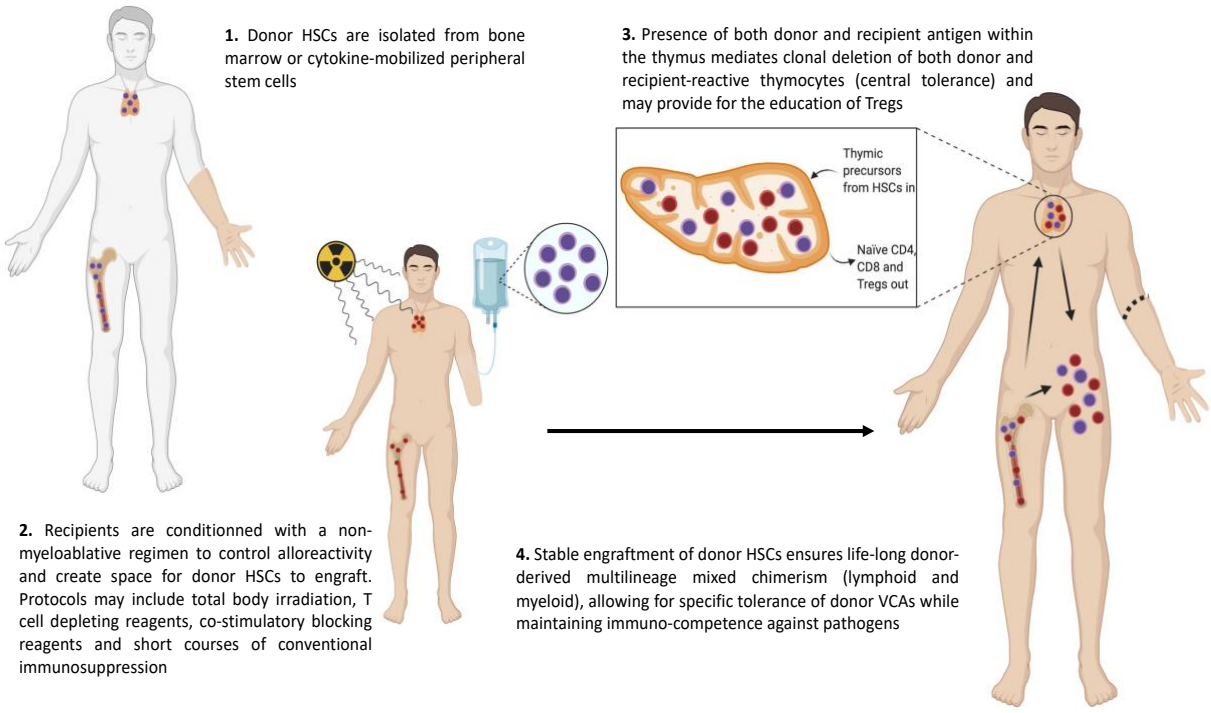


Figure 2. Mechanisms of tolerance in mixed hematopoietic chimerism.

PART B: VASCULARIZED COMPOSITE ALLOTRANSPLANTATION IN HUMANS

Over a million people in France are affected by disfigurements from burns, trauma, infections, malignancies, and congenital anomalies (Haute Autorité de Santé). The most extensive of these have a devastating and costly impact on lives[23]. The reconstructive ladder has provided the principles for surgical management of tissue loss, with free tissue transfer using microsurgical techniques, applied for severe disfigurement[24] (**Figure 3**).

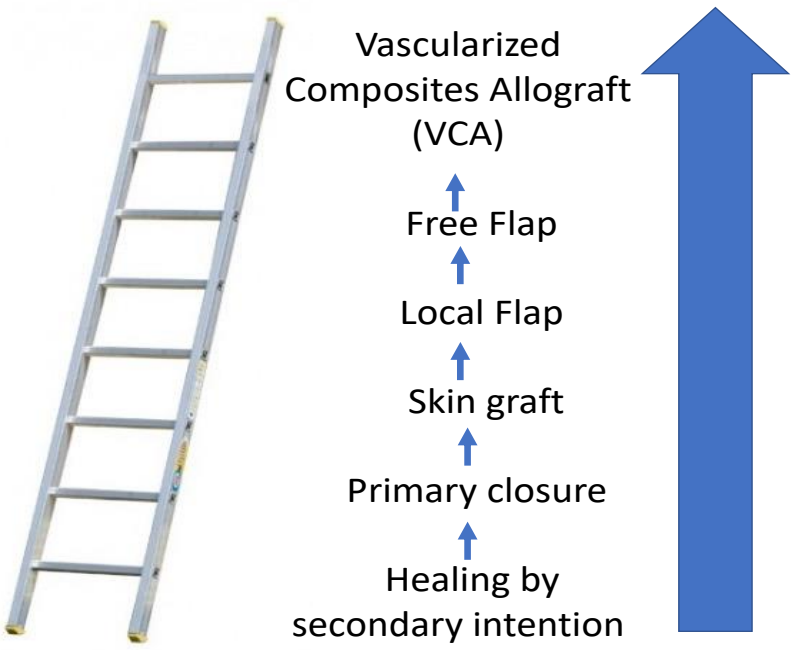


Figure 3: The reconstructive ladder.

A cohort of people, nonetheless, exists for whom adequate restoration of form and function cannot be achieved. The use of transplants for reconstructive purposes would allow single-step reconstruction of complex anatomical structures. Vascularized composite allografts (VCAs) are primarily vascularized units composed of multiple tissue types, including skin, muscle, blood vessel, nerve, and bone. VCA transplants of the face, upper extremities, lower extremities, and abdominal wall have been successfully performed (Figure 4). Significant functional and quality-of-life improvements have been seen following these procedures[25]. However, in the context of life-saving solid organ transplants, a fundamental ethical question remains: are the life-enhancing benefits of VCAs justified by the risks of immunosuppression, which include opportunistic infections, malignancy, and organ failure? This was the discussion of Chapter 7[26]. Strategies to offset the risks of immunosuppression would shift the risk-to-benefit ratio and allow widespread application of this promising reconstructive modality. I contributed to a review of clinical VCA cases performed in survivors of major burns (Chapter 8[27]), 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 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 (Chapter 9[28]). 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 DSA and an estimated time to development of GV was provided at approximately 6 years. Together, the Chapter 8 and 9 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. GV can lead to graft loss, and we had to manage clinically this catastrophic clinical scenario. For the first time, we had to perform a facial retransplantation (Chapter 10[7]). We will also report the management of long-term face transplanted patient and how we handled complications such as facial trauma (Chapter 11[29]). In this part, we will summarize our work in human VCA at European Georges Pompidou Hospital (with Pr Lantieri).

Date	The first :
1954	Kidney transplantation
1964	Hand transplantation (graft loss at 3 weeks)
1998	Hand transplantation (long term)
2001	Abdominal transplantation
2005	Face transplantation
2006	Lower extremity transplantation
2014	Penile transplantation
2018	Face retransplantation

Figure 4: Key dates in VCA

PART C: OXYGENATED MACHINE PERFUSION AND TRANSPLANTATION OF VASCULARIZED COMPOSITE ALLOGRAFT

For over three decades, the clinical state-of-the art in whole organ preservation has been static cold storage (SCS) in an icebox, developed back when solid organ transplantation (SOT) was still a rare and risky procedure. Given the crisis in compatible donor graft availability and the frequent patient's family refusal, the constrained logistics that SCS offers is no longer affordable or acceptable. As with vital organs, ischemic time is the absolute technological constraint: permanent ischemic injury occurs within 2-4 hours of warm ischemia and 6-8 hours of cold ischemia in skeletal muscle, which is abundant in amputated limbs.

In fact, VCAs may degrade faster than some vital organs and already amputated tissues that cannot be salvaged and maintained long enough for replantation are frequently discarded because of the associated, poor functional outcomes in the long-term[30]. For VCA transplants, the matching of gender, skin color, 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 prepped 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/banking of VCAs is needed to provide sufficient time to address the previous concerns, which would then become an enabling technology for the procedure to be more widely available clinically. A key bottleneck to any form of cellular therapy, ranging from cell to organ and tissue transplantation, is the very limited duration of storage [31].

The current gold standard, which is hypothermic preservation on ice (at about 4°C i.e. SCS) in a specialized media (typically University of Wisconsin (UW) solution), limits storage to a few hours for vascular and metabolically active tissues such as the liver, heart and presumably limbs. This restriction creates a major obstacle towards on-demand tissue availability and limits the potential for organ sharing from global down to a regional and/or national level. While SCS has remained in use due to its simplicity and low costs [32], its utility is limited in grafts harvested from Donors after Cardiac Death (DCD). Such DCD grafts remain at or near body temperature without blood or oxygen supply and consequently, sustain further ischemic injury. It is thus well acknowledged that in order to develop enhanced clinical solutions for organ failure, new methods must be developed to enable long-term organ storage and preservation [31,33].

In theory, cryopreservation has the potential to achieve practically infinite storage but successful and viable preservation of vascular tissues and organs has proven elusive and difficult, if not outright impossible [31]. A recent randomized, controlled trial in 2009 was the first to show the superiority of hypothermic (4-60C) machine perfusion (HMP) in kidney grafts – HMP not only significantly decreased the incidence of delayed graft function but results also indicate better graft survival [34]. Through machine perfusion, pulsatile flow can provide nutrients, flush out harmful toxins, decrease the expression of pro-inflammatory endothelial cytokines, and help maintain capillary perfusion [35,36].
Since then, the use of perfusion systems has had a renaissance not only in kidney but liver grafts [37]. A much more practical alternative is sub-zero non-freezing (SZNF) preservation,

which aims to supercool the tissue to temperatures below freezing point (e.g. -6°C) without any phase change, slow down the metabolic and degradation processes beyond what is currently possible at ice-cold temperatures (+4°C, i.e. SCS), and extend the overall duration of preservation [38]. This approach avoids the phase transition issues that exist in cryopreservation and vitrification methods that harm large tissues and render them unsuitable for transplantation subsequently. As the rate of biological reactions are roughly halved for every drop of 10°C in storage temperature, doubling the preservation time with this method seemed achievable. By combining SZNF preservation with subnormothermic (210C) machine perfusion (SNMP) (**Figure 5**) recovery for the stepwise normalization of temperature and metabolic demand.

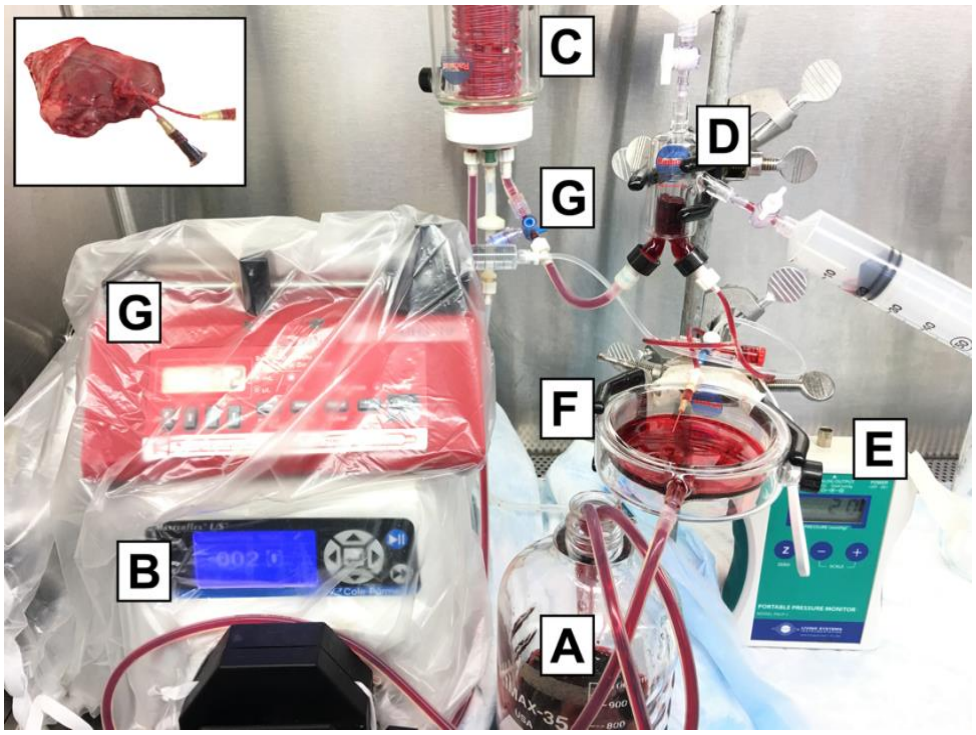


Figure 5. Ex vivo subnormothermic machine perfusion set up with HBOC-201 perfusion solution.

The circuit consists of perfusion solution (A) that is pumped via a roller pump (B) to the oxygenator (C), that is oxygenated with a carbogen mixture (5% CO2 and 95% oxygen). The solution then goes through the bubble trap (D) to prevent air bubbles going into the limb. The pressure is measured (E) at the level of the limb that is laying the basin (F). Inflow samples are measure at the inflow valve (G) with outflow samples are measured directly from the venous outflow canula (as shown in upper left panel).

MGH recently demonstrated successful supercooled rat liver preservation at -6°C with subsequent transplantation, and by doing so, tripled the preservation duration possible with classical SCS in UW solution[39]. Following this discovery, we got the idea to reproduce this protocol in VCA. The first step was to elaborate a surgical model on rats with different characteristics: reproducible, osteomyocutaneous flap, well tolerated by the animal and an easy skin monitoring. Therefore, we described a heterotopic hind limb transplantation

in rat on Chapter 12[40]. This supercooling protocol include 3 phases (**Figure 6**): loading, supercooling preservation and recovery.

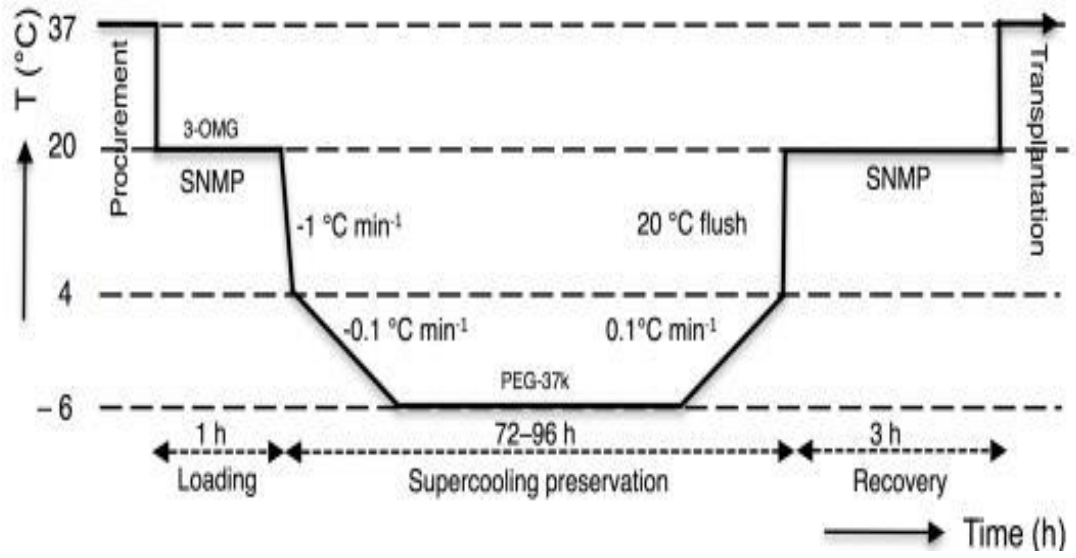


Figure 6. Supercooling protocol developed at MGH

In Chapter 13 we optimized the first step of the supercooling protocol in VCA and elaborate the first patent on it : Application No. PCT/US2020/016840 International Publication number : WO 2020/163500 A1. In Chapter 14[41] we infused biosensors cells as a proof-of-concept study to able to monitor the acute rejection process.

PART D: TISSUE ENGINEERING IN VASCULARIZED COMPOSITE ALLOGRAFT

In this part, I will present 2 processes to recreate biologic tissues:

- Decellularization / recellularization (DE/RE) of natural scaffold
- Recellularization of biomaterials

Given the limitations of autologous skin grafting, the need for a skin substitute has long been evident. Currently, skin substitutes consist of a collection of products which can be categorized as either epidermal, dermal, or combined dermo-epidermal replacements.

Epidermal substitutes consist of a thin layer of keratinocytes typically grown into stratified sheets 2-5 cell layers thick, known as cultured epidermal autografts (CEAs). CEAs are cultured over the course of at least three weeks from a full thickness skin biopsy harvested under sterile conditions. The small sheets (5x10cm) are extremely fragile, easily infected, and average take rates are poor (<50%). The quality of the resulting skin is suboptimal and subject to frequent blistering and wound breakdown. An alternative approach for epidermal replacement has been the use of autologous keratinocyte suspensions, which can be sprayed onto a wound bed. A small biopsy is processed intraoperatively, and keratinocytes are then sprayed directly and immediately onto the wound bed. This procedure is only effective for partial thickness burns, ie. burns which would otherwise heal on their own without the need for skin grafting.

To improve the outcome of STSGs, dermal substitutes were developed. The first commercially available dermal substitute was Integra®, a bilayer composed of a matrix
General Introduction and Aims of this Thesis

containing bovine collagen and chondroitin-6-sulfate covered by a thin polysiloxane layer. More recently developed dermal templates composed of acellular matrices (e.g. AlloDerm, which is human decellularized dermis). All dermal templates necessitate a two-stage procedure in which the product is placed onto the sterile wound bed and allowed to vascularize over 3-4 weeks. The incorporated dermal template then requires coverage with a STSG. Ultimately much of the dermal template is replaced by scar and does little to improve the functional quality of the overlying epidermal autograft. Lastly, dermo-epidermal substitutes consist of two commercially available products: Apligraf and Orcel; both composed of human allogeneic keratinocytes and dermal fibroblasts cultured on a bovine collagen sponge. Each is supplied as a small thin disc (Apligraf-75mm diameter) indicated for the treatment of venous and diabetic foot ulcers though minimal supporting data has been published.

These products have no role in the treatment of larger cutaneous defects, such as those caused by trauma or burns. Current research focused on the creation of skin substitutes typically includes the seeding of acellular dermal matrices or collagen hydrogels with various epidermal cells, fibroblasts, and/or stem cells. Several labs have demonstrated their ability to culture keratinocytes and dermal fibroblasts on collagen hydrogels, creating an appropriately stratified epidermis with basement membrane. Engraftment has been complicated by the period of ischemia whereby the graft must rely on plasmatic imbibition until vascularization via inosculation is complete.

Decellularization / recellularization (DE/RE) of natural scaffold

In Chapter 15[42], we worked on one review explaining the state-of-the-art of this technology in VCA. All the current protocols used has been collected and organized in function of the different tissue decellularized. The most recent efforts to improve engraftment have focused on techniques for pre-vascularization and the creation of microvascular networks within the graft. De novo design of such complex microvascular architecture, however, remains a fundamental challenge of tissue engineering. One way to circumvent this challenge is to utilize acellular matrices with a pre-existing, intact, macro and microvascular network. In recent years tissue decellularization has been

developed as a method for obtaining native ECM scaffolds. Perfusion decellularization, a technique pioneered and optimized by the Ott Laboratory, can generate native ECM scaffolds with intact vasculature and preserved tissue architecture from whole organs. Recellularization of these grafts has generated bioartificial organs (i.e. hearts, lungs, and kidneys[44]) with rudimentary function. The limitation of commercially available acellular human dermis is that it is harvested in sheets from cadaveric donors using a dermatome. Although the pre-existing microvasculature remains intact, there are no macroscopic sized vessels by which to access and perfuse this microvascular network.

This type of dermal template can only be decellularized through its surrounding medium via agitation, and similarly cannot be repopulated with cells via intravascular perfusion

techniques. Perfusion decellularized matrices have been utilized for the regeneration of whole organs; however, it is our belief that the appropriate decellularized platform may also serve as a scaffold for the regeneration of full thickness skin. In fact, the ability to utilize autologous explanted microcirculatory beds as vascularized bio scaffolds in the form of microvascular free flaps has previously been demonstrated in three studies. Only one of these studies, however, utilized perfusion decellularization to generate an acellular scaffold from a fasciocutaneous free flap and recellularization of such a decellularized construct remains novel.

The goal of this work is to utilize perfusion decellularization technology and recellularization techniques on a porcine-based fasciocutaneous flap (FCF)[45] (**Figure 6**) to generate a tissue-engineered, dermo-epidermal skin substitute. The creation of such a product would have an enormous impact on the treatment of cutaneous tissue loss as currently there is still no permanent full thickness skin equivalent that functions close to normal skin and that does not require an autologous donor skin grafting.

An off-the-shelf product that could be pre-vascularized and pre-epithelialized with a patient's own cells could function similar to a full thickness skin graft without the morbidity of a donor site and could be utilized for acute or reconstructive procedures. It would represent a completely novel rung on the reconstructive ladder. In Chapter 16, we will develop a nipple-areolar complex on swine using this technology.

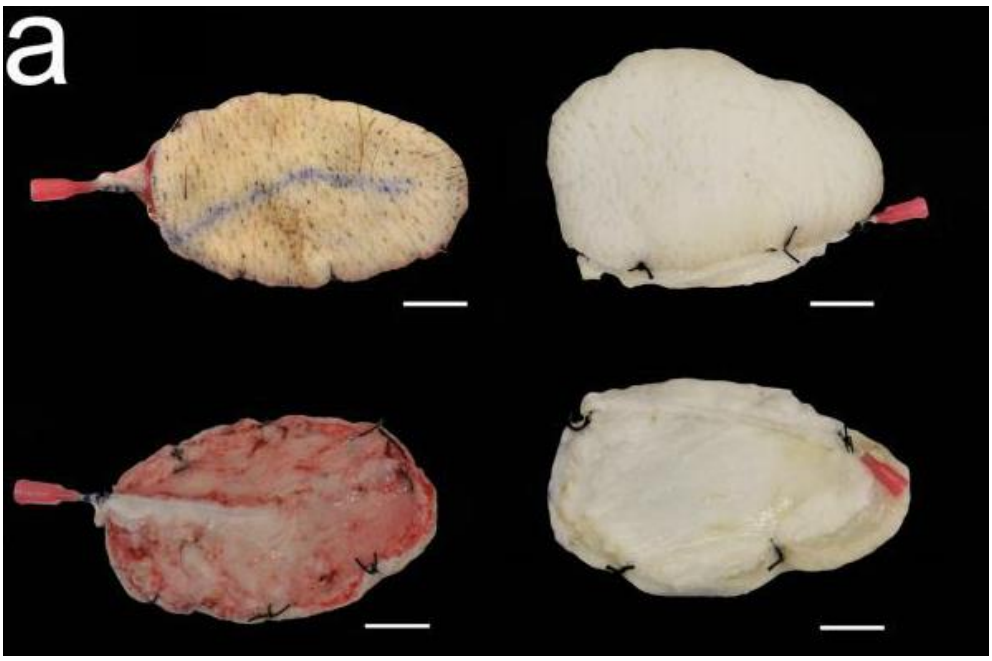


Figure 6. Photographs of isolated FCF, cannulated for perfusion decellularization, top view = epidermal side, bottom view = subcutaneous side. Perfusion decellularization yielding white, acellular scaffolds after 10 days of detergent perfusion

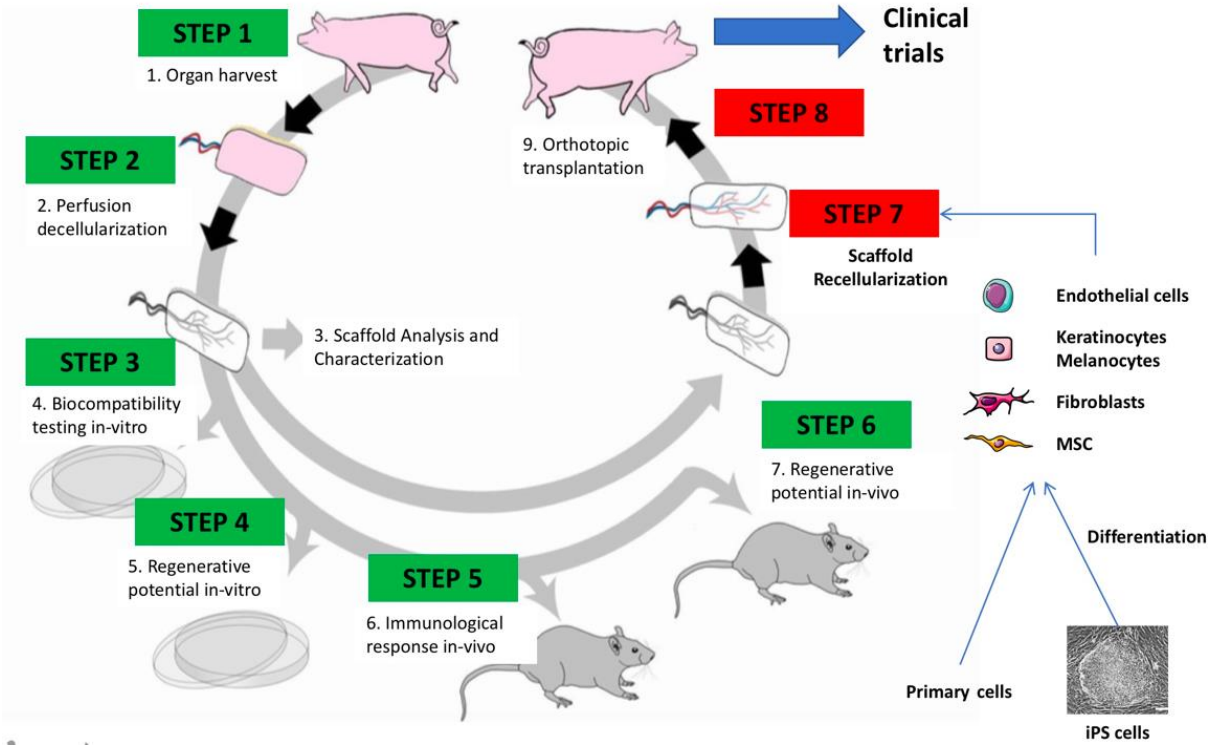


Figure 7 – State-of-the Art in Decellularisation/ Recellularization technology. Patient-specific recellularized tissue scaffold. Green: completed steps. Red: steps in progress. Full thickness skin flaps were harvested with a vascular pedicle from the groin of donor pigs (step 1), and perfusion-decellularized (step 2). Step 7 represent the recellularization process in vitro and step 8 the transplantation of the "recellularized flap''.

Recellularization of biomaterials

The DE/RE is a very interesting concept for composite tissues because the external cellular matrix (ECM) and the vasculature is well-preserved (**Figure 8**).

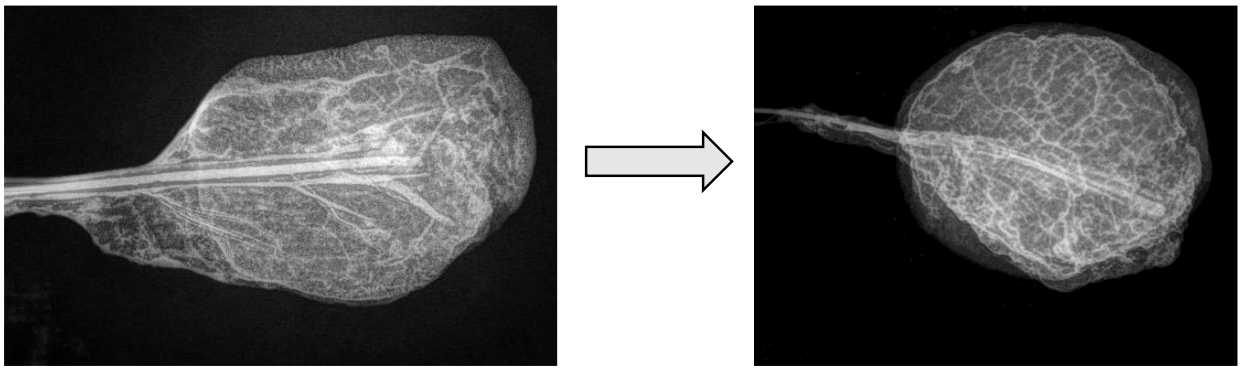


Figure 8. Before (left) and after (right) decellularization of a FCF (cf. **Figure 6**)

On the other hand, for the creation of a single tissue like skin, we faced difficulties for the recellularization process. Indeed, the decellularized dermis is very impermeable for fibroblast colonisation. Therefore, in Chapter 17 we decided to switch to a more porous biomaterial (surgifoam®) to generate dermo-epidermal substitute skin (**Figure 9**).

The particularity of this project is that the optimization of this "artificial skin" was carried out in a multidisciplinary way associating clinicians (plastic surgeon) and scientists. In addition, all the components of this "artificial skin" are GMP (Good Manufacturing Practice) and used primary cells (fibroblast, keratinocyte).

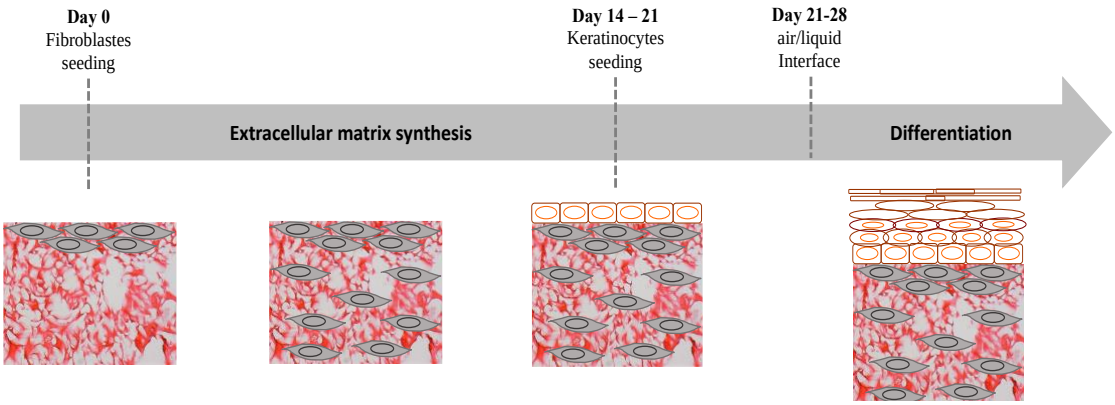


Figure 9. Timeline dermo-epidermal skin substitute using a gelatin sponge (surgifoam®)

The expected benefit of the proposed recovery solution (dermal-epidermal bilayer skin substitute) is healing better than that observed with exclusively dermal or epidermal substitutes, with faster reconstitution of the skin structure. Such a dermo-epidermal substitute would make it possible to overcome the lack of availability of natural skin grafts to cover the lesions of patients with severe burns, and to improve the plastic quality of scars thanks to a more complete cell reconstitution than the current single-cell artificial solutions offered. The study proposes to evaluate this recovery solution on a model of skin wounds produced on the minipig, immunosuppressed by injections of Tacrolimus, by providing the necessary medical care to the animals so that they do not suffer at any time: anaesthesia during surgeries, analgesia during healing of lesions, daily clinical evaluation to ensure the effectiveness of analgesic treatments. This technology is patented since March 2020 (**Figure 10**), and we tested it on swine (Chapter 18). More optimization is still needed to get the most cost-effective skin substitute.

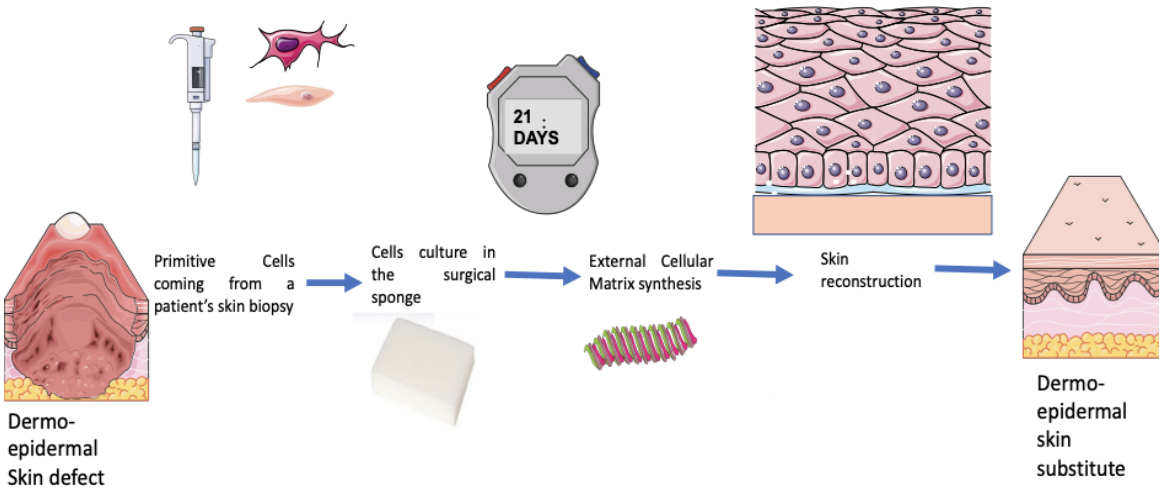


Figure 10. Protocol patented 2020: « Biomaterial comprising a porous resorbable matrix and associated manufacturing method" Application No. PCT/EP2021/058151 International Publication number WO 2021/198177 A1

REFERENCES

1. Ferri J, Piot B, Ruhin B, Mercier J. Advantages and limitations of the fibula free flap in mandibular reconstruction. J Oral Maxillofac Surg. 1997;55(5):440-448.
2. Tessier P, Kawamoto H, Matthews D, et al. Autogenous bone grafts and bone substitutes--tools and techniques: I. A 20,000-case experience in maxillofacial and craniofacial surgery. Plast Reconstr Surg. 2005;116(5 Suppl):6S-24S.
3. GUILD WR, HARRISON JH, MERRILL JP, MURRAY J. Successful homotransplantation of the kidney in an identical twin. Trans Am Clin Climatol Assoc. 67:167-73.
4. Lantieri L, Meningaud J-P, Grimbert P, et al. Repair of the lower and middle parts of the face by composite tissue allotransplantation in a patient with massive plexiform neurofibroma: a 1-year follow-up study. Lancet (London, England). 2008;372(9639):639-45.
5. Lantieri L, Grimbert P, Ortonne N, et al. Face transplant: long-term follow-up and results of a prospective open study. Lancet. 2016;388(10052):1398-1407.
6. Amaral S, Kessler SK, Levy TJ, et al. 18-month outcomes of heterologous bilateral hand transplantation in a child: a case report. Lancet Child Adolesc Heal. 2017;1(1):35-44.
7. Lantieri L, Cholley B, Lemogne C, et al. First human facial retransplantation: 30-month follow-up. Lancet. 2020;396(10264):1758-1765.
8. Messner F, Guo Y, Etra JW, Brandacher G. Emerging technologies in organ preservation, tissue engineering and regenerative medicine: a blessing or curse for transplantation? Transpl Int. 2019;32(7):673-685.
9. Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL. Mixed Chimerism-Based Regimens in VCA. Curr Transplant Reports. 2016.
10. Morelon E, Petruzzo P, Kanitakis J, et al. Face Transplantation: Partial Graft Loss of the First Case 10 Years Later. Am J Transplant. 2017;17(7):1935-1940.
11. Kawai T, Sachs DH, Sprangers B, et al. Long-Term Results in Recipients of Combined HLA-Mismatched Kidney and Bone Marrow Transplantation Without Maintenance Immunosuppression. Am J Transplant. 2014;14(7):1599-1611.
12. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant. 2014;14(2):343-55.
13. Tonsho M, Lee S, Aoyama A, et al. Tolerance of Lung Allografts Achieved in Nonhuman Primates via Mixed Hematopoietic Chimerism. Am J Transplant. 2015;15(8):2231-2239.
14. Lee WP, Yaremchuk MJ, Pan YC, Randolph MA, Tan CM, Weiland AJ. Relative antigenicity of components of a vascularized limb allograft. Plast Reconstr Surg. 1991;87(3):401-11.
15. Siemionow M, Izycki D, Ozer K, Ozmen S, Klimczak A. Role of thymus in operational tolerance induction in limb allograft transplant model. Transplantation. 2006;81(11):1568-76.
16. Lellouch AG, Ng ZY, Rosales IA, et al. Toward Development of the Delayed Tolerance Induction Protocol for Vascularized Composite Allografts in Nonhuman Primates. Plast Reconstr Surg. 2020;145(4):757e-768e.
17. Gama A-R, Ng ZY, Shanmugarajah K, et al. Local Immunosuppression for Vascularized Composite Allografts: Application of Topical FK506-TyroSpheres in a Non-Human Primate Model. J Burn Care Res. April 2020.
18. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant. 2014;14(2):343-55.
19. 19. Leto Barone AA, Kurtz JM, Albritton A, et al. Effects of Transient Donor Chimerism on Rejection of MHC-Mismatched Vascularized Composite Allografts in Swine. Vasc Compos Allotransplantation. 2015;2(1):1-8.
20. Leonard DA, Powell HR, Defazio MW, et al. Cutaneous leukocyte lineages in tolerant large animal and immunosuppressed clinical vascularized composite allograft recipients. Am J Transplant. 2021;21(2):582-592.
21. Shanmugarajah K, Powell H, Leonard DA, et al. The Effect of MHC Antigen Matching Between Donors and Recipients on Skin Tolerance of Vascularized Composite Allografts. Am J Transplant. 2017;17(7):1729-1741.
22. Lellouch AG, Taveau CB, Andrews AR, et al. Local FK506 implants in non-human primates to prevent early acute rejection in vascularized composite allografts. Ann Transl Med. 2021;9(13):1070-1070.
23. Pomares G, Coudane H, Dap F, Dautel G. Psychological effects of traumatic upper-limb amputations. Orthop Traumatol Surg Res. 2020;106(2):297-300.
24. Levin LS. The reconstructive ladder. An orthoplastic approach. Orthop Clin North Am. 1993;24(3):393-409.
25. Fischer S, Kueckelhaus M, Pauzenberger R, Bueno EM, Pomahac B. Functional outcomes of face transplantation. Am J Transplant. 2015;15(1):220-33.
26. Lellouch AG, Lantieri LA. A Second Chance at Life. Cambridge Q Healthc Ethics. 2019;28(3):463-467.
27. Ng ZY, Lellouch AG, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL. Vascularized Composite Allotransplantation-An Emerging Concept for Burn Reconstruction. J Burn Care Res. 2017;38(6):371-378.
28. Ng ZY, Lellouch AG, Rosales IA, et al. Graft vasculopathy of vascularized composite allografts in humans: a literature review and retrospective study. Transpl Int. 2019;32(8):831-838.

29. Goutard M, Lellouch AG, Dussol B, Lantieri LA. Facial Trauma 8 years after a Face Transplantation. Plast Reconstr surgery Glob open. 2021;9(5):e3575.
30. Lin C-H, Aydyn N, Lin Y-T, Hsu C-T, Lin C-H, Yeh J-T. Hand and finger replantation after protracted ischemia (more than 24 hours). Ann Plast Surg. 2010;64(3):286-90.
31. Fahy GM, Wowk B, Wu J. Cryopreservation of complex systems: the missing link in the regenerative medicine supply chain. Rejuvenation Res. 2006;9(2):279-91.
32. McAnulty JF. Hypothermic organ preservation by static storage methods: Current status and a view to the future. Cryobiology. 2010;60(3 Suppl):S13-9.
33. Fowler A, Toner M. Cryo-injury and biopreservation. Ann N Y Acad Sci. 2005;1066:119-35.
34. Moers C, Smits JM, Maathuis M-HJ, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009;360(1):7-19.
35. Khalifian S, Cooney DS, Lee WPA, Brandacher G. The Role of Ischemia-Reperfusion Injury in Reconstructive Transplantation. J Transplant Technol Res. 2013;s3(01).
36. Caterson EJ, Lopez J, Medina M, Pomahac B, Tullius SG. Ischemia-reperfusion injury in vascularized composite allotransplantation. J Craniofac Surg. 2013;24(1):51-6.
37. Schlegel A, Kron P, Dutkowski P. Hypothermic machine perfusion in liver transplantation. Curr Opin Organ Transplant. 2016;21(3):308-14.
38. Matsumoto N, Yoshizawa H, Kagamu H, et al. Successful liquid storage of peripheral blood stem cells at subzero non-freezing temperature. Bone Marrow Transplant. 2002;30(11):777-84.
39. Berendsen TA, Bruinsma BG, Puts CF, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. Nat Med. 2014;20(7):790-3.
40. Goutard M, Randolph MA, Taveau CB, et al. Partial Heterotopic Hindlimb Transplantation Model in Rats. J Vis Exp. 2021;(172).
41. Taveau CB, Lellouch AG, Chin L-Y, et al. In Vivo Activity of Genetically Modified Cells Preseeded in Rat Vascularized Composite Allografts. Transplant Proc. 2021;53(5):1751-1755.
42. Duisit J, Maistriaux L, Bertheuil N, Lellouch AG. Engineering Vascularized Composite Tissues by Perfusion Decellularization/Recellularization: Review. Curr Transplant Reports. 2021;8(2):44-56.
43. Lupon E, Lellouch AG, Acun A, et al. Engineering Vascularized Composite Allografts Using Natural Scaffolds: A Systematic Review. Tissue Eng Part B Rev. July 2021.
44. Guyette JP, Charest JM, Mills RW, et al. Bioengineering Human Myocardium on Native Extracellular Matrix. Circ Res. 2016;118(1):56-72.
45. Jank BJ, Goverman J, Guyette JP, et al. Creation of a Bioengineered Skin Flap Scaffold with a Perfusable Vascular Pedicle. Tissue Eng Part A. June 2017.

CHAPTER 02

Mixed Chimerism-Based Regimens in VCA

Alexandre G. Lellouch
Zhi Yang Ng
Josef M. Kurtz
Curtis L. Cetrulo Jr.

Adapted from Curr Transpl Rep 2016;3(4):390-4. DOI 10.1007/s40472-016-0124-7

ABSTRACT

Amputations and devastating injuries throughout the body can now be reconstructed by transplanting vascularized composite allografts (VCAs) of the extremities, face, penis, scalp, skull, abdominal wall, and even the uterus. While functional and quality of life outcomes have been highly promising, the requirement for, and complications from long-term immunosuppression have predictably been reported in VCA recipients as with solid organ transplantation. Immunologic tolerance represents an alternative strategy to prevent rejection of VCAs without the need for immunosuppression. Clinically, long-term tolerance of allografts without maintenance immunosuppression has been achieved in kidney transplantation patients through the induction of mixed chimerism, whereby both donor and recipient- derived lymphohematopoietic elements co-exist within the recipient. This paper serves to review the currently available evidence from both clinical and experimental studies on mixed chimerism based regimens in VCA.

INTRODUCTION

Compared to solid organ transplantation (SOT), vascularized composite allografts (VCAs) encompass various combinations of tissues including bone, muscle, nerve, tendon, as well as skin. To date, the most commonly transplanted VCAs are the upper extremity[1] and face [2], both of which encompass a significant skin component. Other VCAs that have been performed and reported include that of the abdominal wall[3], lower extremity[4], penis[5], uterus [6], scalp, and skull[7]. Based on the latest report of the International Registry on Hand and Composite Tissue Allotransplantation in 2011, patients compliant with triple immunosuppressive regimens (i.e., calcineurin inhibitor, steroids, mycophenolate mofetil (MMF)) were able to maintain and prevent early loss of the VCA[8]. However, as with the SOT experience, long-term immunosuppression has resulted in various systemic sequelae including metabolic, infectious and neoplastic complications[8, 9]. Furthermore, despite the use of potent immunosuppressive therapy, acute rejection of the VCA with skin as its primary target occurs in up to 90 % of patients, most of whom experience at least one such episode within the first year post- transplantation, with almost 60 % developing multiple epi- sodes[8]. To prevent loss of the VCA, increased immunosuppression is required, representing additional morbidity to the patient. In addition, both experimental[10] and clinical[11] reports have recently described features consistent with chronic rejection in VCA. Therefore, as VCAs are typically performed to improve one's quality of life in contrast to life- saving SOT, it remains difficult to uniformly justify VCA with the expense of life-long immunosuppression and associated risks, and case-by-case determinations will be necessary. Thus, the induction of specific tolerance of the VCA in the absence of immunosuppression remains an ideal that would greatly expand its practice. Mixed chimerism, in which both donor- and recipient-derived lymphohematopoietic elements co-exist in the recipient[12], may potentially negate the spec- ter of chronic rejection, and is a proven strategy to enable immunosuppression withdrawal as demonstrated in clinical trials on renal transplantation[13•]. However, success in clinical VCA through similar attempts has remained elusive[14, 15, 16•] and is the subject of the current review. In addition, we will highlight our laboratory's experience in developing clinically relevant immunologic tolerance protocols with the mixed chimerism strategy in preclinical large animal models of VCA.

Tolerance and Mixed Chimerism

In the field of transplantation, tolerance is defined as a state of specific unresponsiveness to donor antigens[17]. Whereas early descriptions of tolerance distinguished between B central tolerance (defined as induced deletion of alloreactive T and B cells in the thymus) and B peripheral pathways (defined as the suppression of alloreactivity by regulatory cell populations), recent understandings of the mechanisms for both the induction and maintenance of immunological tolerance demonstrate a more complex overlapping of these processes. Hence, it is likely that successful tolerance induction strategies will require multiple levels of immune regulation through both pathways[17]. A myriad of strategies to achieve transplantation tolerance have been reported in murine models including induction with high dose tacrolimus[18], the infusion of recipient-derived dendritic cells[19], and donor bone marrow transplantation (BMT) coupled with myeloablative conditioning regimens to achieve hematopoietic stem cell engraftment [20]. The latter approach, in particular, enables the creation of a mixed chimeric recipient. It has been proposed that the mixed chimerism strategy involves donor hematopoietic stem cell (e.g., from donor BMT) engraftment within the recipient's bone marrow, which results in the maintenance of donor cell lineages and provides continued presentation of donor antigens[21]. Migration to, and maturation within the thymus of antigen presenting cells (of both donor and recipient origin) then facilitates clonal de- letion of donor- and recipient-reactive thymocytes (i.e., central deletion). Regulatory T cells, which may be educated either in the thymus or in the periphery within transplanted tissues, presumably control alloreactive thymocytes that have escaped deletion through its immunosuppressive functions[17]. The balance of deletional and regulatory mechanisms in achieving tolerance is complex and translation into the clinic has re- quired modification of myeolablative conditioning protocols that are potentially lethal into non-myeloablative approaches by introducing additional co-stimulatory blockade[22] or the administration of ex vivo expanded regulatory T cells[23] among others.

Stable Versus Transient Mixed Chimerism

Clinically, tolerance of renal allografts has been achieved via the induction of mixed chimerism. The durability of chime- rism induced varied, however, between stable and transient, depending on the institution's protocol[13]. At Stanford, 21 of 22 HLA-matched kidney transplant patients developed chi- merism and 18 fulfilled the criteria for immunosuppression withdrawal. Of these, 16 (7 stable and 9 transient chimerism) were successfully withdrawn from immunosuppression for periods ranging between 2 and 66 months[13]. Remarkably, at Northwestern, 12 of 19 HLA-mismatched re- nal allograft recipients achieved stable mixed chimerism and have managed to come off immunosuppression for more than a year but longer follow-up will be required to determine the utility and efficacy of their protocol[13]. In contrast, at Massachusetts General Hospital, all 10 HLA-mismatched re- nal transplant recipients developed mixed chimerism, albeit transiently, and 7 had immunosuppression discontinued by 14 months post-transplant and remained so between 5 and 13 years[24, 25]. As described previously, stable mixed chimerism achieved in murine models involves central deletion in maintaining long-term donor-specific tolerance. Due to the myeloablative conditioning protocols involved, such chimerism usually re- sults from near-full donor hematopoietic reconstitution and hence, the associated risks of developing potentially lethal graft versus host disease (GvHD) that would be unacceptable clinically[20]. In clinical[24, 25] and non-

human primate[26, 27] renal transplant studies across HLA-mismatched barriers, despite only achieving transient mixed chimerism (due in part to non-myeloablative conditioning regimens), allograft tolerance could still be induced. It is therefore unlikely that the same mechanisms of central, deletional tolerance observed in mice are operational in the context of transient mixed chime- rism. Studies have since suggested that possible, contributing mechanisms include systemic donor-specific tolerance[25], the role of the kidney itself as T cells from tolerant renal patients were found non-reactive against donor renal epithelial cells[28], and the combination of homeostatic expansion and early thymic emigration of regulatory T cells (B Sprangers, T Morokata, and M Sykes, unpubl.). Supporting evidence for the latter derives from observations of intra-graft levels of FoxP3 (transcription factor associated with regulatory T cells), which were higher in long-term tolerant renal patients com- pared to standard transplant recipients[25]. Application of such mixed chimerism strategies has yet to be trialed in clinical VCA due to the risks of myeloablation and attendant hematological and infective complications. Related studies in SOT though have demonstrated the immu- nomodulatory effect of donor bone marrow cell infusion, in the absence of recipient conditioning, and these renal trans- plant patients could subsequently be maintained on overall lower levels of immunosuppression[29]. Extension of this bone marrow-based, immunomodulatory approach has since been investigated clinically in both facial[14, 15] and upper extremity VCA[16]. Perhaps not surprisingly, there was no evidence of mixed chimerism (transient or stable) in the ab- sence of recipient conditioning. While the upper extremity VCA patients were subsequently able to reduce their immu- nosuppressive requirements to monotherapy with tacrolimus, rejection episodes persisted and donor-specific antibody formation was detected[16], demonstrating the lack of tolerance with this protocol. Of note, these results were demonstrated in earlier, pre-clinical swine VCA studies in which bone marrow cell infusion alone prolonged rejection-free survival of allografts but ultimately rejected in the absence of mixed chimerism[30].

MGH Laboratory Experience

Transient Mixed Chimerism

In order to determine if transient mixed chimerism will be sufficient for tolerance of VCAs as has been demonstrated in renal transplantation, we adopted and modified a previously reported tolerance protocol for hematopoietic stem cell transplantation (HSCT) in miniature swine from our laboratory[31]. Briefly, recipient animals received a conditioning regimen consisting of low-dose (100 to 200 cGy) total body irradiation, T cell depletion with CD3 immunotoxin and a 45-day course (taper to discontinuation ater day 30) of cyclosporine- A together with donor bone marrow cell infusion (target dose 1×10^9 cells/kg). Indeed, with higher doses of total body irradiation and bone marrow cells, transient mixed chimerism was achieved. However, following the loss of chimerism, which coincided with the cessation of immunosuppres- sion (as per protocol), rejection of VCA developed[32]. The period during which mixed chimerism was detectable was associated with in vitro donor-specific unresponsiveness which confirms the immunomodulatory effect of donor bone marrow.

Stable Mixed Chimerism

Based on our results thus far, we hypothesized that stable mixed chimerism will be required to achieve tolerance of VCAs. Using the aforementioned protocol[31], VCAs were first transplanted into established mixed chimeric swine at least 40 days after the withdrawal of all immunosuppression. Indefinite acceptance of the VCAs (up to >400 days) without the requirement for additional immunosuppression suggests that the current protocol was sufficient in achieving tolerance to non-hematopoietic donor antigens that may be found within the VCA itself. In order to translate this result into a clinically applicable protocol, we repeated the experiment but performed VCA contemporaneously to the induction of mixed chimerism with HSCT. This Bday 0^ protocol successfully induced stable mixed chimerism and achieved tolerance of the VCA, which represented an important proof-of-concept for the requirement of stable mixed chimerism. In both cases, VCA tolerance was associated with the absence of rejection clinically and on histology, and corresponding in vitro donor- specific unresponsiveness [33]. A caveat to the applicability of these results is that trans- plantation was performed between haplomatched pairs, which would not be feasible in the clinic due to the requirement for related donors (e.g., parent, sibling). As well, the source of HSC in this protocol was cytokine-mobilized peripheral blood mononuclear cells (CM-PBMCs) rather than bone marrow and the target infusion dose of the former is 15-fold higher (15×10^9 cells/kg vs. 1×10^9 cells/kg) compared to the latter. Of note, preliminary analysis revealed striking differences in cellular composition—the percentage of CD3+ cells in CM- PBMCs is approximately 30 % whereas it is <5 % in BM cells (unpublished data). Pre-conditioning of the donor from day −4 prior to VCA is also required when CM-PBMCs are the cell source for HSCT and would therefore not be applicable clinically because face and hand allografts would have to be procured from deceased donors.

Role of MHC Matching

Clinically, MHC matching has not been an absolute criterion in VCA, unlike in BMT (due to the risk of GvHD)[34] and other considerations such as size, sex, and skin color of the transplanted hand or face would have a more direct impact on functional and esthetic results. The impact of MHC matching on the outcomes of tolerance induction through mixed chime- rism also remains unclear[35]. Attempts in applying our successful VCA tolerance protocol to fully mismatched donor- recipient pairs has led, however, to the development of GvHD and post-transplant lymphoproliferative disorder (PTLD). While a robust tolerance protocol should be sufficient in overcoming full MHC mismatch barriers to reflect the challenge of finding matching donors clinically, such systemic complications resulting from fully allogeneic HSCT necessitated euthanasia and premature termination of experiments (survival from POD 13 to 55) even though all VCAs remained rejection-free up to experimental end point (unpublished data).

The extent of HLA mismatch in clinical VCA has been highly varied though, ranging from 2/6 to 6/6 [4, 8, 9]. To mirror such clinical scenarios, we applied our successful tol- erance protocol to VCAs for transplantation across MHC class I mismatch and MHC class II mismatch barriers. As before, multi-lineage stable mixed chimerism was achieved together with in vitro donor-specific unresponsiveness. However, in MHC class I mismatched (i.e. class II matched) chimeric swine, rather than improved transplantation outcomes as sug- gested from SOT studies[36], there were acute rejection episodes which

would not be acceptable clinically, especially in a face transplant. In contrast, MHC class II mismatched (i.e. class I matched) animals successfully achieved tolerance as seen previously in our haplomatched swine studies[37].

These results have important implications on the extent of genetic matching for donor selection in future clinical trials on the induction of stable mixed chimerism for VCA tolerance, as well as in further studies that are underway to determine countermeasures to the Bsplit tolerance^ [30] observed in Class I mismatched pairs.

CONCLUSIONS

VCA represents a culmination of decades of progress in transplantation immunology and reconstructive microsurgery. Functional results to date have been highly encouraging and immunological outcomes based on studies from both the clinic and laboratory have provided a unique opportunity for further study on the mechanisms underlying tolerance induction via the mixed chimerism strategy. What started as a curious observation by Ray Owen in freemantle cattle has found application to the benefit of renal transplant patients. We anticipate similar progress to eventually achieve successful tolerance of VCA.

REFERENCES

Papers of particular interest, published recently, have been highlighted as:
• Of importance

1. Shores JT, Brandacher G, Lee WP. Hand and upper extremity trans- plantation: an update of outcomes in the worldwide experience. Plast Reconstr Surg. 2015;135(2):351e–60e.
2. Khalifian S, Brazio PS, Mohan R, Shaffer C, Brandacher G, Barth RN, et al. Facial transplantation: the first 9 years. Lancet. 2014;384(9960):2153–63.
3. Giele H, Vaidya A, Reddy S, Vrakas G, Friend P. Current state of abdominal wall transplantation. Curr Opin Organ Transplant. 2016;21(2):159–64.
4. Cavadas PC, Thione A, Carballeira A, Blanes M. Bilateral transfemoral lower extremity transplantation: result at 1 year. Am J Transplant. 2013;13(5):1343–9.
5. Caplan AL, Kimberly LL, Parent B, Sosin M, Rodriguez ED. The ethics of penile transplantation: preliminary recommendations. Transplantation. 2016. doi:10.1097/TP.0000000000001352.
6. Brännström M, Johannesson L, Bokström H, Kvarnström N, Mölne J, Dahm-Kähler P, et al. Livebirth after uterus transplantation. Lancet. 2015;385(9968):607–16.
7. Selber JC, Chang EI, Clemens MW, Gaber L, Hanasono MM, Klebuc M, et al. Simultaneous scalp, skull, kidney, and pancreas transplant from a single donor. Plast Reconstr Surg. 2016;137(6): 1851–61.
8. Petruzzo P, Dubernard JM. The International Registry on Hand and Composite Tissue allotransplantation. Clin Transpl. 2011:247–53.
9. Petruzzo P, Lanzetta M, Dubernard JM, Landin L, Cavadas P, Margreiter R, et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation. 2010;90(12): 1590–4.
10. Mundinger GS, Munivenkatappa R, Drachenberg CB, Ha JS, Vaca EE, Shipley ST, et al. Histopathology of chronic rejection in a nonhuman primate model of vascularized composite allotransplan- tation. Transplantation. 2013;95(10):1204–10.
11. Kaufman CL, Ouseph R, Blair B, Kutz JE, Tsai TM, Scheker LR, et al. Graft vasculopathy in clinical hand transplantation. Am J Transplant. 2012;12(4):1004–16.
12. Sykes M. Mixed chimerism and transplant tolerance. Immunity. 2001;14(4):417–24.
13. Elias N, Cosimi AB, Kawai T. Clinical trials for induction of renal allograft tolerance. Curr Opin organ transplant. 2015;20(4):406–11

Comprehensive review of clinical experience with mixed chime- rism protocols in renal transplantation. Provides background for translation to VCA.

14. Dubernard JM, Lengelé B, Morelon E, Testelin S, Badet L, Moure C, et al. Outcomes 18 months after the first human partial face transplantation. N Engl J Med. 2007;357(24):2451–60.
15. Petruzzo P, Kanitakis J, Testelin S, Pialat JB, Buron F, Badet L, et al. Clinicopathological findings of chronic rejection in a face grafted patient. Transplantation. 2015;99(12):2644–50.
16. Schneeberger S, Gorantla VS, Brandacher G, Zeevi A, Demetris AJ, Lunz JG, et al. Upper-extremity transplantation using a cell- based protocol to minimize immunosuppression. Ann Surg. 2013;257(2):345–51 First clinical series in VCA involving bone marrow cell infusion.
17. Sykes M. Immune tolerance: mechanisms and application in clini- cal transplantation. J Intern Med. 2007;262(3):288–310.
18. Timmermann W, Otto C, Gasser M, Meyer D, Parthum E, Schad J, et al. Long-term small bowel allograft function induced by short- term FK 506 application is associated with split tolerance. Transpl Int. 2000;13(Suppl 1):S532–6.
19. Bériou G, Pêche H, Guillonneau C, Merieau E, Cuturi MC. Donor- specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. Transplantation. 2005;79(8):969–72.
20. Sachs DH, Kawai T, Sykes M. Induction of tolerance through mixed chimerism. Cold Spring Harb Perspect Med. 2014;4(1): a015529.
21. Pilat N, Wekerle T. Transplantation tolerance through mixed chi- merism. Nat Rev Nephrol. 2010;6(10):594–605.
22. Pilat N, Klaus C, Schwarz C, Hock K, Oberhuber R, Schwaiger E, et al. Rapamycin and CTLA4Ig synergize to induce stable mixed chimerism without the need for CD40 blockade. Am J Transplant. 2015;15(6):1568–79.
23. Pilat N, Klaus C, Hock K, Baranyi U, Unger L, Mahr B, et al. Polyclonal recipient nTregs are superior to donor or third-party Tregs in the induction of transplantation tolerance. J Immunol Res. 2015;2015:562935. Kawai T, Sachs DH, Sprangers B, Spitzer TR, Saidman SL, Zorn E, et al. Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance im- munosuppression. Am J Transplant. 2014;14(7):1599–611.

24. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantation with- out maintenance immunosuppression. N Engl J Med. 2008;358(4): 353–61.
25. Kawai T, Cosimi AB, Colvin RB, Powelson J, Eason J, Kozlowski T, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. Transplantation. 1995;59(2):256–62.
26. Kawai T, Sogawa H, Boskovic S, Abrahamian G, Smith RN, Wee SL, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. Am J Transplant. 2004;4(9):1391–8.
27. Fudaba Y, Spitzer TR, Shaffer J, Kawai T, Fehr T, Delmonico F, et al. Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: in vivo and in vitro analyses. Am J Transplant. 2006;6(9):2121–33.
28. Ciancio G, Miller J, Garcia-Morales RO, Carreno M, Burke 3rd GW, Roth D, et al. Six-year clinical effect of donor bone marrow infusions in renal transplant patients. Transplantation. 2001;71(7): 827–35.
29. Hettiaratchy S, Melendy E, Randolph MA, Coburn RC, Neville Jr DM, Sachs DH, et al. Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine. Transplantation. 2004;77(4):514–21.
30. Huang CA, Fuchimoto Y, Scheier-Dolberg R, Murphy MC, Neville Jr DM, Sachs DH. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. J Clin Invest. 2000;105(2):173–81.
31. Leto Barone AA, Kurtz JM, Albritton A, Mallard CA, Shanmugarajah K, Torabi R, et al. Effects of transient donor chi- merism on rejection of MHC-mismatched vascularized composite allografts in swine. Vasc Comp Allotransplantation. 2015;2(1):1–8 First pre-clinical large animal study to demonstrate transient mixed chimerism will not be sufficient for achieving VCA tolerance.
32. Leonard DA, Kurtz JM, Mallard C, Albritton A, Duran-Struuck R, Farkash EA, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant. 2014;14(2):343–55 First pre-clinical large animal study to dem- onstrate the requirement for stable mixed chimerism in achiev- ing VCA tolerance.
33. Greinix HT, Faé I, Schneider B, Rosenmayr A, Mitterschiffthaler A, Pelzmann B, et al. Impact of HLA class I high-resolution mis- matches on chronic graft-versus-host disease and survival of pa- tients given hematopoietic stem cell grafts from unrelated donors. Bone Marrow Transplant. 2005;35(1):57–62.
34. Larsen CP, Page A, Linzie KH, Russell M, Deane T, Stempora L, et al. An MHC-defined primate model reveals significant rejection of bone marrow after mixed chimerism induction despite full MHC matching. Am J Transplant. 2010;10(11):2396–409.
35. Wiebe C, Pochinco D, Blydt-Hansen TD, Ho J, Birk PE, Karpinski M, et al. Class II HLA epitope matching—a strategy to minimize de novo donor-specific antibody development and improve outcomes. Am J Transplant. 2013;13(12):3114–22.
36. Leonard DA, Shanmugarajah K, Powell H, Albritton A, Mallard C, Sachs DH, et al. Abstract 10: the role of skin-specific immunobiology and major histocompatibility complex sharing in vascularized composite allograft tolerance. Plast Reconstr Surg. 2015;135(5S):17.

CHAPTER 03

Tolerance of VCAs via mixed chimerism

Alexandre G. Lellouch
Alec R. Andrews
Gaelle Saviane
Zhi Yang Ng
Ilse M. Schol
Marion Goutard
Amon-Ra Gama
Ivy A. Rosales
Robert B. Colvin
Laurent A. Lantieri
Mark A. Randolph
Gilles Benichou
Curtis L. Cetrulo Jr.

In submission in Frontier In Immunology Journal

ABSTRACT

Background: Vascularized composite allografts (VCAs) allow reconstruction of devastating injuries and amputations, yet require lifelong immunosuppression that is associated with significant morbidity. Induction of immune tolerance of VCAs would permit widespread use of these procedures. VCAs are acquired from deceased donors most likely to be fully-MHC-mismatched (in contrast to living-related renal transplant donor-recipient pairs matched at one MHC haplotype). After achieving VCA tolerance in a swine model equivalent to clinical living-related renal transplants (single-haplotype MHC mismatches: e.g., "mother-daughter"/haploidentical), we tested our protocol on fully MHC-mismatched swine pairs. However, we observed either poor stem cell engraftment or transient chimerism insufficient for tolerance, or morbidity secondary to harsh myeloablative conditioning. We attempted to tease out the swine leukocyte antigens (SLA) responsible for the failure in fully mismatched pairs and test the previously successful haploidentical protocol in both MHC class I-matched/class II-mismatched barriers and vice versa (i.e. MHC class I-mismatched/class II-matched barriers). In these studies, the Class II mismatched results mirrored the haploidentical transplant results: stable mixed chimerism and tolerance. Surprisingly, even though we obtained stable mixed chimerism in the Class I-mismatches, the VCAs were rejected.

Methods: To solve Class-I-mismatch rejection, we moderately increased irradiation of the whole body and thymus, replaced cyclosporine with FK506, added co-stimulatory blockade with CTLA4-Ig, added anti-IL6R mAb and added a vascularized bone marrow component to our VCA by including the femur. Swine were treated with non-myeloablative total body irradiation and thymic irradiation two days prior to infusion of donor bone marrow cells from an MHC class I-mismatched donor. They also received a short-term treatment with CTLA4-Ig (Belatacept®) and anti-IL6R mAb (Tociluzimab®) and were transplanted with an osteomyocutaneous VCA from the same donor.

Results: Stable mixed chimerism and tolerance of MHC class-I-mismatched VCAs in the absence was achieved in 3 swine. Allograft tolerance was associated with a sustained lack of anti-donor T cell response and a concomitant expansion of double negative CD4-CD8- T cells producing IL-10.

Conclusions: This study demonstrates the first successful mixed chimerism-induced VCA tolerance in a large animal model across a Class-I-mismatch. Future studies aimed at fully-mismatched donor-recipient pairs are under investigation with this protocol.

INTRODUCTION

Since the first successful hand transplant was performed in France in 1998 (Dubernard et al., 1999) the field of vascularized composite allotransplantation (VCA) has progressed to a collective, worldwide experience of more than 120 hand transplants, 46 face transplants (Rifkin et al., 2021) and a myriad of other VCAs including lower limb, abdominal wall, penis, laryngeal, scalp and even uterine transplants.(Brannstrom et al., 2015) These remarkable procedures have revolutionized reconstructive surgery with unprecedented outcomes in aesthetic form and functional return after devastating injury or cancer ablation. As with all allotransplants including life-saving solid organ transplants, however, long-term systemic immunosuppression is required to prevent allograft rejection, imparting a risk of significant morbidity that includes metabolic, infectious, renovascular and neoplastic complications.(Petruzzo P, 2017; Conrad et al., 2019)

In order to offer widespread application of these procedures, we and others have pursued the induction of immunologic tolerance by establishing mixed hematopoietic chimerism (Kawai et al., 2013; Kawai et al., 2014); a state in which the transplant recipient's hematopoietic system consists of both donor and recipient elements, such that the transplanted allograft is recognized as "self" and thereby permanently accepted after the cessation of immunosuppression. Mixed chimerism protocols developed in the genetically defined Massachusetts General Hospital (MGH) swine model for renal transplantation were successfully translated to the clinic for living-related donor-recipient kidney transplant pairs with single haplotype major histocompatibility complex (MHC) mismatches (haploidentical).(Elias et al., 2015) Following this blueprint, our laboratory developed a similar protocol for VCA in MGH swine obtaining stable mixed chimerism and long-term VCA tolerance through VCA and co-infusion of cytokine-mobilized peripheral blood donor stem cells (CM-PBMCs) from a haploidentical donor.(Leonard et al., 2014)

Clinically, VCAs are acquired from deceased donors that are most likely to be fully MHC mismatched (unlike a living-related renal transplant donor-recipient pair that are matched at one MHC haplotype). After achieving success in a preclinical VCA swine model equivalent to clinical living-related renal transplant results (single-haplotype MHC mismatches: e.g., "mother-daughter"/haploidentical), we proceeded to test this protocol on fully MHC-mismatched swine pairs. However, we observed either poor stem cell engraftment and only transient chimerism that was insufficient for tolerance, or morbidity secondary to the requirement for harsh myeloablative conditioning.

Using controlled mismatches available with the MGH swine herd, we attempted to tease out the swine leukocyte antigens (SLA) responsible for the failure in fully mismatched pairs and tested the previously successful haploidentical protocol using CM-PBMCs in both MHC class I-matched/class II-mismatched barriers and vice versa (i.e. MHC class I-mismatched/class II-matched barriers). In these studies, the Class II mismatched results mirrored the haploidentical transplant results; the CM-PBMC stem cell source resulted in stable mixed chimerism and tolerance. Surprisingly, even though we obtained stable mixed chimerism in the Class I-mismatches, the VCAs were rejected.(Shanmugarajah et al., 2017)

To solve the Class-I-mismatch rejection problem, we made the following changes to our regimen: we moderately increased irradiation of the whole body and thymus, replaced cyclosporine with FK506, added co-stimulatory blockade with CTLA4-Ig, added

anti-IL6R mAb and added a vascularized bone marrow component to our VCA by including the femur in the transplanted tissue. With this regimen, we achieved reproducible, stable mixed chimerism for the first-time across a Class I mismatch barrier in a large animal model, demonstrating that tolerance to VCA is possible in a clinically relevant large animal model. Importantly, we achieved stable mixed chimerism with use of donor bone marrow as our stem cell source (rather than CM-PBMCs) which resulted in long-term immunosuppression-free tolerance of all components of the VCAs including the epidermis. Additionally, all conditioning medications (FK506, CTLA4-Ig, steroids, and anti-IL6R mAb) employed in this study are FDA-approved for clinical use in various other conditions, and while the protocol tested here was not intended for clinical readiness, these attributes (donor bone marrow and approved drugs), taken together, will be valuable for eventual clinical tolerance protocols across full deceased donor-recipient mismatches (mismatched at Class I and Class II).

MATERIALS AND METHODS

Animals

All animal care and procedures were approved by the MGH Institutional Animal Care and Use Committee (IACUC) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press. This study utilized the MGH miniature swine model.(Sachs et al., 1976) Lines of MGH miniature swine are bred in a pathogen-free facility with defined MHC while maintaining minor antigen variation. Donors and recipients were matched for Class II SLA and mismatched for Class I SLA. Donors were positive for the expression of pig allelic antigen (PAA) to permit analysis of chimerism when transplanted into PAA negative recipients. Osteomyocutaneous VCAs consisting of the distal femur, proximal tibia, muscle cuff, and an island of vascularized skin were performed as previously described.(Hettiaratchy et al., 2004) Donor bone marrow was manually collected from the spine and the long bones of the carcass, filtered, and prepared for intravenous infusion into the recipient at the end of the day of surgery.

Mixed Chimerism Protocol

The timeline and treatment protocol are depicted in Figure 1. Swine were conditioned with 300 cGy total body irradiation (TBI) and 700 cGy thymic irradiation (TI) both on day minus 2. Following donor bone marrow transplantation (target 1x109 cells/kg) on the day of VCA surgery, the animals received co-stimulatory blockade using CTLA4-Ig (Belatacept®, Bristol-Myers-Squibb, Princeton, NJ) (20 mg/kg IV on days 0, 2, 4 and 6) and maintenance on intravenous FK506 for 30 days (target level 10-15 ng/mL). After day 30 the FK506 was reduced in a tapered dose to permit discontinuation by day 45. With the development of idiopathic pulmonary syndrome (IPS) in recipients 1 (R1) and R2, additional methylprednisolone (IM 10 mg) on days -2, -1, 0) and anti-IL6R mAb (Tociluzimab®, Genentech, San Francisco, CA.) (IV 10 mg/kg on days 0, 7, 14, 21, 28) were added to the remaining three recipients. Two untreated control swine (R6 and R7) received the same VCA across class-I-mismatch barriers without conditioning, bone marrow transplantation, or maintenance immunosuppression.

VCA Transplantation

Postoperatively, VCAs were monitored daily for clinical signs of rejection, defined by increased redness, swelling and epidermolysis. Tolerance was defined as rejection-free VCA survival for >100 days without immunosuppression, and with corresponding in vitro evidence of donor-specific unresponsiveness. VCA and host skin biopsies were also taken for histopathological analysis when clinical rejection was suspected, and at postoperative day (POD) 14, 30, 50, and every 50 days thereafter up to the experimental endpoint of POD 250, and secondarily extended to 400 days. Pathologic evaluation of VCA biopsies was performed on sections stained with hematoxylin and eosin (H&E) in accordance with the 2007 Banff Working Classification.(Cendales et al., 2008) The specificity and durability of immune tolerance was tested by applying split-thickness skin grafts at approximately day 150 on the recipients and measuring survival or rejection times. The grafted skin included autologous control skin, skin from the original VCA donor, and skin from a fully mismatched third-party swine.

Chimerism Analysis

Peripheral blood mononuclear cells (PBMCs) were collected weekly to assess chimerism by flow cytometry. Monoclonal antibodies used include PAA (1038H-10-9, mouse IgM) and lineage markers to CD3 (BB23-8E6, mouse IgG2a), CD4 (4-12-4, mouse IgG2b), CD8a (76-2-11, mouse IgG2a), CD21 (B-ly4, mouse IgG1), CD79a (HM47, mouse IgG1), CD172a (74-22-15, mouse IgG1), and FOXP3 (FJK-16s, rat IgG2a). Gating strategy used for characterizing chimeric populations included PAA+ subsets of the following markers: T-lymphocytes (CD3+), B-lymphocytes (either CD3-, CD21+ or CD79a+), Myeloid Cells (CD172a+), NK Cells (CD3-, CD8+), T-regs (CD3+, CD4+, FOXP3+). Flow cytometry data was collected using a FACSVerse (Becton Dickinson, California) and analyzed with FlowJo analysis software (FlowJo, LLC, Ashland, OR, USA).

Mixed Lymphocyte Reaction

Mixed lymphocyte reactions (MLRs) were performed to test T cell allo-responsiveness of transplanted swine.(Thistlethwaite et al., 1984) Briefly, recipient PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) proliferation dye (BioLegend, San Diego, CA) for 10 minutes at 37°C, washed twice in complete RPMI, and plated at a concentration of 4x106 cells/ml in 96-well flat-bottom tissue culture treated plates (Corning, NY). Stimulators cells consisting of either donor, or third-party swine antigen-presenting cells (APCs) were irradiated at 3000rads. Irradiated stimulators were co-incubated with CFSE labeled recipient cells at a 1:1 ratio for 96 hours at 37°C in 5% CO2. After incubation, cells were labeled with fixable viability dye eFluor 780 (eBioscience, ThermoFisher Scientific, Waltham, MA) followed by surface staining with anti-swine CD3 antibody. Samples were acquired with a FACSVerse (Becton Dickinson, California) and data were analyzed with FlowJo analysis software (FlowJo, LLC, Ashland, OR, USA). Viable CD3+ cell populations were assessed for CFSE fluorescence. Proliferation of recipient cells after co-incubation with stimulators was determined by dilution of the CFSE dye.

Cytokine Analysis to Assess Inflammatory Response

The capacity of peripheral blood cells to secrete cytokines was analyzed by intra-

cytoplasmic staining. Recipient PBMCs were incubated for 5 hours with Cell Stimulation Cocktail (Thermo Fisher Scientific, Waltham, MA) and BrefeldinA (Thermo Fisher Scientific, Waltham, MA) solution, to block cytokine secretion, and washed in phosphate buffered saline. Thereafter, the cells were first incubated with surface marker antibodies such as PAA, CD3, CD4, and CD8, before fixation and permeabilization (Thermo Fisher Scientific, Waltham, MA) to allow entry of anti-IL-10 (clone 945A-1A9-26C2, mouse IgG1). The cells were acquired with a FACSVerse flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed with FlowJo (FlowJo LLC, Ashland, OR).

Donor Specific Antibody Detection

The presence of donor specific antibody in peripheral blood was tested using serum from recipient animals obtained at 50-day intervals. Recipient sera were heat-inactivated and incubated with donor, recipient and third-party PBMCs. The binding of serum IgG antibodies to B and T lymphocytes was then analyzed by flow cytometry using FITC-conjugated goat anti-swine IgG (heavy and light chain reactive; Seracare, Milford, MA). Samples were acquired with a FACSVerse flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed with FlowJo (FlowJo LLC, Ashland, OR).

RESULTS

Clinical and Histological Results of Long-term VCA Survival

Recipients R1 and R2 developed an idiopathic pneumonia-like syndrome (IPS) within 2-3 weeks of transplantation and were euthanized. Subsequent recipients were treated with methylprednisolone preoperatively and Tocilizumab® postoperatively. As a result, recipients R3, R4, and R5 survived and had viable accepted grafts for up to 400 days (study end point) (Fig. 2A-F). R4 developed a minor rejection episode on day 256 that resolved spontaneously. It is important to note that none of the recipients R3, R4 and R5 showed any signs of cutaneous or systemic GVHD at any time point post-transplantation. Therefore, all VCAs placed in swine (n=3) treated with our mixed chimerism protocol achieved long-term, immunosuppression-free survival. Class-I-mismatch VCA recipients that did not receive conditioning, bone marrow transplantation, or maintenance immunosuppression promptly rejected their transplants by day 11 (Fig. 2G-H)

Stable Chimerism in the Absence of Ongoing Immunosuppression

Recipient conditioning led to minimal leukocyte depletion in the peripheral blood and both myeloid and lymphoid cell compartments were fully restored within 14 days post-transplantation (Fig. 3). Importantly, all recipients displayed multilineage mixed hematopoietic chimerism during the entire duration of the study (400 days), as detected by staining with anti-PAA antibody(Fuchimoto et al., 1999), including all myeloid and lymphoid subsets (Fig. 3). Virtually all recipient myeloid cells were replaced by donor cells (Fig. 3). Donor chimerism ranging from 30-60% donor cells was detected for all other leukocyte subsets, including NK cells, B and T lymphocytes (Fig. 3). The frequencies of recipient (PAA-) T cells decreased progressively while the proportion of donor (PAA+) T cells increased until around day 100 (Fig. 4). Subsequently, the percentages of donor chimerism among T cells remained stable varying between 50-70% (Fig. 4).

Tolerance of VCAs was Donor-Specific

At day 150 post-transplantation, each long-term recipient received 3 split-thickness skin grafts: an autologous graft, one from the VCA donor, and one skin graft from a third-party swine (MHC-mismatched to both donor and recipient). Both autologous and donor-derived skin grafts were accepted without any immunosuppression until the end of the study. In contrast, all third-party skin grafts were rejected within 9 days after their placement (Fig. 5). These results demonstrate that tolerance of VCAs was donor specific, yet the animals remained immunocompetent.

Tolerance of VCAs was Associated with Lack of Inflammatory T and B Cell Alloresponses

Next, we investigated the status and anti-donor responses of T and B cells in transplanted swine. No significant increase in the frequencies of memory T cells was detected post-conditioning and after VCA transplantation, which indicates a lack of homeostatic expansion after leukodepletion and suggests a lack of sensitization to donor antigens after allotransplantation (data not shown). In addition, it is noteworthy that we did not observe any expansion of FOXP3+ regulatory T cells (data not shown).

Therefore, tolerance of VCAs was associated with a sustained lack of expansion/activation of donor-reactive T cells recognizing donor antigens through the direct allorecognition pathway (Fig. 6A). Also, no donor-specific antibodies were detected at any given time point in serum samples of tolerant swine (data not shown). Finally, starting early post-bone marrow transplantation (day 7), we observed a substantial expansion of T cells producing IL-10 among donor T cells (Fig. 6B). Most of these IL-10 producing T cells were double negative (DN) CD4-CD8- T cells (Fig. 6C).

5. DISCUSSION

Vascularized composite allotransplantation has revolutionized reconstructive surgery with unparalleled aesthetic and functional results achieved in over 120 hand transplants and more than 40 face transplants over the last two decades. These innovative procedures represent a paradigm shift in our ability to reconstruct severe craniofacial deformities and restore function to amputees. Recently this approach has been extended to genitourinary tissue, with 4 successful penis transplants performed over the past three years.(van der Merwe et al., 2017) The VCA field is evolving past the proof-of-concept stage and into the need for reduction of the risk-benefit ratio to allow more widespread application of these revolutionary procedures. VCA patients are as susceptible as solid organ transplant recipients to complications from maintenance immunosuppression that include neoplasm, infection, and renal failure. As the population of long-term VCA recipients increases, so does the observed incidence of chronic rejection—an even more severe complication for which there is currently no solution other than re-transplantation.(Lantieri et al., 2020)

In this context, the need for strategies to induce a state of immunologic tolerance in VCA is more important than ever. The mitigation of both acute and chronic rejection has been achieved with mixed chimerism regimens in large animals and in clinical renal transplant tolerance induction.(Kawai et al., 2013) Mixed chimerism is the coexistence of both donor and recipient hematopoietic cells in a homeostatic state that renders the

recipient tolerant to the donor organs or tissues. Transplanted organs in individuals that have undergone a mixed chimerism based tolerance induction protocol appear pristine by biopsy even decades after transplantation.

In the current study, we have achieved an important milestone towards the goal of a tolerance protocol for VCA based on the induction of mixed hematopoietic chimerism. Such a protocol would need to be safe and reproducible for fully mismatched donor-recipient pairs, which are the most likely matching scenarios in clinical VCA transplantation where more closely matched pairs (such as with living related kidney donors) are not possible (i.e. relatives cannot donate hands or faces). A mixed chimerism protocol would also require a clinically relevant source of hematopoietic stem cells rather than cytokine-mobilized peripheral blood stem cells (CM-PBMC). CM-PBMC would be difficult to acquire in clinical VCA transplantation from a multi-organ donor, as the cytokines given to the donor may affect the suitability of the other organs for transplantation. Furthermore, it is impractical to delay the procurement teams for the various organs while performing leukopheresis to acquire the donor's peripheral blood stem cells.

The use of CM-PBMCs as a stem cell source at supraphysiologic doses led to successful and stable mixed chimerism induction in the lab in our MGH VCA swine model, in which defined SLA permit mismatched pairs that mimic clinical mismatch situations.(Sachs et al., 1976) We obtained immunosuppression-free VCA tolerance across haploidentical mismatches(Leonard et al., 2014) (analogous to a mother-daughter living related donor-recipient transplant pair). However, we encountered several problems when applying this regimen to fully mismatched swine pairs: on one hand, the full mismatch barrier required more ablative conditioning (for ex. higher doses of irradiation) to obtain stem cell engraftment in the recipient with large doses of CM-PBMC- resulting in early toxicity or graft versus host disease. Additionally, the number of bone marrow-derived stem cell and/or quality were not sufficient to engraft to produce stable chimerism, and the VCAs rejected as soon as chimerism disappeared.(Leto Barone AA and et al., 2015)

The mechanisms underlying the induction and maintenance of mixed chimerism for immune tolerance to VCAs remain elusive. Our laboratory recently showed that recipients with VCAs performed directly after (day 3) establishment of mixed chimerism (from days 0 to 2) could be rendered tolerant.(Leonard et al., 2014) Neither T cell anergy nor peripheral regulation by regulatory T cells could be demonstrated to have a contributory role however, which suggests that local mechanism(s) might be responsible.(Leonard et al., 2014)

The features of the MGH Swine herd allowed us to attempt to tease out the SLA responsible for the failure in fully-mismatched pairs and test the previously successful haploidentical protocol (using CM-PBMCs in both MHC class I-matched/class II-mismatched barriers and vice versa i.e. (MHC class I-mismatched/class II-matched barriers).(Shanmugarajah et al., 2017) In these studies, the Class II mismatched results mirrored the haploidentical transplant results- the CM-PBMC stem cell source resulted in stable, mixed chimerism and tolerance. Surprisingly, despite the CM-PBMC stem cell source again resulting in stable, mixed chimerism, the Class I mismatches either partially or fully rejected. Some animals rejected only the allograft epidermis and healed the epidermis with host skin while remaining tolerant to the underlying dermis, whereas other animals fully rejected the entire VCA.

To solve the Class-I-mismatch rejection problem, the following changes were made to the conditioning regimen in this study: irradiation of the whole body and thymus

were increased, cyclosporine was replaced with FK506, co-stimulatory blockade with CTLA4-Ig was added, and anti-IL6R mAb was added, and a vascularized bone marrow component was included by incorporating the swine's femur in the VCA. With this regimen, we achieved reproducible, stable mixed chimerism for the first-time using swine donor bone marrow as our stem cell source rather than CM-PBMCs. The stable mixed chimerism resulted in long-term immunosuppression-free tolerance of all components—epidermis included—of the VCAs.

It should be emphasized that the protocol tested here was not intended for clinical readiness. However, all conditioning medications (FK506, CTLA4-Ig, steroids, and anti-IL6R mAb) employed in this study are FDA-approved for clinical use in various other conditions, and these attributes (use of donor bone marrow rather than CM-PBMCs, as well as FDA-approved drugs), taken together, will be valuable for eventual clinical tolerance protocols across full deceased donor-recipient mismatches (mismatched at Class I and Class II).

In the current study, no significant increase in the number of memory T cells were detected post-conditioning and transplantation, which suggests a lack of homeostatic expansion and sensitization to donor antigens. We also did not observe any expansion of FOXP3+ regulatory T cells. MLR assays also showed a lack of expansion of activated donor specific T cells in tolerant swine. At the same time, the observation that some T cells were still activated via in vitro stimulation with donor APCs suggests that donor-reactive recipient T cells had not been deleted in tolerant swine, which suggests that peripheral mechanisms may be in play. Interestingly, cytokine analysis showed an early and sustained increase in the frequency of CD4-CD8- DN donor T cells producing IL-10, a regulatory cytokine. Similar DN T cells studied in rodents were previously shown to inhibit immune responses mediated by effector CD4+ and CD8+ T cells and prevented allograft rejection(Tizon et al., 2012; Su and Fairchild, 2014) and GVHD(Cooke et al., 2000; Ahmed et al., 2011). In these studies, DN cells mediated their suppressive functions through down regulation of co-stimulatory molecules (CD80, CD86) by Dendritic cells (DCs) and inhibition of their APC functions and by inducing apoptosis of DCs. In humans, DN T cells were shown to acquire MHC peptide complexes from DCs via trogocytosis and induce apoptosis of corresponding CD8+ cytotoxic T cells.(Wang et al., 2010; Miller et al., 2015) Together with our results, it suggests that DN T cells may contribute to VCA tolerance induced via mixed chimerism in our study.

While the regimen used in this study has not yet been tested in Class II or haplo-mismatched pairs, the results demonstrate significant progress in Class I tolerance compared to historical control animals treated with our previous conditioning regimen. Further work will examine which added variables (CTLA4-Ig, FK506, vascularized bone compartment) permitted both engraftment of bone marrow-derived stem cells and stable mixed chimerism and tolerance of the VCA.

REFERENCES

Ahmed, E.B., Wang, T., Daniels, M., Alegre, M.L., and Chong, A.S. (2011). IL-6 induced by Staphylococcus aureus infection prevents the induction of skin allograft acceptance in mice. Am J Transplant 11(5), 936-946. doi: 10.1111/j.1600-6143.2011.03476.x.

Brannstrom, M., Diaz-Garcia, C., Johannesson, L., Dahm-Kahler, P., and Bokstrom, H. (2015). Livebirth after uterus transplantation - Authors' reply. Lancet 385(9985), 2352-2353. doi: 10.1016/S0140-6736(15)61098-4.

Cendales, L.C., Kanitakis, J., Schneeberger, S., Burns, C., Ruiz, P., Landin, L., et al. (2008). The Banff 2007 working classification of skin-containing composite tissue allograft pathology. Am J Transplant 8(7), 1396-1400. doi: 10.1111/j.1600-6143.2008.02243.x.

Conrad, A., Petruzzo, P., Kanitakis, J., Gazarian, A., Badet, L., Thaunat, O., et al. (2019). Infections after upper extremity allotransplantation: a worldwide population cohort study, 1998-2017. Transpl Int 32(7), 693-701. doi: 10.1111/tri.13399.

Cooke, K.R., Hill, G.R., Gerbitz, A., Kobzik, L., Martin, T.R., Crawford, J.M., et al. (2000). Tumor necrosis factor-alpha neutralization reduces lung injury after experimental allogeneic bone marrow transplantation. Transplantation 70(2), 272-279. doi: 10.1097/00007890-200007270-00006.

Dubernard, J.M., Owen, E., Herzberg, G., Lanzetta, M., Martin, X., Kapila, H., et al. (1999). Human hand allograft: report on first 6 months. Lancet 353(9161), 1315-1320. doi: 10.1016/s0140-6736(99)02062-0.

Elias, N., Cosimi, A.B., and Kawai, T. (2015). Clinical trials for induction of renal allograft tolerance. Curr Opin Organ Transplant 20(4), 406-411. doi: 10.1097/MOT.0000000000000211.

Fuchimoto, Y., Huang, C., Shimizu, A., Seebach, J., Arn, S., and Sachs, D.H. (1999). An allelic non-histocompatibility antigen with wide tissue distribution as a marker for chimerism in pigs. Tissue Antigens 54(1), 43-52. doi: 10.1034/j.1399-0039.1999.540105.x.

Hettiaratchy, S., Melendy, E., Randolph, M.A., Coburn, R.C., Neville, D.M., Jr., Sachs, D.H., et al. (2004). Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine. Transplantation 77(4), 514-521. doi: 10.1097/01.tp.0000113806.52063.42.

Kawai, T., Sachs, D.H., Sprangers, B., Spitzer, T.R., Saidman, S.L., Zorn, E., et al. (2014). Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance immunosuppression. Am J Transplant 14(7), 1599-1611. doi: 10.1111/ajt.12731.

Kawai, T., Sachs, D.H., Sykes, M., Cosimi, A.B., and Immune Tolerance, N. (2013). HLA-mismatched renal transplantation without maintenance immunosuppression. N Engl J Med 368(19), 1850-1852. doi: 10.1056/NEJMc1213779.

Lantieri, L., Cholley, B., Lemogne, C., Guillemain, R., Ortonne, N., Grimbert, P., et al. (2020). First human facial retransplantation: 30-month follow-up. Lancet 396(10264), 1758-1765. doi: 10.1016/S0140-6736(20)32438-7.

Leonard, D.A., Kurtz, J.M., Mallard, C., Albritton, A., Duran-Struuck, R., Farkash, E.A., et al. (2014). Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant 14(2), 343-355. doi: 10.1111/ajt.12560.

Leto Barone AA, K.J., Albritton A,, and et al. (2015). Effects of Transient Donor Chimerism on Rejection of MHC-Mismatched Vascularized Composite Allografts in Swine. Vasc Compos Allotransplantation 2(1), 1-8.

Miller, M.L., Daniels, M.D., Wang, T., Chen, J., Young, J., Xu, J., et al. (2015). Spontaneous restoration of transplantation tolerance after acute rejection. Nat Commun 6, 7566. doi: 10.1038/ncomms8566.

Petruzzo P, S.C., Lanzetta M, Dubernard JM. (2017). Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). Curr Transplant Reports 4, 294-303.

Rifkin, W.J., Manjunath, A.K., Kantar, R.S., Jacoby, A., Kimberly, L.L., Gelb, B.E., et al. (2021). A Comparison of Immunosuppression Regimens in Hand, Face, and Kidney Transplantation. J Surg Res 258, 17-22. doi: 10.1016/j.jss.2020.08.006.

Sachs, D.H., Leight, G., Cone, J., Schwarz, S., Stuart, L., and Rosenberg, S. (1976). Transplantation in miniature swine. I. Fixation of the major histocompatibility complex. Transplantation 22(6), 559-567. doi: 10.1097/00007890-197612000-00004.

Shanmugarajah, K., Powell, H., Leonard, D.A., Mallard, C., Albritton, A., Harrington, E., et al. (2017). The Effect of MHC Antigen Matching Between Donors and Recipients on Skin Tolerance of Vascularized Composite Allografts. Am J Transplant 17(7), 1729-1741. doi: 10.1111/ajt.14189.

Su, C.A., and Fairchild, R.L. (2014). Memory T Cells in Transplantation. Curr Transplant Rep 1(3), 137-146. doi: 10.1007/s40472-014-0018-5.

Thistlethwaite, J.R., Jr., Auchincloss, H., Jr., Pescovitz, M.D., and Sachs, D.H. (1984). Immunologic characterization of MHC recombinant swine: role of class I and II antigens in in vitro immune responses. J Immunogenet 11(1), 9-19. doi: 10.1111/j.1744-313x.1984.tb01035.x.

Tizon, R., Frey, N., Heitjan, D.F., Tan, K.S., Goldstein, S.C., Hexner, E.O., et al. (2012). High-dose corticosteroids with or without etanercept for the treatment of idiopathic pneumonia syndrome after allo-SCT. Bone Marrow Transplant 47(10), 1332-1337. doi: 10.1038/bmt.2011.260.

van der Merwe, A., Graewe, F., Zuhlke, A., Barsdorf, N.W., Zarrabi, A.D., Viljoen, J.T., et al. (2017). Penile allotransplantation for penis amputation following ritual circumcision: a case report with 24 months of follow-up. Lancet 390(10099), 1038-1047. doi: 10.1016/S0140-6736(17)31807-X.

Wang, T., Ahmed, E.B., Chen, L., Xu, J., Tao, J., Wang, C.R., et al. (2010). Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model. Am J Transplant 10(7), 1524-1533. doi: 10.1111/j.1600-6143.2010.03066.x.

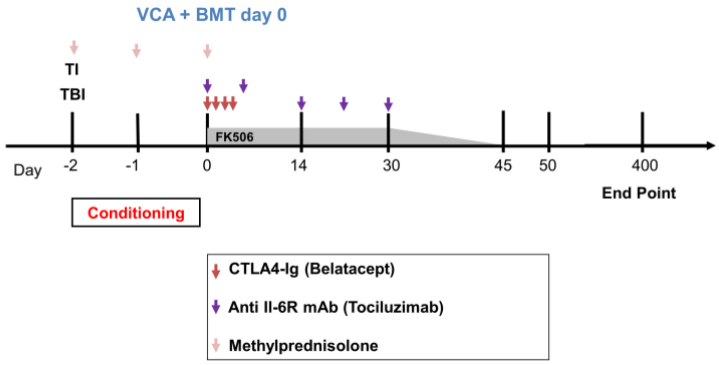


Figure 1. Mixed chimerism protocol for immune tolerance. Recipient conditioning begins prior to transplantation with total body irradiation (TBI, day -2) and thymic irradiation (TI, day -1). Induction is performed with methylprednisolone (days -2, -1, 0) and FK506. Following vascularized composite allotransplantation (VCA), the donor is exsanguinated, and bone marrow is harvested for transplantation (BMT) under the cover of CTLA4-Ig (Belatacept®) and anti-IL6R mAb (Tociluzimab®). FK506 is then continued for 30 days (target trough levels: 10-15 ng/mL) before gradual tapering to discontinuation on day 45; further CTLA4-Ig (Belatacept®) and anti-IL6R mAb (Tociluzimab®) is given I.V. 20 mg/kg (on POD 2, 4, 6) and I.V. 10 mg/kg (on POD 7, 14, 21, 28) respectively.

Figure 2

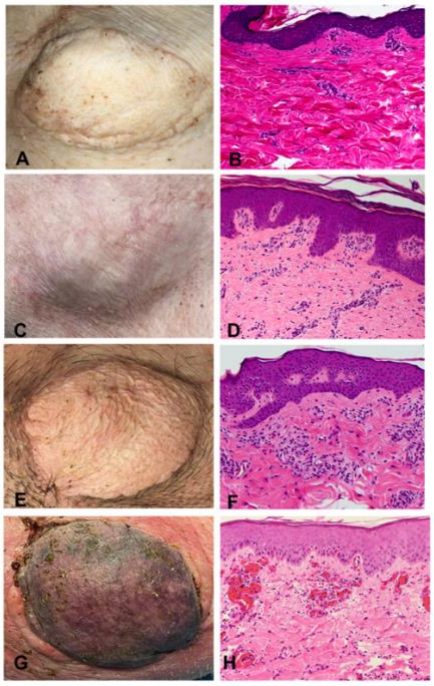


Figure 2. Clinical assessment of successful VCA tolerance. Images of tolerant VCAs from R3 **(A)**, R4 **(C)**, and R5 **(E)** taken on POD 251, 387, and 250, respectively, compared to a representative rejected VCA from untreated control R6 on POD 11 **(G)**. H&E staining of VCA biopsies from R3 **(B)**, R4 **(D)**, and R5 **(F)**, taken on POD 151, 387, and 250

respectively, demonstrating absence of rejection (Banff Grade 0). Whereas H&E staining of VCA skin from class I mismatch untreated control R6 shows focal epidermal necrosis and capillary thrombosis (Banff Grade IV) on POD 11 **(H)**. (POD = post-operative day; VCA = vascularized composite allograft).

Figure 3

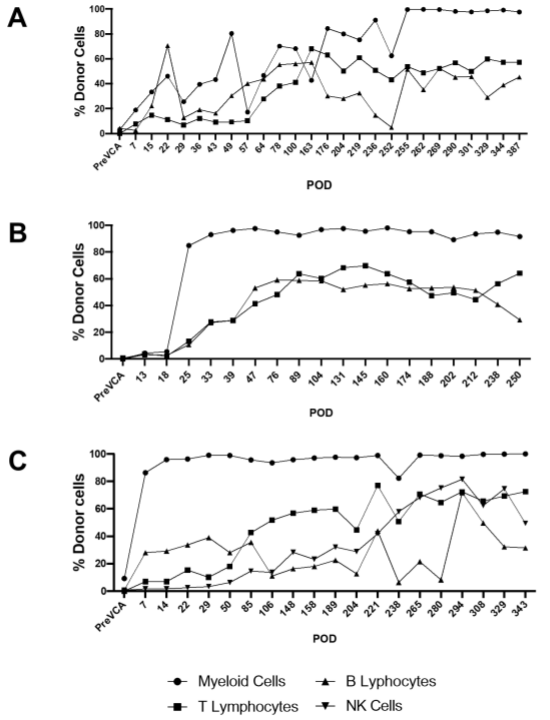


Figure 3. Long-term multi-lineage mixed chimerism in recipient animals R4 **(A)**, R3 **(B)** and R5 **(C)**. Levels of donor chimerism in myeloid cells were >80%, whereas other cell populations (B cells, NK cells, T cells) ranged between 30 to 60%.

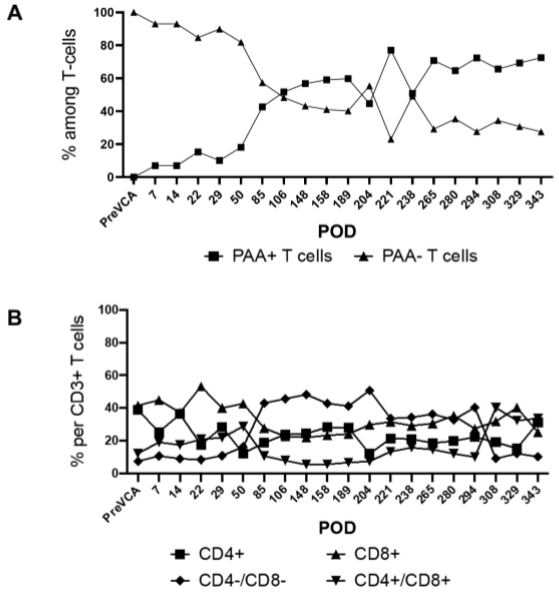


Figure 4. Sub-analysis of T cell subsets in multi-lineage mixed chimerism. Post-transplantation, as bone marrow reconstitution progresses, the percentage of donor (PAA+) and recipient (PAA-) T cells approaches equilibrium at around POD100, remained stable and varied between 50 to 70% of donor T cells to experimental endpoint **(A)**. The population of double negative (CD4-/CD8-) T cells increase from

around POD 50 onwards (following cessation of FK506) and remained at about 20 to 40% of overall CD3+ T cell to experimental endpoint **(B)**.

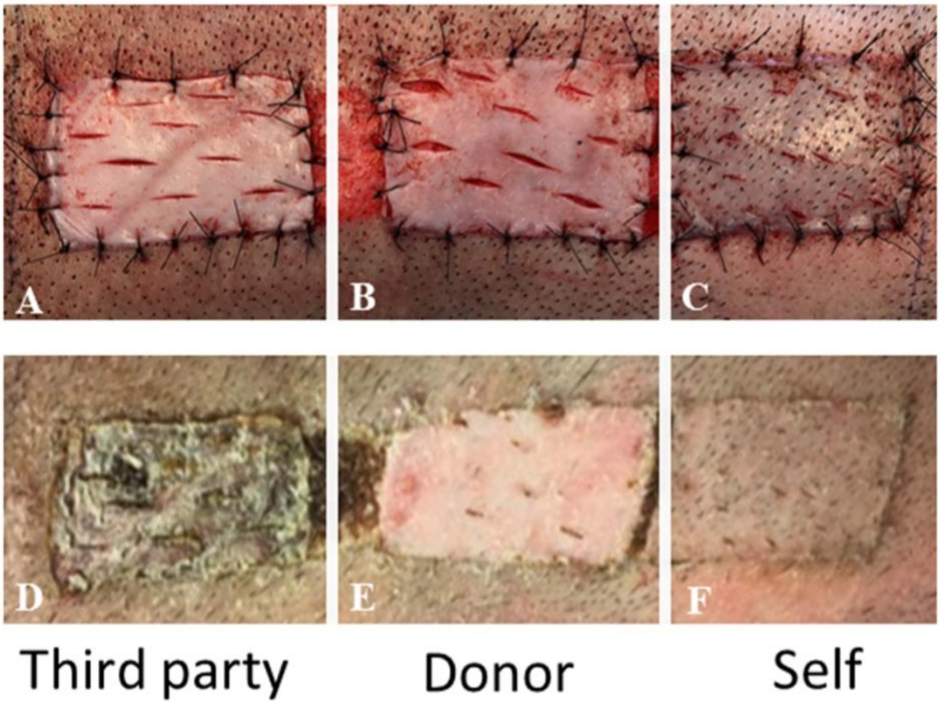


Figure 5. *In vivo* test of immune competence by placement of split-thickness skin grafts from a third-party animal **(A)**, original donor **(B)**, and self **(C)** at POD 150 onto recipient swine tolerant of VCA (representative). By POD 14, there was complete rejection of the skin graft from the third-party animal **(D)** whereas that from the donor **(E)** and self **(F)** had taken.

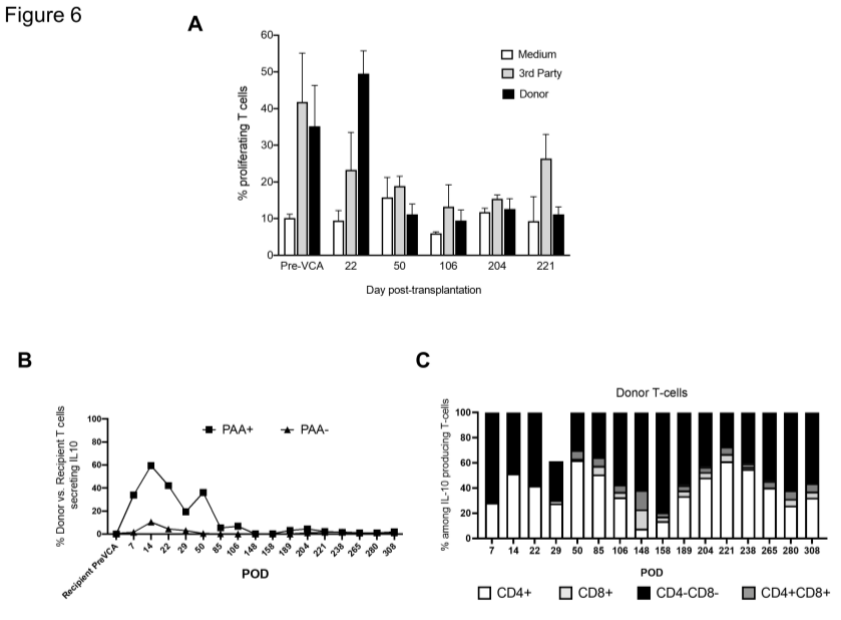


Figure 6. (A) Mixed lymphocyte reaction assay demonstrating proliferation of T cells *in vitro*. From POD 50 onwards, which corresponds more or less to the withdrawal of all immunosuppression and establishment of multi-lineage mixed chimerism, cellular proliferation against donor and self-antigens were comparable to medium, suggesting a state of immune unresponsiveness in peripheral blood. **(B)** Similar to Figure 3 which

showed equilibration of donor (PAA+) and recipient (PAA-) contribution to overall T cell populations, the contribution of IL-10 secreting T cells followed a similar pattern, approaching equilibrium at POD 85. Further analysis revealed that the majority of such IL-10 producing T cells are CD4-CD8- in nature **(C)**.

CHAPTER 04

Toward Development of the Delayed Tolerance Induction Protocol for Vascularized Composite Allografts in Nonhuman Primates

Alexandre G. Lellouch
Zhi Yang Ng
Ivy A. Rosales
Ilse M. Schol
David A. Leonard
Amon-Ra Gama
Robert B. Colvin
Laurent A. Lantieri
Mark A. Randolph
Curtis L. Cetrulo Jr.

Adapted from Plast Reconstr Surg. 2020 Apr;145(4):757e768e.doi:10.1097/PRS.0000000000006676.PMID: 32221215DOI:10.1097/PRS.0000000000006676

ABSTRACT

Background: Transplantation of vascularized composite allografts is limited mainly by the need for life-long immunosuppression. The consequent side effects and looming specter of chronic rejection portend eventual allograft loss. Development of tolerogenic protocols is thus of utmost importance to the field of vascularized composite allograft transplantation.

Methods: With a modified delayed tolerance induction protocol, 10 cynomolgus macaques received hand (n = 2) or face vascularized composite allografts across both full and haploidentical major histocompatibility complex barriers before donor bone marrow transplantation at a later date. Protocol and for-cause allograft skin biopsies were performed for immunohistochemical analysis and analysis of donor-recipient leukocyte contribution; mixed chimerism in peripheral blood and in vitro immune responses were assessed serially.

Results: Before bone marrow transplantation, maintenance immunosuppression for 4 months led to lethal complications, including posttransplant lymphoproliferative disorder (in two of four recipients), which necessitated early study termination. Shortening the maintenance period to 2 months was clinically relevant and allowed all subsequent subjects (n = 6) to complete the delayed tolerance induction protocol. Acute rejection developed within the first 2 to 4 weeks after transplantation, with corresponding near-complete turnover of allograft leukocytes from donor to recipient origin, but donor-specific antibodies remained negative. After bone marrow transplantation, mixed chimerism failed to develop, although carboxyfluorescein succinimidyl ester mixed lymphocyte reaction demonstrated generalized unresponsiveness. However, the accrual of subsequent rejection episodes eventually culminated in graft vasculopathy and irreversible allograft loss.

Conclusions: Despite the various advantages of the delayed tolerance induction protocol, it failed to reliably induce mixed chimerism and thus immunologic tolerance to vascularized composite allografts, given currently available immunosuppression treatment options. Ongoing work shows promise in overcoming these limitations.

Mixed hematopoietic chimerism–based tolerance induction regimens have been clinically successful in haplomatched renal transplant donor-recipient pairs (i.e., living-related or mother-to-daughter).[1] We recently reproduced this result for vascularized composite allografts (hand or face transplants) in haplomatched swine,[2] with the important distinction that stable mixed chimerism was necessary for tolerance of allograft skin[3] (unlike in clinical renal transplantation, in which transient chimerism was sufficient for long-term tolerance of donor kidneys[1]).

Extending these encouraging preclinical results to fully mismatched donor-recipient pairs (analogous to unrelated deceased donors necessary for vascularized composite allograft transplantation) has proven challenging, however, because of the difficulty of achieving mixed chimerism concomitant with transplantation, as well as the increasingly morbid conditioning required of fully mismatched donor-recipient pairs. Attempting to achieve stem cell engraftment and stable mixed chimerism during the immediate perioperative period, when the inflammatory milieu is at its peak, has been likened to planting "flowers in a battlefield"—the marked inflammation generated by surgery does not permit reliable stem cell engraftment (e.g., from donor bone marrow transplantation) and therefore no chimerism is achieved.[4]

Separation of these processes has been examined successfully by investigators at our center using a delayed tolerance induction protocol in nonhuman primates for solid organ transplantation.[5,6] This strategy argues that the transplanted organ can be maintained rejection-free on standard immunosuppression postoperatively until the perioperative inflammatory state subsides and the patient's immunosuppressive regimen has been weaned to low long-term levels. At this juncture then, conditioning and stem cell transplantation may be more feasible.

One of the challenges in adopting current clinical tolerance protocols in solid organ transplantation[7] for vascularized composite allograft is the need for sufficient lead time (e.g., up to 6 days) to adequately precondition the recipient; for example, in a living-related kidney donor tolerance induction protocol, the recipient could undergo such preconditioning before the elective kidney donation.[1] The requirement for deceased donors for vascularized composite allografts makes it unlikely logistically for a patient to receive recipient conditioning in advance of allograft procurement from the donor. The delayed tolerance induction protocol would not only negate the need for this lead-time requirement but also enable a larger potential donor pool, as patients may now foreseeably travel between cities, and countries even,[8] to undergo vascularized composite allograft transplantation with better size and color match of the allograft while maintaining the potential for subsequent tolerance induction (as donor bone marrow may be harvested and cryopreserved for later use[9]). Indeed, the delayed tolerance induction protocol has been demonstrated successfully in small animal vascularized composite allograft transplantation studies.[10] The current study sought to extend our center's successful delayed tolerance induction protocol from solid organ transplantation to vascularized composite allograft transplantation in nonhuman primates.

MATERIALS AND METHODS

Experimental Design

Ten cynomolgus monkeys received vascularized composite allografts from major histocompatibility complex–mismatched donors after induction with equine antithymocyte globulin (ATGAM; Pfizer, New York, N.Y.; 50 mg/kg/day intravenously for 3 days starting on day of surgery), FK506 (0.1 mg/kg intramuscularly), mycophenolate mofetil (CellCept; Genetech, San Francisco, Calif.; 300 mg intravenously), and methylprednisolone (Solu-Medrol; Pfizer, New York, N.Y.; 40 mg intravenously). Maintenance immunosuppression consisted of FK506 (adjusted to keep plasma levels between 20 and 30 ng/ml), mycophenolate mofetil (given parenterally every day mixed into the animal's daily feed provided ad libitum, with reduction to 100 to 200 mg daily by postoperative day 14), and methylprednisolone (gradual taper over 14 days to 1 g given intramuscularly once a day for maintenance) (Fig. 1).

In the initial cohort (n = 4), each monkey would undergo bone marrow transplantation 4 months later, with recipient conditioning as described previously.6 Two monkeys developed posttransplant lymphoproliferative disorder before bone marrow transplantation, however, and had to be removed from the study. Thus, we altered our delayed tolerance induction protocol such that in the second cohort (n = 6), monkeys underwent bone marrow transplantation after 2 months, not only to be more clinically relevant but also to reduce the risk of immunosuppression-related complications, such as sensitization and posttransplant lymphoproliferative disorder.[11]

Animals

Cynomolgus monkeys (Macaca fascicularis) weighing 5 to 10 kg were used (Charles River Primates, Wilmington, Mass.). Transplant pairs were selected for compatible ABO blood types and major histocompatibility complex mismatching. Experiments were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Cynomolgus Major Histocompatibility Complex Genotyping

Peripheral blood mononuclear cells were obtained from animals and sent to the Wisconsin National Primate Research Center for major histocompatibility complex genotyping, based on established protocols.[12,13]

Vascularized Composite Allograft Transplantation and Bone Marrow Procurement

Orthotopic upper extremity[14] (n = 2) and heterotopic partial face[15] (n = 8) allograft transplants were performed as previously described. After euthanasia, donor vertebrae were harvested, fragmented, and incubated in RPMI medium with 1% DNAse (1 hour at room temperature). Bone marrow cells were extracted from the lysate, resuspended in cryopreservation media containing 58% pentastarch, 10% dimethylsulfoxide, and 32% human albumin, and kept frozen in liquid nitrogen.

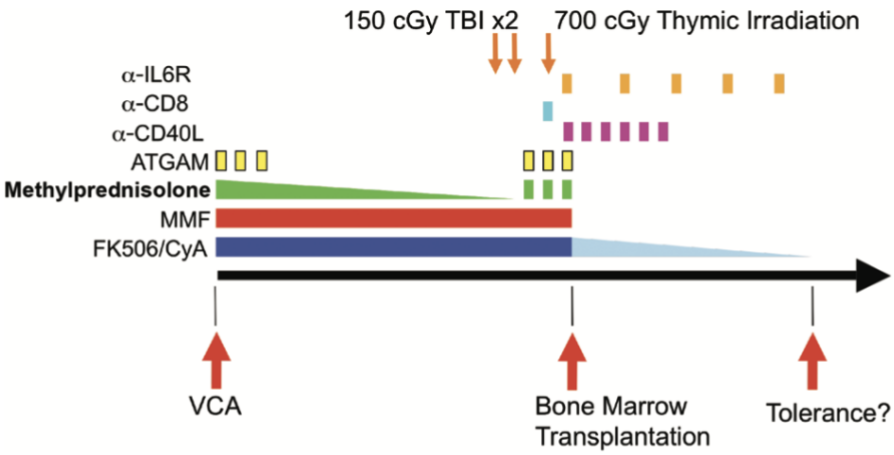


Fig. 1. Delayed tolerance induction protocol for vascularized composite allograft transplantation. The sequence and timing of the protocol interventions are depicted relative to the timing of allografting and bone marrow transplantation. After induction with ATGAM (due to its general anti-T-cell effect, with the aim of averting early acute rejection), FK506 (which appears to amplify the utility of ATGAM when administered concurrently[49]), mycophenolate mofetil, and methylprednisolone, the allografts were maintained with conventional triple-drug immunosuppression to mimic the majority of current clinical vascularized composite allograft practice. This was followed by bone marrow transplantation at 4 or 2 months later by using a nonmyeloablative conditioning regimen (proven successful in clinical mixed chimerism trials for kidney transplantation[1]) that included total body irradiation (150 cGy, on days −6 and −5 from bone marrow transplantation), thymic irradiation (700 cGy on day −1 from bone marrow transplantation), ATGAM (50 mg/kg intravenously on days −2, −1, and 0 from bone marrow transplantation), methylprednisolone (5 mg intravenously on days −2, −1, and 0 from bone marrow transplantation), and anti-CD8 monoclonal antibody (cM-T807; Centocor, Inc., Horsham, Pa.; 5 mg/kg intravenously on day −1 from bone marrow transplantation; for memory T-cell depletion). After bone marrow transplantation, the recipients were treated with short courses of anti-CD40L (h5C8; 20 mg/kg intravenously on posttransplantation days 0 and 2, and 10 mg/kg on posttransplantation days 5, 7, 9, and 12; for costimulatory blockage) and anti-IL-6 receptor monoclonal antibody (tocilizumab, Actemra; Genentech, San Francisco, Calif.;10 mg/kg intravenously on posttransplantation days 0, 7, 14, 21, and 28), along with a 28-day course of cyclosporine (target trough, 200 to 400 ng/ml) to overcome highly alloreactive recipient responses to achieve engraftment of donor bone marrow cells. After this, no further immunosuppression was given. *ATGAM*, equine anti-thymocyte globulin; *CyA*, cyclosporine A; *MMF*, mycophenolate mofetil; *VCA*, vascularized composite allograft.

Rejection Monitoring and Immunohistopathological Examination

After transplantation, allografts were monitored twice daily for the first 72 hours and once daily subsequently. Protocol and for-cause allograft skin biopsies were performed using a standard 6-mm punch biopsy kit at approximately 30-day intervals. When rejection was diagnosed clinically (i.e., increased erythema, swelling, ulceration, and so on), treatment was initiated with steroid bolus and gradual taper over 14 days to 1 g intramuscularly once a day (same as maintenance).

All allograft skin biopsy samples were fixed in formalin and stained with hematoxylin and eosin. Immunohistopathological evaluation, including staining for C4d, was performed by transplant pathologists (I.A.R and R.B.C) blinded to the study. The severity of acute rejection was graded according to the 2007 Banff scale for composite tissues.[16]

Nonmyeloablative Conditioning and Delayed Bone Marrow Transplantation

After vascularized composite allograft transplantation, donor bone marrow cells that were previously harvested and cryopreserved were infused (target dose, 3 × 108 cells/kg intravenously)[17,18] following recipient conditioning at 4 months (in the initial cohort) and 2 months (subsequent cohort), as described in the delayed tolerance induction protocol (Fig. 1).

Flow Cytometric Analyses of Vascularized Composite Allograft Skin Leukocytes

Allograft skin biopsies were prepared and analyzed as described previously,[19] with modification for nonhuman primate cells. (**See Document, Supplemental Digital Content 1**, which shows vascularized composite allograft skin flow cytometry assay, ***http://links.lww.com/PRS/D997***.)

Flow Cytometric Analysis of Peripheral Blood for Detection of Chimerism

Transplant pairs were preselected based on their differential reactivity to major histocompatibility complex class I antigens in nonhuman primates using a specific antibody, H38 (One Lambda, Inc., Canoga Park, Calif.). Chimerism (percentage of donor leukocytes) was assessed in peripheral blood via flow cytometry twice weekly during the first week and once every fortnight thereafter. (**See Document, Supplemental Digital Content 2**, which shows peripheral blood mixed chimerism assay, ***http://links.lww.com/PRS/D998***.)

Measurement of T Cell–Mediated Responses by

Mixed Lymphocyte Reaction Assays

The systemic immune status of recipient animals was assessed by proliferation dye-based mixed lymphocyte reaction assays approximately every 30 days. (**See Document, Supplemental Digital Content 3**, which shows the carboxyfluorescein succinimidyl ester mixed lymphocyte reaction assay, ***http://links.lww.com/PRS/D999***.)

Detection of Donor-Reactive Antibodies

Donor-specific antibody formation was tested by flow cytometry, as previously described.[20]

RESULTS

Triple Immunosuppression Failed to Abrogate Acute Rejection of Vascularized Composite Allografts

Vascularized composite allograft transplantation was performed across both haploidentical and full major histocompatibility complex–mismatch barriers (Tables 1 and 2). Results for upper extremity vascularized composite allografts (M1413 and M4213) were reported previously.[14] Despite the use of a clinical triple immunosuppression regimen (FK506, mycophenolate mofetil, and methylprednisolone) that was able to maintain solid organ transplantation allografts rejection-free in previous nonhuman primate studies from our center,[5,6] this was not the case for vascularized composite allografts, even with FK506 levels that were almost double those typically used in clinical patients[21–23] (Tables 1 and 2).

Rejection episodes were highly variable, ranging from mild and subtle edema to a maculopapular rash of varying intensities, and tended to present clinically for the first time around post-operative day 30, regardless of the preceding and corresponding systemic levels of FK506 and extent of major histocompatibility complex mismatch. Of note, acute rejection at this time point had a tendency to recur 2 weeks later despite treatment and necessitated further steroid bolus and gradual taper. Therefore, we included an additional surveillance allograft biopsy at around postoperative day 14 in subsequent nonhuman primate recipients and indeed identified subclinical rejection even with adequate, corresponding FK506 levels at this time point. Steroid bolus and gradual taper, though, were able to prevent early recurrence, and subjects remained rejection-free (both clinically and histologically) up to the point of bone marrow transplantation at postoperative day 60 (Fig. 2).

Acute Rejection Episodes on Triple Immunosuppression Did Not Result in Donor-Specific Antibody Formation

At 1 and 2 months after vascularized composite allograft transplantation, despite clinical and histological confirmation of rejection in almost all nonhuman primate recipients, donor-specific antibody was not detected in any subject (data not shown). This finding is in contrast to observations in nonhuman primate renal transplantation experiments at our center using the delayed tolerance induction protocol, in which acute rejection episodes usually result in donor-specific antibody formation and an inability to obtain tolerance.[18] The lack of donor-specific antibody also reflects the ability to detect skin rejection quite early in the rejection process (relative to the kidney), as the skin of the face and hand allograft is clinically visible.

The cell-mediated arm of the immune response was assessed by carboxyfluorescein diacetate succinimidyl ester mixed lymphocyte reaction based on the proliferation of both CD4+ and CD8+ T cells. Before bone marrow transplantation, in vitro responsiveness against donor and third-party antigens was maintained andcomparable, which suggests that T cell activation was also adequately suppressed during the period

of maintenance immunosuppression. [**See Figure, Supplemental Digital Content 4**, which shows that in vitro evidence from carboxyfluorescein diacetate succinimidyl ester mixed lymphocyte reaction suggests that the presence of vascularized bone marrow

Table 1. Study Outcomes on Initial Cohort of Animals on 4-Month Maintenance Immunosuppression

Recipient	VCA	MHC Mismatch	Complications	DSA/C4d	Average FK506 Dose (ng/ml)	Creatinine, Glucose (mg/dl)	Survival	Reason for Euthanasia
M1413	Hand	Full	Banff II (POD 97)	–/–	28.8 (16.7–40.2)	0.6–0.9, 53–157	POD 136	Necrotizing fasciitis of VCA
M4213	Hand	Full	Banff I (POD 30)	–/–	25.7 (5.7–48.7)	1.1–1.4, 47–175	POD 51	VCA loss (Banff IV)
M6014	Face	Full	Banff 0 (POD 19, 48, 78)	–/–	23.3 (9.0–38.9)	0.6–1.1, 74–157	POD 107	PTLD
M6714	Face	Haploidentical	—	–/–	18.7 (8.7–47.5)	0.8, 123	POD 79	PTLD

VCA, vascularized composite allograft; MHC, major histocompatibility complex; DSA, donor-specific antibody; POD, postoperative day; PTLD, posttransplant lymphoproliferative disorder.

as part of a vascularized composite allograft (VCA) did not provide additional immunomodulation while on maintenance immunosuppression, as the antidonor and anti-third-party responses were comparable; at 30 days after donor bone marrow transplantation (DBMT), antidonor responses were reduced while anti-third-party responses were maintained; at around 60 days after donor bone marrow transplantation (1 month off all immunosuppression, no rejection yet), there was generalized unresponsiveness; however, at around 72 days after donor bone marrow transplantation (after rejection had developed), it appears that antidonor and anti-third-party responsiveness had recovered and may have also developed against self-antigens (representative data shown; no difference observed between full major histocompatibility complex-mismatch and haploidentical recipients). PHA, phytohemagglutinin (positive control for assay); ***http://links.lww.com/ PRS/D1000***.]

Rapid Recipient Leukocyte Infiltration of Vascularized Composite Allografts

Flow cytometric analysis of vascularized composite allograft skin biopsy samples showed near-complete (>90 percent) turnover of skin leukocytes (CD4+, CD8+) in allograft dermis from donor to recipient-origin T cells by the first protocol biopsy around postoperative day 30. At this same time point, however, the kinetics of this turnover was slower in the epidermis, where a variable amount (75 percent to 93 percent) of CD207+ T cells (Langerhans cell phenotype) were of donor origin (not shown). Similar results were obtained after additional surveillance biopsies were introduced at week 2. [**See Figure, Supplemental Digital Content 5**, which shows in vitro analysis of leukocytes from vascularized composite allograft skin biopsies at 2 and 4 weeks after transplantation. Donor-origin Langerhans cell populations were nearly 90 percent at week 2 in the epidermis, with corresponding CD4+ and CD8+ populations of more than 90 percent recipient origin by this time in the dermis. By week 4, the donor-origin Langerhans cell population decreased to about 60 percent, but the CD4+ and CD8+ dermal populations remained at more than 90 percent recipient origin, suggesting tissue residence. No difference was observed between full major histocompatibil- ity complex–mismatch and haploidentical recipients (not shown), ***http://links.lww.com/PRS/E2***.] No quantitative differences in cellular kinetics were observed between the upper extremity and facial during the period allograft recipients, or between full major histocompatibility complex-

Table 2. Study Outcomes on Subsequent Cohort of Animals on 2-Month Maintenance Immunosuppression

Recipient	VCA	MHC Mismatch	Complications	DSA/C4d	Average FK506 Dose (ng/ml)	Creatinine, Glucose	Survival to POD/Days off IS	Reason for Euthanasia
M6514	Face	Full	Banff I (POD 36)	−/−	21.2 (9.6–33.0)	0.6–0.8, 59–151	74/–	Sepsis
			Banff II (POD 46)	−/+				
M4415	Face	Full	Banff II (POD 14*)	−/−	20.5 (9.0–29.8)	0.6–0.8, 94–131	76/–	PTLD
M4515	Face	Full	Banff I (POD 15)	−/−	23.4 (10.2–34.8)	0.7–0.9, 73–140	119/25	PTLD
M3316	Face	Haploidentical	Banff I (POD 27)	−/−	23.1 (11.0–34.3)	0.7–0.7, 114–119	93/4	PTLD
M3516	Face	Haploidentical	Banff I (POD 12*)	−/−	21.2 (10.2–33.7)	1.0–1.1, 111–131	100/10	Self-mutilation
			Banff I (POD 100†)	−/−				
M3815	Face	Haploidentical	Banff II (POD 126*)	−/−	29.1 (17.0–40.6)	0.7–0.9, 73–115	224*†/36	VCA loss (Banff IV)
			Banff II (POD 172†)	−/−				
			Banff II (POD 195*)	−/+				

VCA, vascularized composite allograft; MHC, major histocompatibility complex; DSA, donor-specific antibody; POD, postoperative day; IS, immunosuppression; PTLD, posttransplant lymphoproliferative disorder.
*Endothelialitis.
†Graft vasculopathy.

mismatch and haploidentical recipients (data not shown).

Delayed Bone Marrow Transplantation Failed to Establish Mixed Chimerism After vascularized composite allograft transplantation and before bone marrow transplantation, no evidence of mixed chimerism (either transient or stable) was detected peripherally, despite the incorporation of vascularized bone marrow as part of the allograft (data not shown). After bone marrow transplantation, there was no evidence of mixed chimerism in all recipient nonhuman primates except for one time point in M3815 at approximately 5 weeks after bone marrow transplantation, with transient low levels of donor monocytes and lymphocytes detected. [**See Figure, Supplemental Digital Content 6**, which shows a flow cytometry plot suggestive of transient mixed chimerism in M3815 (H38+ recipient) at 5 weeks after bone marrow transplantation (1 week after complete withdrawal of all immunosuppression). There is evidence of donor-origin (H38−) cells within the circulation after infusion of donor bone marrow cells. Gating from left to right, ***http://links.lww.com/PRS/E3***.]

Immunohistological Evaluation of Rejected and Long-Term Surviving Vascularized Composite Allografts

Acute rejection episodes varied from Banff I to IV and had varying features of endothelialitis, C4d staining, and graft vasculopathy (Tables 1 and 2). Severe graft vasculopathy was detected in our sole long-term survivor, M3815. Before conditioning and receiving donor bone marrow cells, this animal was rejection-free clinically and on histologic analysis. Following gradual taper and complete withdrawal of a bridging course of cyclosporine on postoperative day 90, the animal remained rejection-free for 5 weeks before developing Banff II rejection with endothelialitis on postoperative day 126.

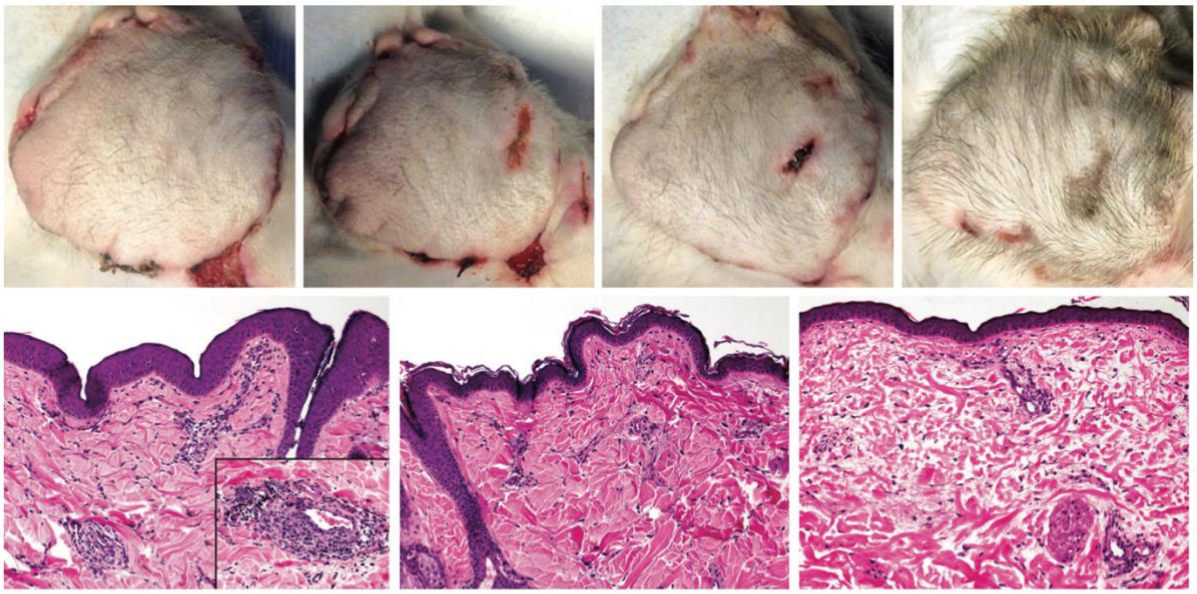


Fig. 2. Clinical course for M4415 (full major histocompatibility complex mismatch recipient). (*Above, left*) Equivocal appearance of vascularized composite allograft on postoperative day 14. Complete clinical resolution after steroid bolus and taper between postoperative day 18 (*above, second from left*) and postoperative day 25 (*above, second from right*). (*Above, right*) No evidence of recurrence up to postoperative day 60 before bone marrow transplantation. (*Below, left*) Corresponding histologic analysis shows focal, moderate-to-severe deep perivascular inflammation (Banff grade II) with endothelialitis (*inset*) on postoperative day 14, and no evidence of rejection on postoperative day 32 (*below, center*) and postoperative day 60 (*below, right*).

Although rejection was treated successfully with steroid bolus and gradual taper and FK506, at 2 weeks after re-withdrawal of immunosuppression, the allograft became erythematous again on postoperative day 172 and biopsy showed Banff II rejection. This episode was not reversible with steroid bolus and gradual taper and FK506, and it persisted, with biopsy on postoperative day 195 showing Banff II rejection with endothelialitis; it culminated in necrosis of the allograft on postoperative day 224, after veterinary-imposed withdrawal of treatment. Graftectomy (on post- operative day 224) analysis showed severe Banff IV rejection and C4d deposition in capillaries and arteriolar and arterial endothelium. Severe graft vasculopathy was also present in arteries (Fig. 3).

DISCUSSION

As long-term outcomes of clinical vascularized composite allograft transplantation accumulate, we are now witnessing the expected morbidity and mortality predictable from our transplant colleagues' experience in solid organ transplantation, namely, the devastating effects of chronic immunosuppression, such as renal failure, cancer, and fatal infections.[21–23] Episodes of graft failure due to noncompliance with maintenance immunosup- pression and graft loss due to chronic rejection have also become more frequent, demonstrating limitations of our current ability to safely suppress the human immune system. Indeed, vascularized composite allograft transplantation has evolved from the "proof-of-concept" or "demonstration of technical success" phase to a pressing need for immunological advances to allow further evolution.

Development of clinical tolerance induction protocols in vascularized composite allograft transplantation is therefore more important than ever, particularly with regard to the

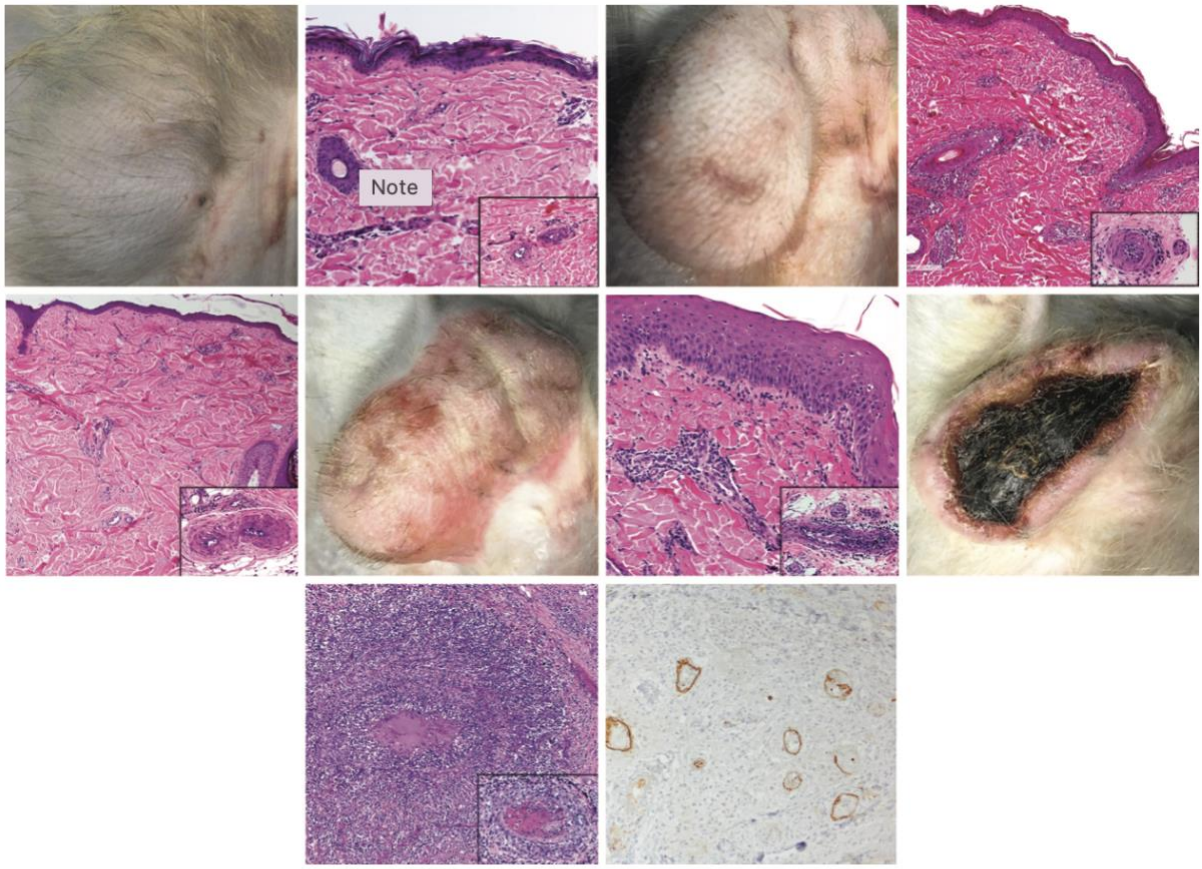


Fig. 3. Clinical course for M3815 (haploidentical recipient) with corresponding histological analysis. (*Above, left*) Clinical appearance at postoperative day 92 before bone marrow transplantation; (*above, second from left*) corresponding histologic section shows no evidence of rejection. (*Above, second from right*) The first episode of rejection occurred on postoperative day 126; (*above, right*) biopsy showed acute cell-mediated rejection characterized by moderate perivascular inflammation with mild epidermal involvement and endothelialitis (*inset*). (*Center, left*) Biopsy at postoperative day 143 showed resolution of rejection, but a subcutaneous artery with features of graft vasculopathy was seen (*inset*). (*Center, second from left*) Clinical recurrence of rejection was seen at postoperative day 172; (*center, second from right*) biopsy showed acute cellular rejection with moderate perivascular inflammation, epidermal involvement, and prominent endothelialitis in arterioles. (*Center, right*) Graftectomy at postoperative day 224 showed severe transmural vasculitis and graft vasculopathy when (*below, left*) the allograft was no longer salvageable and (*below, right*) C4d stained positive in the deep vessels.

specter of chronic rejection.[24] An underappreciated aspect of tolerance entails the homeostatic balance of immunology that results when a patient is rendered tolerant of a transplant. Biopsy specimens from renal tolerance patients show clean histologic sections decades after transplantation; in contrast, subclinical chronic rejection requires retransplantation.[25] Prevention of chronic rejection of faces and hands through tolerance induction would arguably be more important than abrogation of acute rejection.

Mixed chimerism tolerance protocols have been developed at our institution in mice, swine, and nonhuman primates for solid organ transplantation.[26] The present nonhuman primate study allowed us to study the applicability of an established delayed tolerance induction protocol in solid organ transplantation for developing mixed chimerism in vascularized composite allografts. However, nonhuman primate models are extremely challenging for vascularized composite allograft transplantation in general and tolerance induction in particular. The immune system of the cynomolgus macaque is more complex and robust than a human's,[27] with chimerism difficult to induce and rejection frequent.

However, overcoming these challenges will instill a necessary rigor that will make clinical translation of such protocols safer. Indeed, this paradigm has been realized clinically in haplomatched1 and full-mismatched renal transplant patients at Massachusetts General Hospital. We report a number of lessons learned for future work.

Control of Acute Rejection

Compared with clinical solid organ transplantation data in which major histocompatibility complex class II sharing (i.e., haploidentical) improved graft outcomes,[28] nonhuman primate recipients in this vascularized composite allograft study suffered early rejection episodes frequently during the delay period on maintenance immunosuppression, despite standard or even high levels of immunosuppression (based on established solid organ transplantation protocols),[21–23] with only a few haploidentical subjects avoiding a rejection episode (M6714 and M3815). In reviewing the literature on nonhuman primate vascularized composite allograft studies, major histocompatibility complex mismatch data were not always provided, and some report selection of transplant pairings based on the least degree of proliferation by mixed lymphocyte reaction, which suggests that the true immunologic challenge of a full major histocompatibility complex–mismatch barrier may not have been fully explored,[29–32] unlike in the current study (Table 3).

As have other groups using this model,[31,32] we utilized historical experimental vascularized composite allograft data as controls (i.e., groups without bone marrow transplantation, bone marrow transplantation without preconditioning, or bone marrow transplantation after a different induction or maintenance immunosuppression regimen) for this study due to the high costs and ethics involved in nonhuman primate studies.

Encouragingly, we have recently extended our ability to limit rejection episodes in the delay period (see "T Cell Turnover in the Skin") to successful prevention of rejection altogether, even in fully mismatched pairs in this nonhuman primate model, through the addition of local immunosuppression technology (data not shown).

Management of Posttransplant Lymphoproliferative Disorder

The high incidence of posttransplant lymphoproliferative disorder, both in the initial and subsequent cohort of nonhuman primates, limited our ability to obtain reproducible assessments of chimerism and tolerance of allografts. Previous studies have attributed this to simian lymphocryptovirus, which is genomically equivalent to human Epstein-Barr virus.[31,33] Routine testing of lymphocryptovirus status before transplant in nonhuman primates would be ideal but is not widely available. Local immunosuppression technologies that allow lower systemic levels have also shown promise in mitigating this difficult complication (see previous mention).

T Cell Turnover in Skin

Recent studies have demonstrated that human skin contains almost twice the number of circulating leukocytes.[34] In a series of clinical face transplant patients, acute rejection episodes were postulated to be accounted for by the persistence of donor-origin lymphocytes from the allograft itself.[35] The analysis of acute rejection in vascularized

composite allografts may, however, be confounded by variation in the induction and maintenance immunosuppressive protocols utilized at different centers.[36] In the current study, by the time rejection was clinically apparent at postoperative day 30, flow cytometric analyses demonstrated nearly complete turnover of leukocyte populations within allograft dermis from donor to recipient origin by postoperative day 14 (as noted in the added postoperative day 14 surveillance biopsy). The numbers of these recipient-origin T cells then remained at approximately the same levels (>90 percent) up to the end of the experiment, which suggests that these cells may be skin-resident in nature, such as memory T cells, especially in the context of vascularized composite allografts.[35,37] Such memory T cells are also more resistant to conventional immunosuppression,[38] as used in this study and clinically, and may account for the development, recurrence, and progression of rejection episodes, as seen in M4213 and M6514. Of note, the nonhuman primates used in this study were 5 to 8 years old, the typical reproductive age group in adults,[39] and their profile of skin immune cells has been found to be similar to that of humans,[40] lending further support to our observations.

Unfortunately, the low cellular yield from our vascularized composite allograft skin assays (2-3-day process) precluded further mechanistic studies. When early subclinical acute rejection was diagnosed by week 2 in this study, steroid boluses effectively prevented further progression (e.g., M4213) and recurrence (e.g., M6514), at least up to the point of bone marrow transplantation in this nonhuman primate experimental setting. Perhaps induction with even higher dosages of steroids and/or prolonged maintenance at larger doses may potentially avert the early onset of acute rejection. Such findings also question the validity of steroid-sparing protocols that are currently in use at certain vascularized composite allograft centers, especially when these patients have subsequently required reinstatement or repeated bolus dosing of steroids

Table 3. Related Nonhuman Primate Studies Investigating Various Immunologic Protocols and Outcomes on Acute Rejection

Authors	No.	VCA	MHC Mismatch	Induction	Maintenance	Rejection	Alloantibody
Cendales et al.[29]	7	Hand	"Nonidentical"	No	FK506, MMF, steroid	Median POD 17 (range, 5–76)	Yes (6/7)
Silverman et al.[30]	3	Face	?	ATG	FK506	POD 45, 70	Not checked
Barth et al.[31]	5	Face	?	High-dose FK506	FK506	No (but developed PTLD from POD 60 to 177)	No
	3	Face	?	High-dose FK506	Rapamycin (after 28 days)	POD 28, 49, 70	No
Barth et al.[32]	4	Face	?	High-dose FK506	FK506, MMF	POD 44, 163	No

VCA, vascularized composite allograft; MHC, major histocompatibility complex; FK506, tacrolimus; MMF, mycophenolate mofetil; POD, postoperative day; ATG, antithymocyte globulin; PTLD, posttransplant lymphoproliferative disorder.

because of multiple rejection episodes.[41,42] More recently, it appears that induction with both T and B cell depletion (using rituximab), followed by triple immunosuppressive therapy for maintenance, can allow vascularized composite allograft patients to survive rejection-free for at least 2 years.[43] Further work aimed at characterization of these skin-resident T cells is ongoing in our (manuscript in progress) and others' laboratories.

No Sensitization after Rejection Episodes

Interestingly, and surprisingly, donor-specific antibodies were not detected after rejection episodes in our nonhuman primate recipients, a finding that mirrors our clinical experience in two human allograft patients (hand and penile) after a total of four rejection episodes. Related experience from nonhuman primate tolerance studies at our center suggest that with the development of donor-specific antibodies after an acute rejection episode in renal transplantation, the development of mixed chimerism and tolerance will not be successful.[1,4,5]

Lack of Chimerism

While transient mixed chimerism was sufficient for tolerance of kidney allografts clinically, our group has previously shown that stable mixed chimerism is required for vascularized composite allograft tolerance in preclinical swine studies.[1,3,44] Similarly, although Barth et al.[32] have reported detection of "macrochimerism" (>1 percent circulating donor cells), with levels ranging between 1.27 and 11.7 percent, rejection still developed. In our current study, M3815 had evidence of transient mixed chimerism (38.9 percent donor origin CD3+ cells at 5 weeks after bone marrow transplantation; **see Figure, Supplementar y Digital Content 6**, ***http://links.lww.com/PRS/E3***) but failed to develop tolerance to the allograft, a result that is consistent with findings in our swine model. That its allograft was able to survive immunosuppression-free for 36 days after cessation of all immunosuppression may potentially be accounted for by the residual effect of the peri–bone marrow transplantation bridging course of cyclosporine and tocilizumab.[6,45] Interestingly, carboxyfluorescein succinimidyl ester mixed lymphocyte reaction suggested that after bone marrow transplantation, the immunomodulatory effect of donor bone marrow cells led to persistent and generalized unresponsiveness (based on in vitro assessment). Clinically, however, rejection episodes still developed with increasingly shorter intervals (from 5 weeks to 2 weeks) and decreased response to standard immunosuppressive treatment. Agents to help engraftment of bone marrow for long-term repopulating cells are undergoing investigation in our laboratory, with encouraging results (recent chimerism levels of 30 percent donor-origin cells have been obtained at 30 days after bone marrow transplantation in nonhuman primate allografts; data not shown).

Chronic Rejection in Nonhuman Primate Vascularized Composite Allografts

Progressive worsening of M3815's vascularized composite allograft appearance and eventual nonresponse to treatment of acute rejection episodes led to a diagnosis of chronic rejection that was verified histologically. Although circulating donor-specific antibodies remained negative throughout, terminal histologic analysis detected both C4d and graft vasculopathy in deep vessels, which suggests ongoing, chronic, active antibody-mediated rejection. Certainly, non-HLA antibodies may account for this donor-specific antibody-negative/C4d-positive phenomenon,[46] but a contributory, local mechanism at the level of the skin may also be responsible and remains to be elucidated. This finding in and of itself is exciting, as it represents a potential model for the study and treatment of chronic rejection in a large animal vascularized composite allograft model.

Future Work

For successful transition to the tolerance induction period, the delayed tolerance induction protocol may require novel immunosuppression strategies to avert the development of acute rejection in the early post–vascularized composite allograft period. This requirement is in stark contrast to solid organ transplantation, where the incidence of acute rejection of kidney allografts is less than 10 percent following transplantation under similar immunologic protocols,[47] a finding that may account for the higher success rate of achieving mixed chimerism through the delayed tolerance induction protocol in nonhuman primate solid organ transplantation studies. Alternative options include local delivery of FK50648 to enable therapeutic levels at the skin (but not systemically) to ameliorate rejection episodes and sensitization. This strategy has promise for the prevention of acute rejection, avoidance of overimmunosuppression and consequent post-transplant lymphoproliferative disorder. Such a technology is currently under evaluation, with encouraging early outcomes in our laboratory. In addition, new agents to reduce the barrier to stem cell engraftment are also under investigation and have resulted in successful chimerism induction in nonhuman primate models of solid organ transplantation. Finally, further study of vascularized composite allograft skin immunobiology, especially with regard to specific and candidate cell populations responsible for acute rejection episodes and consequent sensitization, may yet provide invaluable and novel insights.

CONCLUSIONS

Tolerance is more critical than ever at this time for vascularized composite allograft transplantation in light of the expected and observed long-term sequelae of chronic immunosuppression that are being recognized with more frequency in our patients. The portent of chronic rejection of vascularized composite allografts, for which tolerance represents a solution, only amplifies the directive to develop safe tolerance protocols in large animal models. Nonhuman primate models, while challenging, represent a rigorous platform for the engineering of mixed chimerism-based regimens for vascularized composite allograft patients. The data herein demonstrate significant “lessons learned” toward eventual successful translation of clinical tolerance for vascularized composite allografts.

REFERENCES

1. Kawai T, Sachs DH, Sykes M, Cosimi AB; Immune Tolerance Network. HLA-mismatched renal transplantation with- out maintenance immunosuppression. *N Engl J Med.* 2013;368:1850–1852.
2. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized com- posite allograft tolerance across MHC barriers in a large ani- mal model. *Am J Transplant.* 2014;14:343–355.
3. Leto Barone AA, Kurtz JM, Albritton A, et al. Effects of tran- sient donor chimerism on rejection of MHC-mismatched vascularized composite allografts in swine. *Vasc Compos Allotransplantation* 2015;2:1–8.
4. Yamada Y, Benichou G, Cosimi AB, Kawai T. Tolerance induction after organ transplantation, "delayed tolerance," via the mixed chimerism approach: Planting flowers in a battle field. *Chimerism* 2012;3:24–28.
5. Yamada Y, Boskovic S, Aoyama A, et al. Overcoming memory T-cell responses for induction of delayed tolerance in non- human primates. *Am J Transplant.* 2012;12:330–340.
6. Tonsho M, Lee S, Aoyama A, et al. Tolerance of lung allografts achieved in nonhuman primates via mixed hema- topoietic chimerism. *Am J Transplant.* 2015;15:2231–2239.
7. Elias N, Cosimi AB, Kawai T. Clinical trials for induction of renal allograft tolerance. *Curr Opin Organ Transplant.* 2015;20:406–411.
8. Ben-Amotz O, Kruger EA, McAndrew C, et al. Logistics in coordinating the first adult transatlantic bilateral hand trans- plant: Lessons learned. *Plast Reconstr Surg.* 2018;142:730–735.
9. Gorantla VS, Schneeberger S, Moore LR, et al. Development and validation of a procedure to isolate viable bone marrow cells from the vertebrae of cadaveric organ donors for com- posite organ grafting. *Cytotherapy* 2012;14:104–113.
10. Chen B, Xu H, Corbin DR, Ildstad ST. A clinically fea- sible approach to induce delayed tolerance in recipients of prior kidney or vascularized composite allotransplants. *Transplantation* 2012;94:671–678.
11. Huh K, Sommer W, Robinson K, et al. Two months delayed induction of mixed chimerism after heart and kidney cotransplantation in nonhuman primates. *J Heart Lung Transplant*. 2017;36(Suppl):S192–S193.
12. O'Connor SL, Blasky AJ, Pendley CJ, et al. Comprehensive characterization of MHC class II haplotypes in Mauritian cynomolgus macaques. *Immunogenetics* 2007;59:449–462.
13. Pendley CJ, Becker EA, Karl JA, et al. MHC class I character- ization of Indonesian cynomolgus macaques. *Immunogenetics* 2008;60:339–351.
14. Leonard DA, Powell H, Albritton A, et al. Upper extrem- ity transplantation in non-human primates: An ortho-topic model for translational research. *Vasc Compos Allotransplantation* 2015;2:17–25.
15. Barth RN, Bluebond-Langner R, Nam A, et al. Facial sub- unit composite tissue allografts in nonhuman primates: I. Technical and immunosuppressive requirements for pro- longed graft survival. *Plast Reconstr Surg.* 2009;123:493–501.
16. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tis- sue allograft pathology. *Am J Transplant.* 2008;8:1396–1400.
17. Kawai T, Sogawa H, Boskovic S, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant.* 2004;4:1391–1398.
18. Koyama I, Nadazdin O, Boskovic S, et al. Depletion of CD8 memory T cells for induction of tolerance of a previously trans- planted kidney allograft. *Am J Transplant.* 2007;7:1055–1061.
19. Shanmugarajah K, Powell H, Leonard DA, et al. The effect of MHC antigen matching between donors and recipients on skin tolerance of vascularized composite allografts. *Am J Transplant.* 2017;17:1729–1741.
20. Boskovic S, Kawai T, Smith RN, et al. Monitoring antido- nor alloantibodies as a predictive assay for renal allograft tolerance/long-term observations in nonhuman primates. *Transplantation* 2006;82:819–825.
21. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. *Transplantation* 2010;90:1590–1594.
22. Petruzzo P, Dubernard JM. The International Registry on Hand and Composite Tissue Allotransplantation. *Clin Transpl*. 2011:247–253.
23. Petruzzo P, Sardu C, Lanzetta M, Dubernard JM. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). *Curr Transplant Reports* 2017;4(4):294–303.
24. Ng ZY, Lellouch AG, Rosales IA, et al. Graft vasculopathy of vascularized composite allografts in humans: A literature review and retrospective study. *Transpl Int.* 2019;32:831–838.
25. Kawai T, Sachs DH, Sprangers B, et al. Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance immunosup- pression. *Am J Transplant.* 2014;14:1599–1611.
26. Sachs DH, Sykes M, Kawai T, Cosimi AB. Immuno- intervention for the induction of transplantation tolerance through mixed chimerism. *Semin Immunol.* 2011;23:165–173.
27. Zitsman JS, Alonso-Guallart P, Ovanez C, et al. Distinctive leukocyte subpopulations according to organ type in cyno- molgus macaques. *Comp Med.* 2016;66:308–323.
28. Nojima M, Ihara H, Kyo M, et al. The significant effect of HLA-DRB1 matching on acute rejection in kidney trans- plants. *Transpl Int.* 1996;9(Suppl 1):S11–S15.
29. Cendales LC, Xu H, Bacher J, Eckhaus MA, Kleiner DE, Kirk AD. Composite tissue allotransplantation: Development of a preclinical model in nonhuman primates. *Transplantation* 2005;80:1447–1454.

30. Silverman RP, Banks ND, Detolla LJ, et al. A heterotopic pri- mate model for facial composite tissue transplantation. *Ann Plast Surg.* 2008;60:209–216.
31. Barth RN, Nam AJ, Stanwix MG, et al. Prolonged survival of composite facial allografts in non-human primates associated with posttransplant lymphoproliferative disorder. *Transplantation* 2009;88:1242–1250.
32. Barth RN, Rodriguez ED, Mundinger GS, et al. Vascularized bone marrow-based immunosuppression inhibits rejection of vascularized composite allografts in nonhuman primates. *Am J Transplant.* 2011;11:1407–1416.
33. Schmidtko J, Wang R, Wu CL, et al. Posttransplant lymphop- roliferative disorder associated with an Epstein-Barr-related virus in cynomolgus monkeys. *Transplantation* 2002;73:1431–1439.
34. Clark RA, Chong B, Mirchandani N, et al. The vast majority of CLA+ T cells are resident in normal skin. *Immunol.* 2006;176:4431–4439.
35. Lian CG, Bueno EM, Granter SR, et al. Biomarker evaluation of face transplant rejection: Association of donor T cells with target cell injury. *Mod Pathol.* 2014;27:788–799.
36. Sarhane KA, Tuffaha SH, Broyles JM, et al. A critical analysis of rejection in vascularized composite allotransplantation: Clinical, cellular and molecular aspects, current challenges, and novel concepts. *Front Immunol.* 2013;4:406.
37. Ng ZY, Read C, Kurtz JM, Cetrulo CL. Memory T cells in vascularized composite allotransplantation. *Vasc Compos Allotransplantation* 2015;2:75–79.
38. Krummey SM, Ford ML. Heterogeneity within T cell memory: Implications for transplant tolerance. *Front Immunol.* 2012;3:36.
39. Jong G, Ruiter JR, Haring R. Genetic structure of a popu- lation with social structure and migration. In: *Conservation Genetics*. Basel: Birkhäuser Basel; 1994:147–164.
40. Adam L, Rosenbaum P, Cosma A, Le Grand R, Martinon F. Identification of skin immune cells in non-human primates. *J Immunol Methods* 2015;426:42–49.
41. Kaufman CL, Ouseph R, Blair B, et al. Graft vasculopa- thy in clinical hand transplantation. *Am J Transplant.* 2012;12:1004–1016.
42. Diaz-Siso JR, Fischer S, Sisk GC, et al. Initial experience of dual maintenance immunosuppression with steroid with- drawal in vascular composite tissue allotransplantation. *Am J Transplant.* 2015;15:1421–1431.
43. Gelb BE, Diaz-Siso JR, Plana NM, et al. Absence of rejection in a facial allograft recipient with a positive flow crossmatch 24 months after induction with rabbit anti-thymocyte globu- lin and anti-CD20 monoclonal antibody. *Case Rep Transplant.* 2018;2018:7691072.
44. Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL. Mixed chi- merism-based regimens in VCA. *Curr Transplant Reports* 2016;3:390–394.
45. Aoyama A, Tonsho M, Smith RN, et al. Non-human pri- mate lung allograft survival is prolonged by IL-6 Inhibition and ATG treatment possibly through expansion of periph- eral regulatory T cells. *Am J Transplant.* 2016;16(suppl 3):203–404.
46. Shinoda K, Akiyoshi T, Chase CM, et al. Depletion of foxp3(+) T cells abrogates tolerance of skin and heart allografts in murine mixed chimeras without the loss of mixed chime- rism. *Am J Transplant.* 2014;14:2263–2274.
47. Kaufman DB, Leventhal JR, Axelrod D, Gallon LG, Parker MA, Stuart FP. Alemtuzumab induction and prednisone-free maintenance immunotherapy in kidney transplantation: Comparison with basiliximab induction–long-term results. *Am J Transplant.* 2005;5:2539–2548.
48. Mastroianni M, Ng ZY, Goyal R, et al. Topical delivery of immunosuppression to prolong xenogeneic and allo-geneic split-thickness skin graft survival. *J Burn Care Res*. 2018;39:363–373.
49. Sewgobind VD, van der Laan LJ, Kho MM, et al. The cal- cineurin inhibitor tacrolimus allows the induction of func- tional CD4CD25 regulatory T cells by rabbit anti-thymocyte globulins. *Clin Exp Immunol.* 2010;161:364–377.

CHAPTER 05

Local Immunosuppression for Vascularized Composite Allografts: Application of Topical FK506-TyroSpheres in a Nonhuman Primate Model

Amon-Ra Gama
Zhi Yang Ng
Kumaran Shanmugarajah
Melissa Mastroianni
Mark A. Randolph
Alexandre G. Lellouch
Joachim Kohn
Curtis L. Cetrulo, Jr.

Adapted from Model J Burn Care Res. 2020 Apr 30;iraa062. doi: 10.1093/jbcr/iraa062. PMID: 32352521

ABSTRACT

Transplantation of vascularized composite allografts (VCAs) provides a means of restoring complex anatomical and functional units following burns and other disfigurement otherwise not amenable to conventional autologous reconstructive surgery. While short- to intermediate-term VCA survival is largely dependent on patient compliance with medication, the myriad of side effects resulting from lifelong systemic immunosuppression continue to pose a significant challenge. Topical immunosuppression is therefore a logical and attractive alternative for VCA. Current formulations are limited though, by poor skin penetration but this may be mitigated by conjugation of immunosuppressive drugs to TyroSpheres for enhanced delivery. Therefore, we investigated the topical application of FK506-TyroSpheres (in the form of a gel dressing) in a clinically relevant nonhuman primate VCA model to determine if allograft survival could be prolonged at reduced levels of maintenance systemic immunosuppression. Six Major Histocompatibility Complex (MHC)- mismatched cynomolgus macaques (Macaca fascicularis) served as reciprocal donors and recipients of radial forearm fasciocutaneous flaps. Standard Bacitracin ointment and FK506-TyroSpheres were applied every other day to the VCAs of animals in groups 1 (controls, n = 2) and 2 (experimental, n = 4), respectively, before gradual taper of systemic FK506. Clinical features of VCA rejection still developed when systemic FK506 fell below 10 ng/ml despite application of FK506-TyroSpheres and prolonged VCA survival was not achieved. However, unwanted systemic FK506 absorption was avoided with TyroSphere technology. Further refinement to optimize local drug delivery profiles to achieve and maintain therapeutic delivery of FK506 with TyroSpheres is underway, leveraging significant experience in controlled drug delivery to mitigate acute rejection of VCAs.

INTRODUCTION

Transplantation of vascularized composite allografts (VCAs) provides a means of restoring complex anatomical and functional units, such as the hands or face, to patients with severe disfigurement from burns or limb loss otherwise not amenable to conventional autologous reconstructive surgery.[1,2] To date, over 43 facial and 120 upper extremity transplants have been successfully performed worldwide[3,4] with about 20% occurring in burn survivors.[5] However, in contrast to solid organ transplantation (SOT) which are life-saving procedures, VCA is life-enhancing by restoring both a patient's dignity[6] and quality of life through functional and psychosocial rehabilitation.[7] Because of this important distinction, efforts to develop tolerance protocols[8] as well as means for reducing the burden of immunosuppression required by VCA recipients are of paramount importance for further evolution of this field.

Currently, maintenance immunosuppression regimens in VCA are lifelong and largely based on the SOT experience, which typically consist of combination triple therapy—prednisone, mycophenolate mofetil, and tacrolimus (FK506). The latter belongs to the class of drugs called calcineurin inhibitors and has been the mainstay in preventing and treating acute rejection episodes in liver, renal, and pancreatic transplants since the 1980s; among calcineurin inhibitors, FK506 has proven more effective than cyclosporine.[9–11] While short-to-intermediate-term VCA survival has been near 100% in patients compliant with medication, the myriad of side effects resulting from chronic immunosuppression include metabolic complications, medication toxicities, malignancies, and infections that have all predictably been reported.[12–15]

Furthermore, despite strict adherence to treatment regimens, up to 85% of VCA patients experience acute rejection of the skin in the first year after transplantation[16,17] and require additional treatment to prevent progression of rejection and irreversible loss of the VCA. This represents an increased overall burden of immunosuppression and associated risk of developing the aforementioned side effects. Naturally, there has been growing interest in alternatives to systemic administration such as topical delivery of immunosuppression to the VCA itself, given its comparative ease of access unlike the typical intra-abdominal location of SOT. The topical route of administration also has the following theoretical advantages: 1) improves recipient compliance, 2) potentially reduces systemic dose requirements and the risk of systemic complications, and 3) increases local delivery of the therapeutic to the vicinity of immunologic cellular interactions between donor and recipient skin. Indeed, there have been clinical reports of the use of various topical immunosuppressive agents (protopic, clobetasol propionate) in various combinatory regimens with systemic therapy (eg, steroid pulse, increasing maintenance dosages) for the treatment of rejection episodes in VCA, albeit with inconsistent results.[18,19]

Mastroianni et al have previously shown that topical delivery of slow-release immunosuppression through TyroSpheres technology could lead to nontake of both allo- and xeno-skin grafts due to impaired inflammation, angiogenesis, and vascularization.[20] Briefly, TyroSpheres is a Polymeric Local Delivery System with a fully degradable

ABA-type triblock copolymer made up of three subunits—desaminotyrosyltyrosine ethyl ester, desaminotyrosyltyrosine, and polyethylene glycol (1K) (Figure 1).[21] Polymer synthesis consists of two key steps: chemical synthesis of the monomers, followed by preparation of the polymer itself. Therefore, the objective of this study was to assess whether FK506-TyroSpheres in the form of a gel dressing could achieve rejection-free allograft survival in a clinically relevant nonhuman primate (NHP) model, and reduce overall systemic maintenance immunosuppression requirements when applied to the skin of primarily vascularized VCA transplants.

METHODS

FK506-TyroSpheres Design

TyroSpheres were loaded with FK506 as described previously.[20] A gel formulation was prepared specifically for this study by suspending TyroSpheres in 1%-hydroxypropyl methylcellulose gel to provide better handling properties for topical applications.

Animals

Three full Major Histocompatibility Complex (MHC) mismatched cynomologus macaque (Macaca fascicularis) pairs (total n = 6) were used as reciprocal donors and recipients of fasciocutaneous radial forearm flaps similar to that described previously by Cendales et al.[22] The study was approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Guide for the Care and Use of Laboratory Animals under strict veterinary supervision.

Experience with large animal models for transplantation research over the past 30 years at this center has demonstrated that approximately three transplant recipients per experimental group are sufficient to provide statistical power for detection of survival differences, and to achieve statistical significance in the comparison of categorically scored histological specimens using nonparametric statistical tests (eg, Mann–Whitney U) with an alpha level of 5% and a beta level of 80%.[23]

Radial Forearm Flap Allotransplantation

Animals in both control (n = 2) and experimental (n = 4) groups received a cast on the left upper extremity 14 days prior to VCA transplantation for acclimatization. On the day of surgery, animals were premedicated with intramuscular (IM) glycopyrolate and sedated using IM ketamine. After endotracheal intubation, general anesthesia was maintained with a continuous infusion of ketamine and isoflurane 0.5% to 3%. Radial forearm fasciocutaneous flaps (6 × 4 cm) were designed with careful attention to the position and course of the cephalic vein. The skin and underlying fascia were raised based on the radial artery and cephalic vein. Flap inset and microsurgical anastomosis were then performed before wound closure in layers (Figure 2).

Experimental Protocol

Following VCA transplantation, all animals (n = 6) received initial systemic immunosuppression with intravenous (IV) methylprednisolone 40 mg, IM FK506 0.2 mg/kg, and IV mycophenolate mofetil 300 mg. Standard Bacitracin ointment was applied to cover the entire surface of the VCAs of control animals in group 1 (n = 2); FK506-TyroSpheres were similarly applied (~0.5 ml) every other day to the VCAs of experimental animals in group 2 (n = 4).

Both groups commenced taper of methylprednisolone (to maintenance of IM 1 mg/day after postoperative day [POD] 14) and FK506 from POD 1 and 7, respectively (Figure 3). VCAs were followed until complete clinical rejection, defined as less than 10% of viable skin remaining, was observed and the corresponding systemic levels of FK506 noted. Once complete rejection had developed, skin biopsies were taken, fixed in formalin, and sent for routine histopathological analysis by a board-certified pathologist blinded to the study. The animals were then killed and removed from study at this experimental time point.

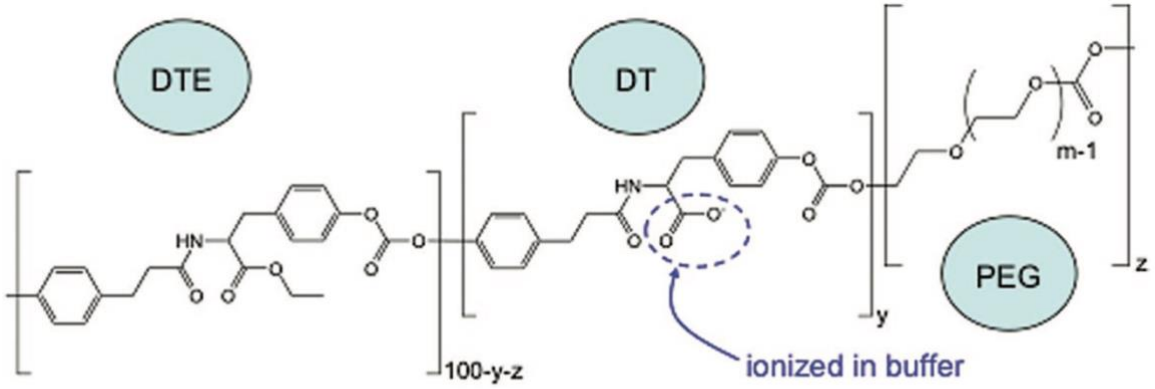


Figure 1. Schematic design of poly(DTE-co-y%DT-co-z% PEG_{1k} carbonate) copolymers used to discover an optimized composition for local drug delivery by varying the relative abundance of the three basic ingredients within the polymer matrix. *DT*, desaminotyrosyl-tyrosine; *DTE*, desaminotyrosyl-tyrosine ethyl ester; *PEG*, polyethylene glycol.

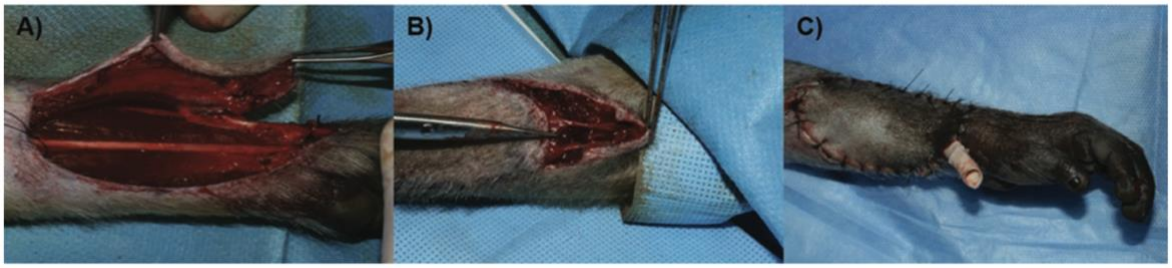


Figure 2. Radial forearm fasciocutaneous VCA model. (A) Dissection down to fascia, (B) anastomosis of cephalic vein and radial artery, (C) after flap inset and closure with a penrose drain left in situ. *VCA*, vascularized composite allograft.

RESULTS

FK506-TyroSpheres Absorption Profile in Human Cadaveric Skin

Humans and NHPs have similar topical skin absorption profiles.[24] Skin distribution studies were performed as previously described[25] and used to quantify the penetration of FK506 into the dermal layer of human cadaver skin (due to relative ease of procurement compared with ethical and resource considerations in NHPs) with 30% drug-loaded TyroSpheres-gel. Drug levels were consequently measured in the tissue and

showed a substantial increase in FK506 concentration within the epidermis, from 36 µg/g in the first hour after application to 606 µg/g after 24 h; there was little penetration of FK506 into the dermis, suggesting no systemic uptake (Table 1). Indeed, this was the case in the current study, whereby systemic FK506 levels in NHPs were tapered successfully according to protocol without further spikes (Figure 4).

Current Topical Formulation of FK506-TyroSpheres Did Not Prolong VCA Survival in NHPs

Two animals in the experimental group (FK506-TyroSpheres) lost their VCAs in the immediate postoperative period secondary to unsalvageable venous thrombosis, attributed to small vessel size. The remaining animals in both control (Bacitracin) and experimental groups (n = 2 each) displayed clinical features of rejection, such as increasing erythema, darkening of the skin, edema, and necrosis, when FK506 trough levels approached 10 ng/ml (Figures 4 and 5; target level for successful maintenance = 20–30 ng/ml).

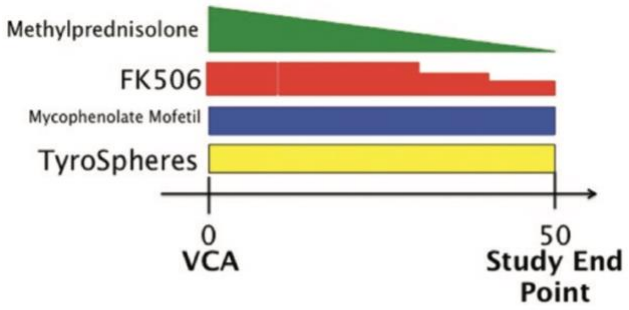


Figure 3. Schematic illustrating maintenance immunosuppression regimen of the current study following VCA. Methylprednisolone and FK506 undergo gradual taper from POD 1 and 7, respectively, while FK506-TyroSpheres are applied every other day liberally to cover the entire surface of the VCA in recipient animals. *FK506*, tacrolimus; *POD*, postoperative day; *VCA*, vascularized composite allograft.

Table 1. Distribution profile of FK506-TyroSpheres in the epidermis and dermis of human cadaveric skin

	FK506	
Time (h)	Epidermis (µg/g) ± SD	Dermis (µg/g) ± SD
1	36 ± 9	8 ± 5
2	43 ± 6	16 ± 17
4	186 ± 29	6 ± 1
24	606 ± 8	12 ± 12

FK506, tacrolimus.

VCAs first displayed signs of rejection on POD 8 and 20 in the control group, and POD 12 and 14 in the experimental group. Progression to full VCA loss occurred by POD 20 and 22 in the former, and POD 20 and 24 in the latter group (Table 2 and Figure 5). There were no significant clinical or histological differences between treatment and control groups observed (Figure 6). No maceration of the skin nor wound-related complications were noted locally; opportunistic infections and clinical signs of cardio- or nephrotoxicity were not observed systemically in the experimental group.

DISCUSSION

Local delivery of FK506 has been investigated as a potential maintenance agent in VCA and has so far yielded encouraging results in mitigating rejection in rodent models.[26,27] Gharb et al showed significant allograft survival in rodent hemiface transplants using topical FK506 alone while also achieving a lower incidence of complications both locally and systemically when used in combination with systemic immunosuppression.[26] Similarly, Solari et al demonstrated the efficacy of combining topical FK506 with systemic immunosuppression whereby four of six animals achieved post-VCA survival of 100 days without developing grade III rejection compared with animals treated with topical FK506 alone in which grade III rejection developed by POD 9.[27] Most recently, Unadkat et al were able to prolong survival of rat

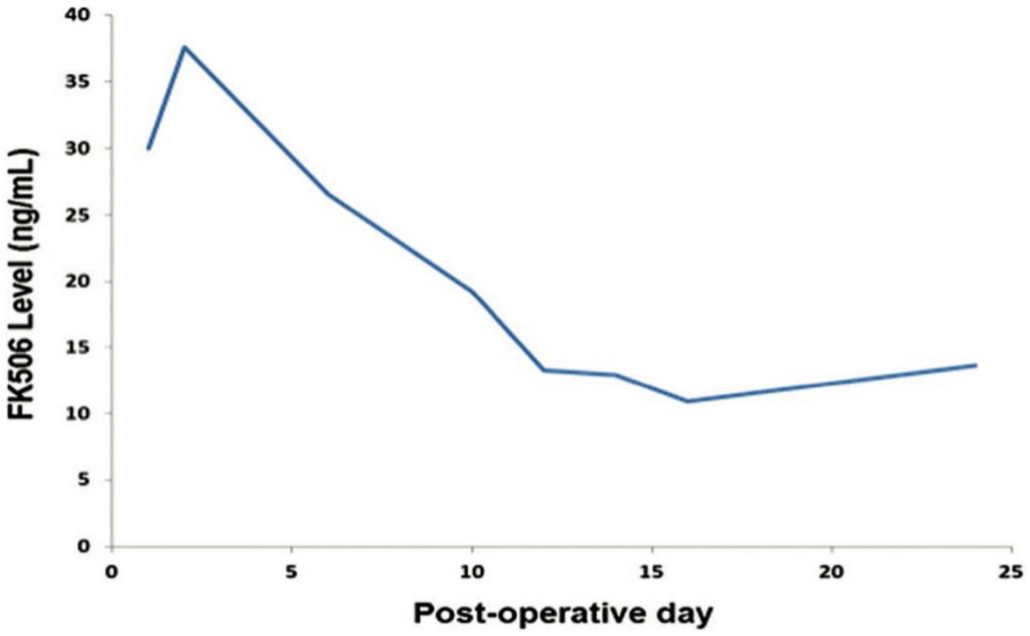


Figure 4. Representative systemic levels of FK506 during study. Immunosuppressive medications were tapered (without further spikes despite topical FK506-TyroSpheres application) to allow clinical demonstration of VCA rejection, which could be appreciated clinically when systemic FK506 levels were around 10 ng/ml. *FK506*, tacrolimus; *VCA*, vascularized composite allograft.

hindlimb VCA models, using a single implantable FK506 disk, for up to 180 days. Levels were maintained between 5 and 15 ng/ml, indicating minimal systemic absorption whilst maintaining the allograft free of rejection.28 Although these results are encouraging in small animal studies, further investigation is required to elucidate the benefits of local FK506 therapy in large animal models prior to clinical application.

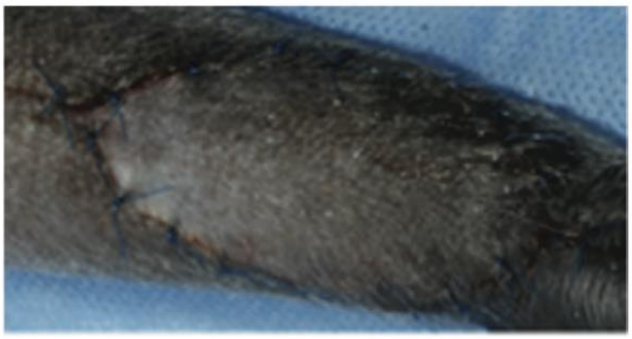
Due to its strong lipophilicity, FK506 is poorly absorbed across the stratum corneum layer of skin. In order to overcome this limitation, FK506 can be conjugated to tyrosine-derived block copolymers which spontaneously self-assemble in aqueous media into TyroSpheres. This approach has previously been shown to 1) be nontoxic in vitro[29] and in vivo,[30] [2]) bind and deliver hydrophobic drugs,[31] and [3]) significantly enhance the permeation of hydrophobic agents into the epidermis32 and allow investigators to achieve sustained and localized drug release.[25,33] Additionally, previous studies from this laboratory investigating the use of topical immunosuppression based on TyroSpheres technology led to very high local drug (FK506) levels that appeared to interfere with angiogenesis, neovascularization and "take" of split thickness skin grafts.[20] Hence, the drug-releasing parameters of the technology were modified in this study. In addition, this study evaluated the efficacy of FK506-TyroSpheres in preventing rejection of primarily vascularized allogenic skin flaps (ie, VCA), in contrast to skin grafts which undergo a significant period of ischemia, inflammation and reperfusion injury before inosculation and vascularization that can complicate evaluation of the effect of local immunosuppression for VCAs.

Unfortunately, the primary aim of rejection-free and prolonged VCA survival was not achieved in this NHP study. Nevertheless, important steps in the development of local immune therapy were achieved. First, unwanted systemic absorption of FK506 was

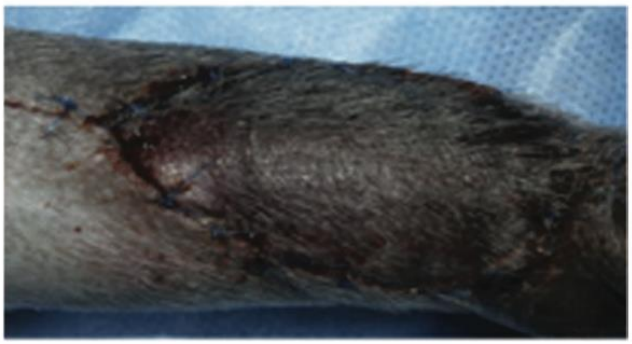
successfully avoided through TyroSpheres technology. Second, the drug release profile of FK506-TyroSpheres showed that at 24 h after application, the concentration of FK506 in human cadaveric epidermis is approximately 50 times that of the dermis (Table 1) but rejection of VCAs still developed despite repeat application of FK506- TyroSpheres every other day. This suggests that dermal tissue levels of FK506 were inadequate and likely due, in part, to insufficient skin penetration in the NHP model. This observation is important because, while the exact mechanisms underlying acute rejection of skin in VCA remain undetermined,[16] earlier work by Dvorak et al suggests that the microvascular endothelium located within the dermis is the major target of immunological responses in vascularized skin allografts,[34] which contrasts with observations of the Cuono technique for burn reconstruction in which allograft rejection is believed to originate from allogenic epidermis instead.[35] Recent work by Lian et al utilizing biomarker-based immunohistochemistry studies of clinical face transplant recipients' biopsy samples provide further evidence—perivascular accumulation of lymphocytes within the dermis was followed by epidermal infiltration and persistence during and after rejection episodes.[36] Related NHP studies from this laboratory have also enabled further characterization of the kinetics and nature of these cellular subsets in VCA skin which demonstrate a rapid, near complete turnover to recipient-origin cells involving the dermis and subsequently, the epidermis in the early posttransplantation period (Lellouch and Ng, unpublished data).

With regard to transplantation tolerance, a successful strategy for local immunosuppression could augment future tolerance induction protocols in an important way: recent large animal studies have suggested that transient high-dose FK506 for 60 days coupled with nonmyeloablative conditioning (50 cGy total body irradi- ation and 350 cGy thymic irradiation) could achieve im- munologic tolerance of VCA across full MHC-mismatch barriers (to mirror unrelated donor–recipient transplant pairs) with immunosuppression-free survival beyond POD 300.37 However, induction with prolonged, high-dose FK506 systemically is not clinically feasible because of the associated risks of cardio-38 and nephrotoxicity39; topical delivery of high-dose FK506 through TyroSpheres tech- nology to substitute for the high systemic doses in this protocol may thus be an option. An alternative approach to achieve transplantation tolerance is based on delayed mixed chimerism40 but success is predicated on the successful abrogation of rejection episodes during the delay period (Lellouch and Ng, unpublished data). The current clin- ical experience in VCA suggests though that translation of delayed tolerance induction protocols (DTIP) would likely be unsuccessful given the high incidence of acute rejection episodes in the first year posttransplantation as compared with SOT (85% vs 10%) despite the use of similar immu- nosuppressive regimens.13–15 Most encouragingly, this lab- oratory has managed to overcome this limitation through further modification of FK506-TyroSphere technology for a successful rejection-free delay period and successful gen- eration of mixed chimerism in a NHP DTIP study (data not shown). Further studies are currently underway to de- termine reproducibility of results.

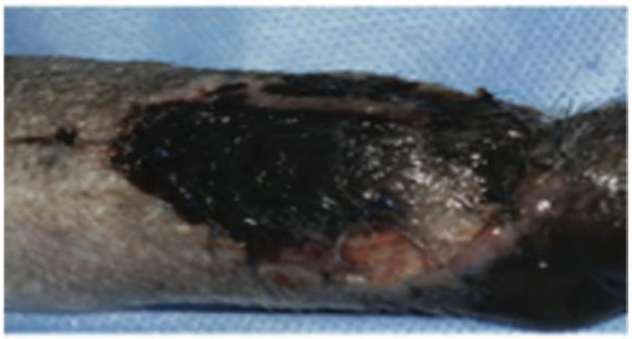
Finally, an alternative to the topical delivery of FK506 is intragraft administration. Gajanayake et al showed that a one-time injection of subdermal proteolytic enzyme-



Day 2



Day 10



Day 24

Figure 5. Representative time course of radial forearm fasciocutaneous flap VCA undergoing rejection following tapering of systemic immunosuppression; rejection was seen as erythema, swelling and progressed to frank necrosis. *VCA*, vascularized composite allograft.

activated FK506 hydrogel in rodent limb VCA transplants was a feasible alternative to topical therapy by prolonging allograft survival beyond 100 days.[41] Fries et al then showed prolonged VCA survival through subcutaneous injection of an enzyme-activated FK506 hydrogel (both low- and high-dose formulations) with overall survival ranging from 24 to 93 days. Three of four animals in the high-dose group had to be killed between POD 24 and 42 however, due to failure to thrive with weight loss and poor feeding, which probably reflects untoward systemic absorption of FK506.[42] Various other routes of drug delivery include subdermal penetration, direct subdermal delivery, and even intravascular therapies but their utility remain to be determined in the context of VCA.

For now, the ability to reproducibly control and achieve adequate local levels of FK506 to abrogate VCA rejection would represent a major advance in the field of VCA in particular, especially for burn patients who have exhausted autologous reconstructive options, and SOT in general for many reasons. If VCA patients can be maintained rejection-free with topical application of FK506- TyroSpheres in the form of a regular gel dressing alone, or in combination with overall reduced levels of systemic immunosuppression, such a technology may successfully allow the avoidance or minimization of rejection episodes that result from noncompliance with oral medications and the subsequent alloantibody formation that may lead to chronic rejection and negate potential tolerance induction strategies.[43]

CONCLUSION

While the current gel formulation of FK506-TyroSpheres was unable to mitigate the development of acute rejection following vascularized composite allotransplantation in a clinically relevant NHP model, invaluable mechanistic insights were gained, albeit indirectly. Such knowledge gained has enabled, and will continue, to be areas of active investigation in this laboratory for further development of therapeutics to address this most pressing of issues in the field of VCA.

Table 2. Summary of current study

Animal	Treatment	First Signs of Rejection (POD)	Full Rejection (POD)	Complication
Group 1—controls				
M714	Bacitracin	20	22	None
M814	Bacitracin	8	20	None
Group 2—experimental				
M414	FK506-TyroSpheres	12	20	None
M514	FK506-TyroSpheres	14	24	None
M314	FK506-TyroSpheres	N/A	N/A	Technical failure (venous thrombosis)
M614	FK506-TyroSpheres	N/A	N/A	Technical failure (venous thrombosis)

FK506, tacrolimus; N/A, Not Applicable; POD, postoperative day.

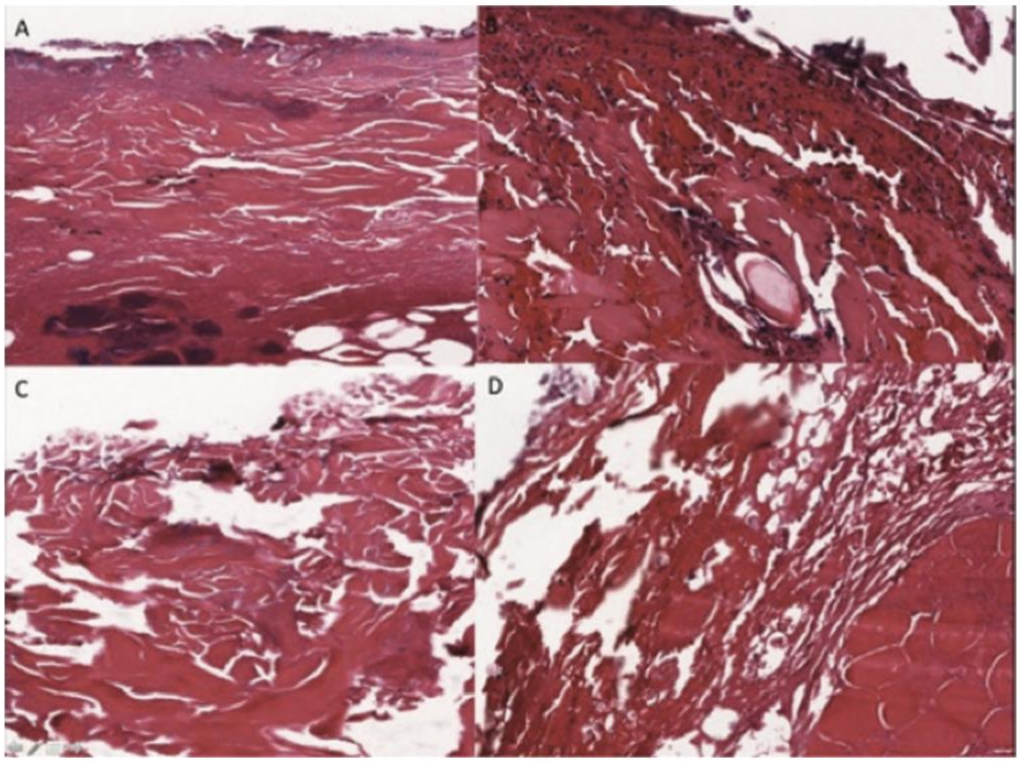


Figure 6. Histology from VCA biopsies taken at time of complete rejection (A) M714, nonviable skin and subcutaneous tissue, thrombosed vessels. (B) M814, necrosis and thrombus. (C) M414, nonviable skin. (D) M514, nonviable skin and muscle. *VCA*, vascularized composite allograft.

REFERENCES

1. Diaz-Siso JR, Bueno EM, Sisk GC, Marty FM, Pomahac B, Tullius SG. Vascularized composite tissue allotransplantation—state of the art. Clin Transplant 2013;27:330–7.
2. Pribaz JJ, Caterson EJ. Evolution and limitations of conventional autologous reconstruction of the head and neck. J Craniofac Surg 2013;24:99–107.
3. Sosin M, Rodriguez ED. The face transplantation update: 2016. Plast Reconstr Surg 2016;137:1841–50.
4. Shores JT, Brandacher G, Lee WP. Hand and upper extremity trans- plantation: an update of outcomes in the worldwide experience. Plast Reconstr Surg 2015;135:351e–60e.
5. Ng ZY, Lellouch AG, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL Jr. Vascularized composite allotransplantation—an emerging concept for burn reconstruction. J Burn Care Res 2017;38:371–8.
6. Lellouch AG, Lantieri LA. A second chance at life. Camb Q Healthc Ethics 2019;28:463–7.
7. Kaufman CL, Breidenbach W. World experience after more than a decade of clinical hand transplantation: update from the Louisville hand trans- plant program. Hand Clin 2011;27:417–21, vii.
8. Lellouch AG, Ng ZY, Kurtz JM et al. Mixed chimerism-based regimens in VCA. Curr Transplant Rep 2016;3:390–4.
9. Starzl TE, Todo S, Fung J, Demetris AJ, Venkataramman R, Jain A. FK 506 for liver, kidney, and pancreas transplantation. Lancet 1989;2:1000–4.
10. Filler G. Calcineurin inhibitors in pediatric renal transplant recipients. Paediatr Drugs 2007;9:165–74.
11. Bolin P Jr, Shihab FS, Mulloy L et al.; OPTIMA Study Group. Optimizing tacrolimus therapy in the maintenance of renal allografts: 12-month results. Transplantation 2008;86:88–95.
12. Broyles JM, Alrakan M, Ensor CR et al. Characterization, prophylaxis, and treatment of infectious complications in craniomaxillofacial and upper extremity allotransplantation: a multicenter perspective. Plast Reconstr Surg 2014;133:543e–51e.
13. Petruzzo P, Lanzetta M, Dubernard JM et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation 2010;90:1590–4.
14. Petruzzo P, Dubernard JM. The International Registry on Hand and Composite Tissue Allotransplantation. Clin Transpl 2011;247–53.
15. Petruzzo P, Sardu C, Lanzetta M et al. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). Curr Transplant Rep 2017;4:294–303.
16. Sarhane KA, Tuffaha SH, Broyles JM et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and mo- lecular aspects, current challenges, and novel concepts. Front Immunol 2013;4:406.
17. Kueckelhaus M, Fischer S, Seyda M et al. Vascularized composite allotransplantation: current standards and novel approaches to pre- vent acute rejection and chronic allograft deterioration. Transpl Int 2016;29:655–62.
18. Chen Y-T, Huang S-C, Chen C-C et al. Topical tacrolimus and steroids modulate T cells in acute rejection of hand allotransplantation: two case reports. Microsurgery 2020;40:217–23.
19. Diaz-Siso JR, Fischer S, Sisk GC et al. Initial experience of dual mainte- nance immunosuppression with steroid withdrawal in vascular composite tissue allotransplantation. Am J Transplant 2015;15:1421–31.
20. Mastroianni M, Ng ZY, Goyal R et al. Topical delivery of immunosup- pression to prolong xenogeneic and allogeneic split-thickness skin graft survival. J Burn Care Res 2018;39:363–73.
21. Bhatnagar D, Dube K, Damodaran VB et al. Effects of terminal ster- ilization on PEG-based bioresorbable polymers used in biomedical applications. Macromol Mater Eng 2016;301:1211–24.
22. Cendales LC, Xu H, Bacher J, Eckhaus MA, Kleiner DE, Kirk AD. Composite tissue allotransplantation: development of a preclin- ical model in nonhuman primates. Transplantation 2005;80: 1447–54.
23. Kawai T, Cosimi AB, Sachs DH. Preclinical and clinical studies on the induction of renal allograft tolerance through transient mixed chimerism. Curr Opin Organ Transplant 2011;16:366–71.
24. Godin B, Touitou E. Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models. Adv Drug Deliv Rev 2007;59:1152–61.
25. Batheja P, Sheihet L, Kohn J, Singer AJ, Michniak-Kohn B. Topical drug delivery by a polymeric nanosphere gel: formulation optimiza- tion and in vitro and in vivo skin distribution studies. J Control Release 2011;149:159–67.
26. Gharb BB, Rampazzo A, Altuntas SH et al. Effectiveness of topical immunosuppressants in prevention and treatment of rejection in face allotransplantation. Transplantation 2013;95:1197–203.
27. Solari MG, Washington KM, Sacks JM et al. Daily topical tacrolimus therapy prevents skin rejection in a rodent hind limb allograft model. Plast Reconstr Surg 2009;123(2 Suppl):17S–25S
28. Unadkat JV, Schnider JT, Feturi FG et al. Single implantable FK506 disk prevents rejection in vascularized composite allotransplantation. Plast Reconstr Surg 2017;139:403e–14e.

29. Mondon K, Zeisser-Labouèbe M, Gurny R, Möller M. Novel cyclosporin A formulations using MPEG-hexyl-substituted polylactide micelles: a suitability study. Eur J Pharm Biopharm 2011;77:56–65.
30. Sheihet L, Dubin RA, Devore D, Kohn J. Hydrophobic drug de- livery by self-assembling triblock copolymer-derived nanospheres. Biomacromolecules 2005;6:2726–31.
31. Nardin C, Bolikal D, Kohn J. Nontoxic block copolymer nanospheres: design and characterization. Langmuir 2004;20:11721–5.
32. Sheihet L, Garbuzenko OB, Bushman J, Gounder MK, Minko T, Kohn J. Paclitaxel in tyrosine-derived nanospheres as a potential anti-cancer agent: in vivo evaluation of toxicity and efficacy in comparison with paclitaxel in Cremophor. Eur J Pharm Sci 2012;45:320–9.
33. Kilfoyle BE, Sheihet L, Zhang Z, Laohoo M, Kohn J, Michniak- Kohn BB. Development of paclitaxel-TyroSpheres for topical skin treat- ment. J Control Release 2012;163:18–24.
34. Dvorak HF, Mihm MC Jr, Dvorak AM, Barnes BA, Galli SJ. The microvasculature is the critical target of the immune response in vascularized skin allograft rejection. J Invest Dermatol 1980;74:280–4.
35. Cuono C, Langdon R, McGuire J. Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury. Lancet 1986;1:1123–4.
36. Lian CG, Bueno EM, Granter SR et al. Biomarker evaluation of face transplant rejection: association of donor T cells with target cell injury. Mod Pathol 2014;27:788–99.
37. Wang HD, Swanson EW, Cheng H-T et al. 2523: vascularized composite allograft tolerance with transient high-dose tacrolimus across a full MHC mismatch in a large animal model. Vasc Compos Allotransplantation 2016;3:8. 38. Miller LW. Cardiovascular toxicities of immunosuppressive agents. Am J
38. Transplant 2002;2:807–18.
39. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009;4:481–508.
40. Chen B, Xu H, Corbin DR, Ildstad ST. A clinically feasible approach to induce delayed tolerance in recipients of prior kidney or vascularized composite allotransplants. Transplantation 2012;94:671–8.
41. Gajanayake T, Olariu R, Leclère FM et al. A single localized dose of enzyme-responsive hydrogel improves long-term survival of a vascularized composite allograft. Sci Transl Med 2014;6:249ra110.
42. Fries CA, Lawson SD, Wang LC et al. Graft-implanted, enzyme respon- sive, tacrolimus-eluting hydrogel enables long-term survival of orthotopic porcine limb vascularized composite allografts: a proof of concept study. PLoS One 2019;14:e0210914.
43. Ng ZY, Lellouch AG, Rosales IA et al. Graft vasculopathy of vascularized composite allografts in humans: a literature review and retrospective study. Transpl Int 2019;32:831–8.

CHAPTER 06

Local FK506 implants in non-human primates to prevent early acute rejection in vascularized composite allografts

Alexandre G. Lellouch
Corentin B. Taveau
Alec R. Andrews
Joseph Molde
Zhi Yang Ng,
Philipp Tratnig-Frankl
Ivy A. Rosales
Marion Goutard
Elise Lupon
Laurent A. Lantieri
Robert B. Colvin
Mark A. Randolph
Joachim Kohn
Curtis L. Cetrulo Jr.

Adapted from Ann Transl Med 2021. doi: 10.21037/atm-21-313

ABSTRACT

Background: Previous vascularized composite allograft (VCA) studies from our laboratory have shown that topical FK506 delivery in non-human primates (NHPs) was limited by inadequate dermal penetration and rejection persisted. Herein, we report the first utilization of FK506 via subcutaneously implanted discs to mitigate VCA rejection in NHPs.

Methods: Full major histocompatibility complex (MHC)-mismatched NHP pairs underwent partial-face VCA and FK506 disc implantation along the suture line. All allotransplants were maintained post-operatively for two months on the FK506 discs, methylprednisolone, mycophenolate mofetil, and supplemented with intramuscular FK506 if necessary. Group 1 (n=4) was used for optimization of the implant, while Group 2 (n=3) underwent delayed bone marrow transplantation (DBMT) after two months. VCA skin biopsies and peripheral blood samples were obtained for serial assessment of rejection and mixed chimerism by histopathology and flow cytometry respectively.

Results: In Group 1, two technical failures occurred. Of the remaining two NHPs, one developed supratherapeutic levels of FK506 (50–120 ng/mL) and had to be euthanized on postoperative day (POD) 12. Reformulation of the implant resulted in stable FK506 levels (20–30 ng/mL) up to POD12 when further intramuscular (IM) FK506 injections were necessitated. In Group 2, two NHPs survived to undergo conditioning and one successfully developed chimerism at 2–3 weeks post-DBMT (96–97% granulocytes and 7–11% lymphocytes of recipient-origin). However, all three NHPs had to be terminated from study at POD64, 77 and 86 due to underlying post-transplant lymphoproliferative disorder. All VCAs remained rejection-free up to study endpoint otherwise.

Conclusions: This study shows preliminary results of local FK506 implants in potentially mitigating VCA acute rejection for tolerance protocols based on mixed chimerism approach.

INTRODUCTION

It has been more than 20 years since the first successful hand transplant1 and the utility of vascularized composite allografts (VCAs) remains plagued by the same fundamental problem, lifelong systemic immunosuppression and the attendant sequelae of life-threatening complications[2-4]. Compared to solid organ transplantation (SOT), VCAs differ in the composition of the transplanted tissues including the skin that presents a higher antigenic load potentially requiring even more immunosuppression[5,6]. Based on the latest report of the International Registry on Hand and Composite Tissue Allotransplantation in 2017, patients compliant with triple immunosuppressive regimens (i.e., calcineurin inhibitor, steroids, mycophenolate mofetil (MMF) were able to maintain and prevent early loss of the VCA. However, acute rejection of the VCA occurs in up to 90% of patients, over the first year post transplantation, with almost 60% developing multiple episodes[2]. The exteriorized skin component of VCAs could present an opportunity for earlier detection and prompt treatment of rejection with topical immunosuppression. The clinical experience in VCA however, has more or less confirmed that while topicals may help reverse rejection episodes, further titration and boluses of systemic immunosuppression (typically steroids) were often necessary[7,8]. Indeed, our laboratory has recently reported that topical FK506 did not allow the reduction of systemic maintenance immunosuppression in VCAs in a non-human primate (NHP) model and could only penetrate into the epidermis[9]. Additionally, we have demonstrated through in vitro analysis of NHP VCA skin biopsies that by week 2, skin leukocyte populations (CD4+, CD8+) within the dermis have undergone near-complete (>90%) turnover from donor (VCA) to recipient-origin[10]. Nonetheless, VCA rejection episodes were unavoidable and rendered subsequent immune tolerance protocol attempts through mixed chimerism using bone marrow transplantation impossible[10].

Inducing a state of immunologic tolerance to allotransplants in the absence of immunosuppression would greatly expand the clinical application of VCA. Mixed chimerism, in which both donor-and recipient-derived lymphohematopoietic elements co-exist in the recipient[11], may negate the spectre of chronic rejection, and is a proven strategy to enable immunosuppression withdrawal as demonstrated in clinical trials on renal transplantation[12]. Many reported strategies to induce mixed chimerism rely on 1–2 days of recipient preconditioning, including viable donor cell infusions, which would not be logistically possible for VCAs from cadaveric donors. Using a delayed tolerance induction protocol (DTIP), whereby the donor cells are temporarily stored for infusion 1–2 months after VCA, could be one approach if acute rejection was mitigated during the delay period.

A variety of research efforts are underway to develop drug delivery systems (DDS) that may mitigate rejection in VCA[13,14]. All previous DDS studies have been limited to small animal models that arguably, have limited translational potential, especially in the context of VCA15, except for a recent porcine study by Fries et al.[16]. Those authors showed that tacrolimus-loaded hydrogel implanted into the allograft delayed the onset of Grade IV acute rejection in porcine forelimb VCAs. In this study, we describe our experience in replacing our previous topical FK506- TyroSpheres (in the form of a gel

dressing acting mainly on the epidermis[9] with subcutaneously implanted discs for direct and sustained release into VCA dermis. The primary and secondary aims of our study are thus mitigation of acute rejection, and the successful development of delayed mixed chimerism respectively. The attainment of the former is expected to enable the latter to be achieved. We present the following article in accordance the ARRIVE reporting checklist.

Methods

All experiments were performed in accordance with the NIH's Guide for the Care and Use of Laboratory Animals with approval from the IACUCs of Rutgers University and Massachusetts General Hospital (2015N000146).

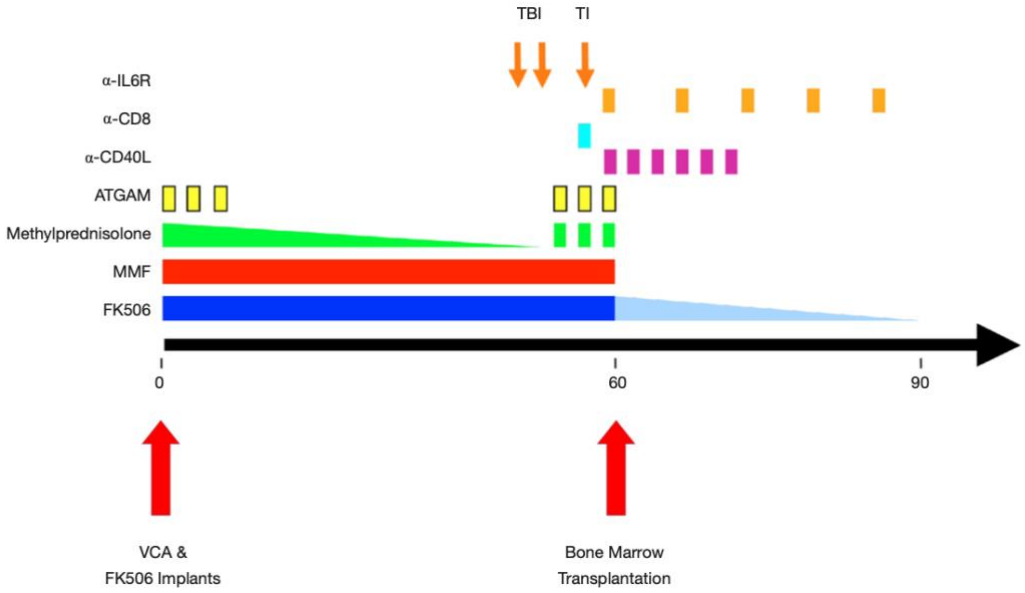


Figure 1 Delayed tolerance induction protocol for VCA. The sequence and timing of the protocol interventions are depicted relative to the timing of VCA and DBMT. The VCAs were maintained with conventional triple-drug immunosuppression for 60 days, followed by DBMT using a nonmyeloablative conditioning regimen including total body irradiation (TBI, 150 cGy, on POD 54 and 55), thymic irradiation (TI, 700 cGy on POD 59), ATGAM (IV 50 mg/kg on POD 58, 59 and 60), methylprednisolone (IV 5 mg on POD 58, 59 and 60) and anti-CD8 mAb (cM-T807, Centocor, Inc., Horsham, PA; 5 mg/kg IV on POD 59). After DBMT, the recipients were treated with short courses of anti-CD40L (h5C8; 20 mg/kg IV on POD 60 and 62, and 10 mg/kg on POD 65, 67, 69, and 72) and anti-IL-6 receptor mAb (tocilizumab, ACTEMRA®; Genentech, San Francisco, CA;10 mg/kg IV on POD 0, 67, 74, 81 and 88). After this, no further immunosuppression was given. ATGAM, equine anti-thymocyte globulin; DBMT, delayed bone marrow transplantation; mAb, monoclonal antibody; POD, post-operative day; TBI, total body irradiation; TI, thymic irradiation; VCA, vascularized composite allograft.

Experimental design

Adult, male cynomolgus monkeys (Macaca fasicularis) weighing 5 to 10 kg were chosen for this study (Charles River Primates, Wilmington, MA). Transplant pairs were selected for compatible ABO blood types and full major histocompatibility complex (MHC)-mismatching[17,18] before undergoing heterotopic, partial face VCA[19].

In Group 1, four NHPs were used to optimize the pharmacokinetics of FK506 delivery. In Group 2, three NHPs received the optimized FK506 implants and were maintained on

supplemental FK506 as necessary along with standard methylprednisolone, and MMF. Animals in the latter group underwent the DTIP involving preoperative thymic and total body irradiation along with other conditioning agents, as described previously10 and detailed in Figure 1, prior to receiving delayed bone marrow transplantation (DBMT) at 60 days.

Optimization of local FK506 implant design

Details of the in vitro and in vivo testing and manufacturing of the FK506 implants in Sprague-Dawley rats are beyond the scope of the current study and have been reported elsewhere (20). Essentially, scale-up iterations of the FK506 implants were cycled through Group 1 before arriving at the final formulation for Group 2, and all were distributed evenly along the suture line between donor (VCA) and host skin.

The initial iteration of FK506 implants (15 wt% film measuring 10 mm × 10 mm each) in Group 1 was based on the release profile, tissue distribution and standard intravenous (IV) or intramuscular (IM) FK506 dosing protocol of 0.1 mg/kg/day. Based on the timeline of the DTIP where immunosuppression lasts up to 90 days (60 days maintenance and 30 days bridging post-DBMT), total dosages were:

- IV/IM FK506 = 0.1 mg/kg/day × 10 kg/NHP × 90 days = 90 mg/NHP.

To provide the equivalent dose via FK506 implants,

- Implant FK506 = 0.15 mg/mg film × 60 mg film/ implant × 10 films/NHP = 90 mg/NHP.

Therefore, ten 15% FK506-loaded films were sterilized intra-operatively with povidone-iodine before implantation (in the first two NHPS in Group 1) at least 20 mm apart around the transplant site (Figure 2A,B,C,D). The in vivo implant release profile (see Results section) was not consistent with that predicted by in vitro data, with a high initial release of FK506 followed by a second spike which required redesign. This was addressed (in the next two NHPs, M3 and M4, in Group 1) through lamination to seal in the drug and provide a diffusion barrier to prevent the initial burst release of FK506. Two implants were then placed in M3, while only one was used in M4; this was equivalent to a dosing of approximately 1.8 and 0.9 mg/ kg respectively and models the daily IV/IM dose with the secondary goal of also extending local immunosuppression for as long as possible.

The final version of the FK506 implants in Group 2 was that of 4 mm circular implants (24 total) loaded with 30 wt% tacrolimus and 1 wt% vitamin E (stabilizer to prevent degradation following sterilization and storage) totaling 3 cm2 (based on in vivo drug release studies in rodents at Rutgers University). These implants were also sterilized pre-operatively by gamma irradiation to mirror actual clinical application. Such a design aimed to provide therapeutic levels of FK506 via controlled release over the first 7–10 days to combat acute rejection of the VCA with further IM supplementation thereafter as necessary. Despite the likely lower systemic levels, the presumed higher FK506

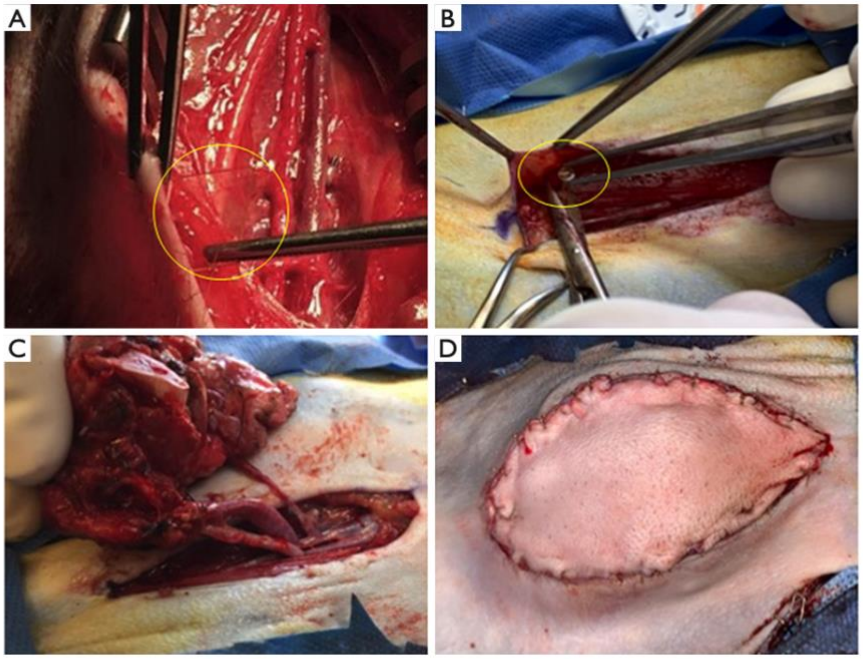


Figure 2 Development of local FK506 implant technology: (A) initial, unsuccessful iterations consisting of 15% drug-loaded devices measuring 10 mm × 10 mm each (10× film and 1× laminated versions), and (B) final, successful iteration consisting of 4 mm diameter circular implants loaded with 30 wt% tacrolimus and 1 wt% vitamin E (stabilizer to prevent degradation following sterilization and storage) totaling 3 cm^2 (C) placed along the suture line and (D) final appearance on skin closure. Implants circled in yellow to aid visualization.

concentration within the soft tissue (based on rodent studies, no validated method in NHPs) surrounding the implants is hypothesized to provide adequate local immunosuppression throughout the lifetime of the implant to prevent acute rejection. Historical NHP subjects from our laboratory without FK506 implantation were previously reported and utilized as controls for this study[10].

Immunological monitoring

Post-transplantation, VCAs were monitored twice daily for the first 72 hours and once daily subsequently, or more frequently if requested. Protocol and for-cause VCA skin biopsies were performed using a standard 6 mm punch biopsy kit at approximately 30-day intervals. When rejection was suspected clinically (i.e., increased erythema, swelling, ulceration etc.), treatment was initiated with steroid bolus and gradual taper over 14 days to 1 mg per day (same as maintenance). All VCA skin biopsies were fixed in formalin, stained with hematoxylin and eosin (H&E), and subjected to pathologic analysis according to the 2007 Banff Working Classification for composite tissues[21,22] by two pathologists (I.A.R and R.B.C) blinded to the study.

Peripheral blood chimerism was evaluated chronologically post-VCA by flow cytometry as described previously at approximately 2-week intervals[10,23]. Systemic levels of FK506 were measured chronologically post-VCA with an Abbott ARCHITECT Immunoassay Analyzer.

Statistical analysis

Experience with large animal models for transplantation research over the past 30 years at MGH has demonstrated that three transplant recipients per experimental group are sufficient to provide statistical power for the detection of survival differences, and to achieve statistical significance in the comparison of categorically-scored histological specimens using non-parametric statistical tests (e.g., Mann-Whitney U) with an alpha risk level of 5% and a beta risk level of 80%[24].

Results

Optimization of FK506 delivery

In Group 1, M1 developed venous thrombosis of the VCA and lost the graft by POD 4. Systemic FK506 levels were measured up to POD 12 in M2 and ranged from 50–120 ng/mL. These levels were 2–6 fold higher than the therapeutic target range of 20–30 ng/mL (for NHPs) and were thought to have arisen from the combination of the induction dose of IM FK506 (0.2 mg/kg × 9 kg NHP =1.8 mg) and high early release levels from FK506 implants that had been observed during the first few days from earlier in vitro studies in rats (not shown). The sustained and high release of FK506 past the first week, however, was cause for serious concern. Clinical assessment of M2 suggested potential tacrolimus toxicity including poor appetite, weight loss, and a slight tremor, resulting in removal of this animal from the study on POD 12.

The burst release of FK506 was addressed by lamination and reducing the number of implants (from ten) as described in Materials & Methods, and implemented for the next two NHPs (M3, M4) in Group 1. M4 received two implants, developed venous thrombosis and lost the graft by POD 5. M3 received two implants, which reduced systemic levels of FK506 to within the desired therapeutic range up to POD 11 before additional IM dosing was required. M3 was then removed from study on POD 13 as it had reached the experimental end point of rejection (corresponding FK506 levels <20 ng/mL) (Figure 3).

In Group 2 NHPs (n=3), initial systemic FK506 levels at POD 3 were 60.0, 79.1 and 112.4 ng/mL and subsequent IM FK506 administration was stopped. In M5, FK506 levels fell rapidly to 26.6 ng/mL by POD 10, which necessitated reinstatement of IM FK506. In contrast, M6 did not require additional IM FK506 up to the point of DBMT (at POD 60). In this animal systemic FK506 levels had a second peak at week two (100 ng/mL) before gradually tapering to and maintaining just above the target range at 30–40 ng/mL from approximately POD 30 onwards. M7 had a similar course to M6 and only required additional IM FK506 from around POD 45 onwards when systemic FK506 levels fell below the target range (Figure 4).

Successful mitigation of acute rejection

Acute rejection episodes were avoided in all three Group 2 NHPs. However, routine CBC from M7 on POD 12 revealed a raised granulocyte count (14.5×109/L) that manifested with a cutaneous rash on the VCA associated with pruritus. This was treated

presumptively as rejection by titrating methylprednisolone back up to 40 mg IV, and symptomatically with diphenhydramine (50 mg/kg/day) until clinical resolution was achieved. All Group 2 NHPs had VCA biopsies taken on POD 30 and POD 60 that returned negative for rejection (Figure 5).

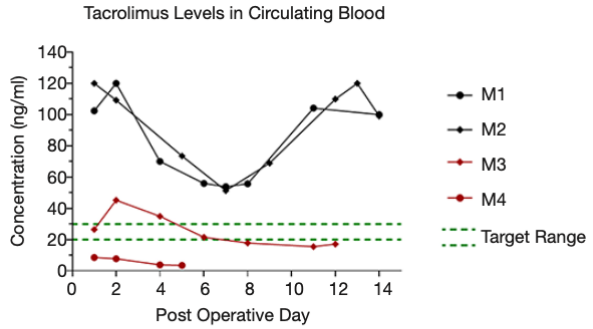


Figure 3 Systemic FK506 concentration comparison in Group 1: for the first iteration of NHP studies, M1 and M2, against the second iteration of NHP studies, M3 and M4. By using fewer laminated implants, systemic FK506 levels were brought to the target range for up to 11 days *in vivo* without requiring additional IM administration. NHP, non-human primate.

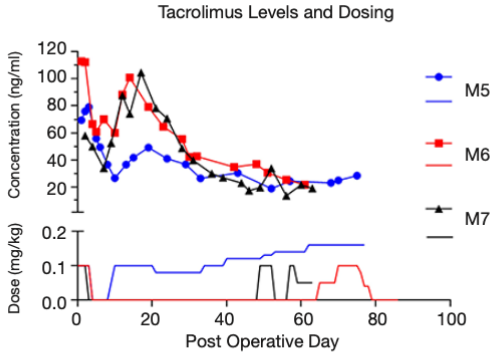


Figure 4 Systemic levels of FK506 (lines with symbols) in Group 2 following transplantation, and corresponding dosages of IM FK506 (solid lines). M5 required additional IM FK506 throughout the study whereas M6 did not, and M7 had a similar course until POD 45.

Detection of donor cells after DTIP but PTLD remained rampant

All three NHPs in Group 2 successfully underwent the DTIP and received DBMT but developed progressive weight loss of between 30–35% of total body weight and worsening leukocytosis (14×109/L–37.4×109/L) shortly after. Of note, M6 developed a period of impaired glucose tolerance with random blood sugar at 389 mg/dL on POD 29 while FK dosing was being maintained by only the subcutaneous implant. This was treated with a diabetogenic diet and insulin until POD 50. At a similar timepoint, M7 which also had FK dosing maintained solely by subcutaneous implant, developed lymphadenopathy of the right inguinal lymph node on POD 39. The lymph node was surgically excised and histology confirmed underlying post-transplant lymphoproliferative disorder (PTLD). Although the animal did go on to receive DBMT, it had lost >20% of its total body weight by POD 64 and was also removed from study per veterinary recommendation. During this timeframe, all three NHPs were maintained with comparable systemic tacrolimus levels despite the varying clinical courses. Following DBMT, there was strong clinical suspicion for PTLD in M5 and M6, which was confirmed at the end of study on POD 77 and 86, respectively. PTLD were all likely of recipient-origin (Figure 6).

Final chimerism analysis showed a very low percentage of donor cells (~2–5%) at experimental endpoint in M5 and M7. M6 on the other hand successfully developed mixed chimerism (96–97% granulocytes and 7–11% lymphocytes of donor-origin) at 2–3 weeks post-DBMT (Figure 7).

Discussion

A plethora of drug delivery systems have been reported in small animal VCA models to mitigate acute rejection using various immunosuppressants including FK506, steroids, and rapamycin[13,14]. These include direct topical application[25,26]; intra-graft administration with various functionalities such as "on-demand" drug release in response to inflammation[27] and "on-cue" release upon ultrasound stimulation[28]; and local biodegradable implants with sustained regional release[29]. Most encouragingly, these reports have all had varying degrees of long-term (100–280 days) VCA survival, minimal systemic toxicity and related complications, and the potential for immunoregulation through chimerism and even increased levels of Tregs[30].

Our laboratory is focused on developing a similar approach to local immunosuppression but instead, we utilized a translational large animal model (NHPs). We have previously shown that topical FK506 was limited by inadequate dermal penetration in NHP when given alongside reduced levels of systemic immunosuppression[9]. This outcome reflected the clinical experience in VCA, whereby topicals could only augment systemic therapy [2,8]. Hence, our current study was focused on directing local FK506 delivery to the dermis, similar to the reported experience in rats[29]. Our main challenges, however, were to translate successes in small animal models for scale-up of in vivo testing in a pre-clinical NHP VCA model.

To the best of our knowledge, there has only been one prior large animal study on local immunosuppression. Fries et al.[16] investigated the use of high (91 mg/limb) and low-dose (49 mg/limb) FK506-loaded hydrogels alone, administered subdermally prior to skin closure in a porcine VCA model. Both experimental groups had similar early burst release of FK506 (range, 33.18–42.16 ng/mL and 11.91–35.62 ng/mL for high and low-dose groups respectively on POD 1) followed by a second spike around POD7–10. However, animals in the high-dose group had to be euthanized at POD 24, 30 and 42 due to failure to thrive, while low-dose group animals survived up to POD 56, 63, 91 and 93 before Grade IV rejection developed. Similarly, NHP recipients in Group 2 of the current study developed a myriad of side effects, likely from the high dose of systemic FK506, including diabetes and the development of recipient-origin PTLD (in association with BMT). VCAs in the study by Fries et al. were performed across only a single haplotype mismatch rather than a full MHC-mismatch as in our current study. Furthermore, Fries' study was designed to consider graft survival as up to Grade IV rejection (i.e., necrosis). However, Grade I-II rejection developed from as early as POD 4 in both groups, an observation that is arguably of greater clinical importance, which underlies the premise of topical and/or local immunosuppressive therapy (i.e., to prevent the onset of Grade I-II rejection episodes). While Fries' results are encouraging with the use of the FK506-loaded hydrogels alone, our study represents an alternative approach towards FK506 treatment. We utilized FK506 implants, standard induction and maintenance triple immunosuppression concurrently (in Group 2), and provided proof-of-concept of the

Local FK506 implants in non-human primates to prevent early acute rejection in vascularized composite allografts

efficacy of our technology in mitigating acute rejection across the highest MHC mismatch barrier (i.e., full) possible. Additionally, 2 of 3 NHPs in Group 2 of the current study also

demonstrated rejection-free VCA survival without additional IM FK506 dosing for 45–60 days (Figure 4).

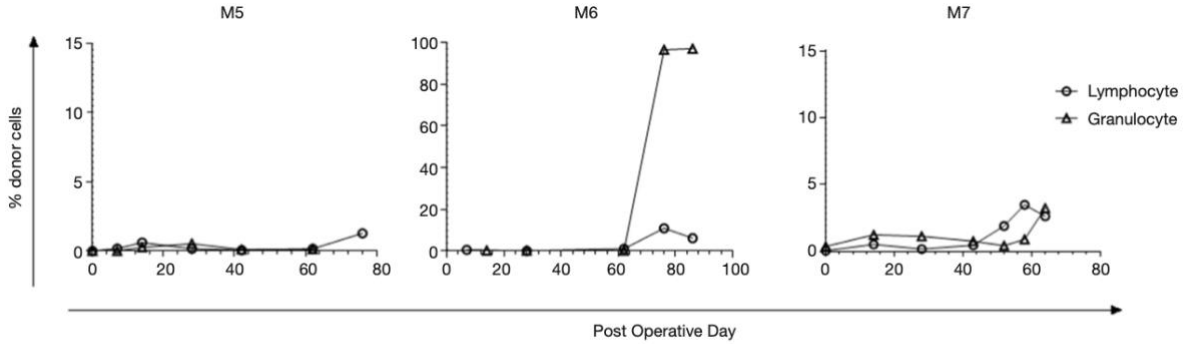


Figure 7 Flow cytometry plots of chimerism analysis of Group 2 recipients. Large spike in donor cells within the granulocyte population of M6 whole blood post-DBMT; donor cells in this gate are negative for almost all lineage markers. Very low percentage of donor cells was detected for M5 and M7 throughout the study. DBMT, delayed bone marrow transplantation.

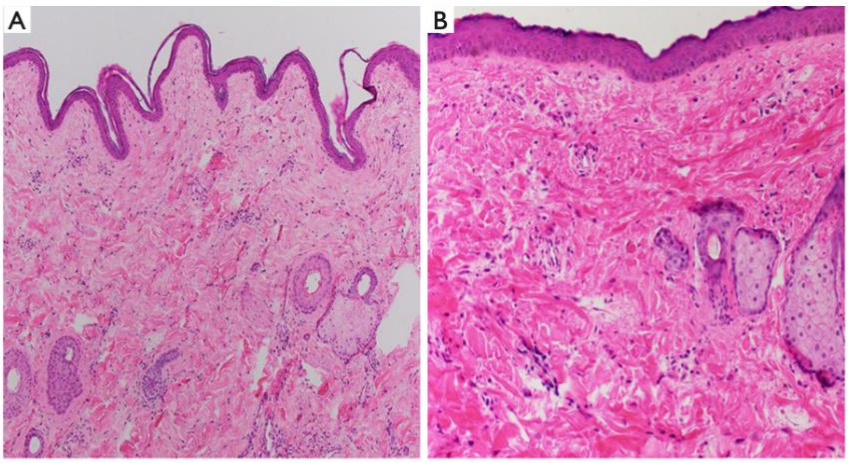


Figure 5 (A) M5 (H&E, 100×) and (B) M7 (H&E, 400×). The skin from the VCA shows no evidence of rejection at end of study. The epidermis is intact and shows no inflammation. VCA, vascularized composite allograft.

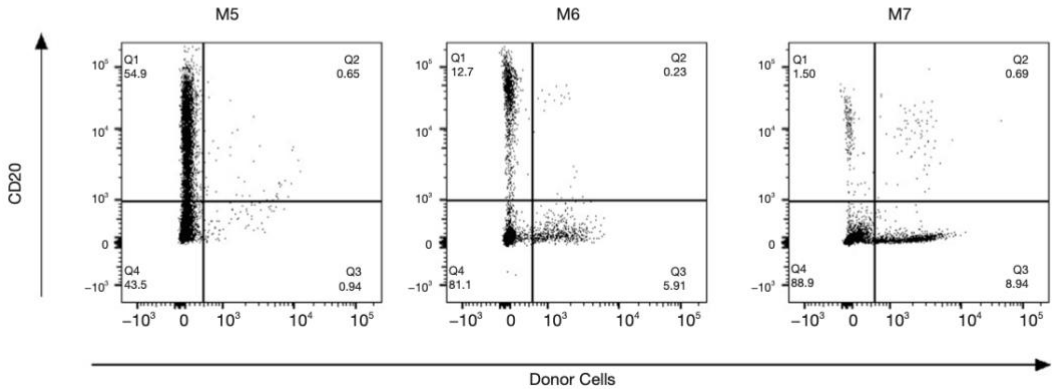


Figure 6 End-of-study whole blood data for each Group 2 recipient. PTLD likely of recipient origin in all recipients since there was minimal double positive staining in the CD20+ H38+ population (Q2). Majority of B-cells are recipient origin. PTLD, post-transplant lymphoproliferative disorder.

This study is a proof-of-concept for clinical application of local FK506 implant technology. Additionally, it may prevent cases of VCA rejection that are due to patient noncompliance with oral immunosuppression. We do acknowledge that the current formulation, despite

our best efforts, can lead to unacceptably high levels of systemic FK506. However, the surgically accessible subcutaneous position permits removal of some, or all of the implants in the event of unacceptably high levels.

Interestingly, high-dose FK506 has been explored as part of induction therapy prior to VCA in both NHP[29] and swine[30] models with varying results. In NHPs, high dose FK506 (systemic levels of 30–50 ng/mL) was maintained for 28 days before maintenance on once-daily IM dosing (target trough levels of 10–20 ng/mL) and 5 of 6 NHPs demonstrated clinically rejection-free (but histology revealed mild rejection) survival of up to 177 days (mean, 113 days). All five NHPs subsequently developed PTLD and no evidence of chimerism was detected throughout the study. In swine, full MHC-mismatch transplant pairs underwent VCA following conditioning with 50 cGy total body and 350 cGy thymic irradiation (i.e., a "day 0" protocol rather than a DTIP) and results were highly variable. High dose FK506 (15–20 ng/mL) maintenance prevented VCA rejection in swine (3 of 3) but all developed major infectious complications leading to death. Swine that only received transient high dose FK506 for 60 days (3 of 3) had indefinite VCA survival (>300 days) while off all immunosuppression without any mention of the presence or absence of chimerism.

Previous SOT studies in swine utilizing high-dose FK506 as induction over 12-days[31-33] have led to immune tolerance, so a species-specific mechanism may explain the differences in NHP versus swine experiments. Also, we have previously shown in swine VCA studies that transient mixed chimerism was insufficient for tolerance[34] and that stable mixed chimerism would be required instead[35], but this was dependent on the MHC barriers involved[36]. Building on these mechanistic studies in swine, our laboratory has been focused on developing a successful mixed chimerism protocol in NHPs. Our recent work showed that DTIP, in which animals are kept on immunosuppression until healed and recovered from the initial tissue transplant procedure, then conditioned and given stem cells for tolerance induction months later, while successful in SOT[37], was not able to mitigate acute VCA rejection during the delay period. The rejection episodes negated development of mixed chimerism in NHPs[10], similar to the experience in clinical renal tolerance trials38. While recent clinical developments have shown the intriguing promise of adding rituximab as part of induction to abrogate rejection episodes for up to two years post-VCA (39), the need for strict patient compliance with maintenance immunosuppression remains and demonstrates the potential utility of our local FK506 implants (Figure 8). Most encouragingly, there is now emerging evidence to support the use of rituximab prophylactically against PTLD (40), which is a strategy under consideration in our laboratory.

In conclusion, the recent development of DDS has shown much promise in controlling acute rejection of VCA. Our current study has shown that such proof-of-concept work in small animals can be scaled up into a clinically relevant large animal model (NHP). Future efforts will be focused on optimizing our local FK506 implant technology to minimize

systemic morbidity by reducing the initial peak of burst release, in pursuance of the DTIP strategy for mixed chimerism and immune tolerance to VCA.

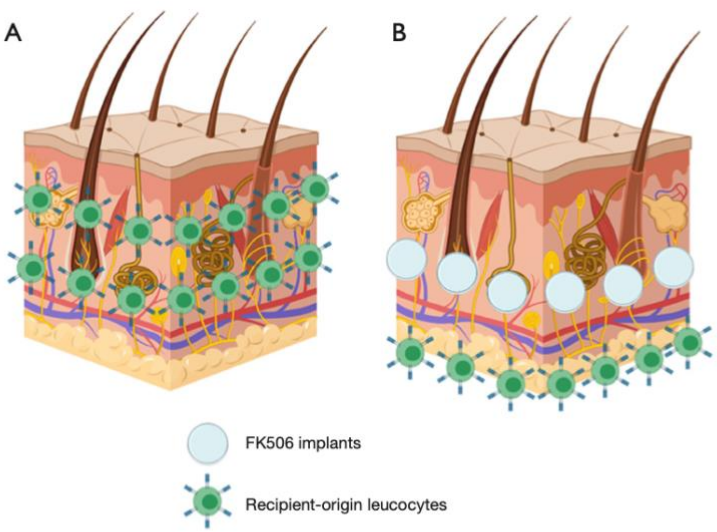


Figure 8 Proposed mechanism of action of local FK506 implant technology in averting rejection in vascularized composite allografts (VCA). (A) Previous studies from our laboratory suggest that skin cells within VCA dermis undergoes near complete turn-over and becomes infiltrated by recipient-origin leukocytes which likely results in rejection. (B) By delivering FK506 into the dermis directly through subcutaneous implantation, this infiltration of recipient-origin leukocytes may be delayed and/or negated completely, to mitigate the development of acute VCA rejection.

REFERENCES

1. Dubernard JM, Owen E, Herzberg G, et al. Human hand allograft: report on first 6 months. Lancet 1999;353:1315-20.
2. Petruzzo P SC, Lanzetta M, Dubernard JM. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). Curr Transplant Reports 2017;4:294-303.
3. Petruzzo P, Dubernard JM. The International Registry on Hand and Composite Tissue allotransplantation. Clin Transpl 2011:247-53.
4. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation 2010;90:1590-4.
5. Lee WP, Yaremchuk MJ, Pan YC, et al. Relative antigenicity of components of a vascularized limb allograft. Plast Reconstr Surg 1991;87:401-11.
6. Kueckelhaus M, Fischer S, Seyda M, et al. Vascularized composite allotransplantation: current standards and novel approaches to prevent acute rejection and chronic allograft deterioration. Transpl Int 2016;29:655-62.
7. Diaz-Siso JR, Fischer S, Sisk GC, et al. Initial experience of dual maintenance immunosuppression with steroid withdrawal in vascular composite tissue allotransplantation. Am J Transplant 2015;15:1421-31.
8. Chen YT, Huang SC, Chen CC, et al. Topical tacrolimus and steroids modulate T cells in acute rejection of hand allotransplantation: Two case reports. Microsurgery 2020;40:217-23.
9. Gama AR, Ng ZY, Shanmugarajah K, et al. Local Immunosuppression for Vascularized Composite Allografts: Application of Topical FK506-TyroSpheres in a Nonhuman Primate Model. J Burn Care Res 2020;41:1172-8.
10. Lellouch AG, Ng ZY, Rosales IA, et al. Toward Development of the Delayed Tolerance Induction Protocol for Vascularized Composite Allografts in Nonhuman Primates. Plast Reconstr Surg 2020;145:757e-68e.
11. Sykes M. Mixed chimerism and transplant tolerance. Immunity 2001;14:417-24.
12. Kawai T, Sachs DH, Sykes M, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. N Engl J Med 2013;368:1850-2.
13. Taddeo A, Tsai C, Vogelin E, et al. Novel targeted drug delivery systems to minimize systemic immunosuppression in vascularized composite allotransplantation. Curr Opin Organ Transplant 2018;23:568-76.
14. Dhayani A, Kalita S, Mahato M, et al. Biomaterials for topical and transdermal drug delivery in reconstructive transplantation. Nanomedicine (Lond) 2019;14:2713-33.
15. Leonard DA, Gordon CR, Sachs DH, et al. Immunobiology of face transplantation. J Craniofac Surg 2012;23:268-71.
16. Fries CA, Lawson SD, Wang LC, et al. Graft-implanted, enzyme responsive, tacrolimus-eluting hydrogel enables long-term survival of orthotopic porcine limb vascularized composite allografts: A proof of concept study. PLoS One 2019;14:e0210914.
17. O'Connor SL, Blasky AJ, Pendley CJ, et al. Comprehensive characterization of MHC class II haplotypes in Mauritian cynomolgus macaques. Immunogenetics 2007;59:449-62.
18. Pendley CJ, Becker EA, Karl JA, et al. MHC class I characterization of Indonesian cynomolgus macaques. Immunogenetics 2008;60:339-51.
19. Barth RN, Bluebond-Langner R, Nam A, et al. Facial subunit composite tissue allografts in nonhuman primates: I. Technical and immunosuppressive requirements for prolonged graft survival. Plast Reconstr Surg 2009;123:493-501.
20. Molde Joseph MA, Steele Joseph AM, Lima Mariana RN, et al. Localized tacrolimus delivery for transplant rejection prevention via tyrosphere-loaded rapidly soluble films. Transactions of the Annual Meeting of the Society for Biomaterials and the Annual International Biomaterials Symposium April 2019;Volume 40:5104.
21. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. Am J Transplant 2008;8:1396-400.
22. Schneider M, Cardones AR, Selim MA, et al. Vascularized composite allotransplantation: a closer look at the banff working classification. Transpl Int 2016;29:663-71.
23. Boskovic S, Kawai T, Smith RN, et al. Monitoring antidonor alloantibodies as a predictive assay for renal allograft tolerance/long-term observations in nonhuman primates. Transplantation 2006;82:819-25.
24. Kawai T, Cosimi AB, Sachs DH. Preclinical and clinical studies on the induction of renal allograft tolerance through transient mixed chimerism. Curr Opin Organ Transplant 2011;16:366-71.
25. Solari MG, Washington KM, Sacks JM, et al. Daily topical tacrolimus therapy prevents skin rejection in a rodent hind limb allograft model. Plast Reconstr Surg 2009;123:17S-25S.
26. Gharb BB, Rampazzo A, Altuntas SH, et al. Effectiveness of topical immunosuppressants in prevention and treatment of rejection in face allotransplantation. Transplantation 2013;95:1197-203.
27. Gajanayake T, Olariu R, Leclere FM, et al. A single localized dose of enzyme-responsive hydrogel improves long-term survival of a vascularized composite allograft. Sci Transl Med 2014;6:249ra110.

28. Feturi FG, Wang H, Brudno Y, et al. Abstract 23: Ultrasound-mediated on-demand release from ionically cross-linked hydrogel: New approach for targeted Immunotherapy in Vascularized Composite Allotransplantation. Plast Reconstr Surg Glob Open 2017;5:18-9.
29. Unadkat JV, Schnider JT, Feturi FG, et al. Single Implantable FK506 Disk Prevents Rejection in Vascularized Composite Allotransplantation. Plast Reconstr Surg 2017;139:403e-14e.
30. Sutter D, Dzhonova DV, Prost JC, et al. Delivery of Rapamycin Using In Situ Forming Implants Promotes Immunoregulation and Vascularized Composite Allograft Survival. Sci Rep 2019;9:9269.
31. Barth RN, Nam AJ, Stanwix MG, et al. Prolonged survival of composite facial allografts in non-human primates associated with posttransplant lymphoproliferative disorder. Transplantation 2009;88:1242-50.
32. Leto Barone AA WH, Swanson EW, et al. Abstract 45: Vascularized Composite Allograft Tolerance Across A Full MHC Mismatch Is Possible With Transient High Dose Tacrolimus. Plast Reconstr Surg - Glob Open 2017;5:34-5.
33. Madariaga ML, Michel SG, Tasaki M, et al. Induction of cardiac allograft tolerance across a full MHC barrier in miniature swine by donor kidney cotransplantation. Am J Transplant 2013;13:2558-66.
34. Madariaga ML, Spencer PJ, Michel SG, et al. Effects of Lung Cotransplantation on Cardiac Allograft Tolerance Across a Full Major Histocompatibility Complex Barrier in Miniature Swine. Am J Transplant 2016;16:979-86.
35. Utsugi R, Barth RN, Lee RS, et al. Induction of transplantation tolerance with a short course of tacrolimus (FK506): I. Rapid and stable tolerance to two-haplotype fully mhc-mismatched kidney allografts in miniature swine. Transplantation 2001;71:1368-79.
36. Leto Barone AA KJ, Albritton A, et al. Effects of Transient Donor Chimerism on Rejection of MHC-Mismatched Vascularized Composite Allografts in Swine. Vasc Compos Allotransplantation 2015;2:1-8.
37. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant 2014;14:343-55.
38. Shanmugarajah K, Powell H, Leonard DA, et al. The Effect of MHC Antigen Matching Between Donors and Recipients on Skin Tolerance of Vascularized Composite Allografts. Am J Transplant 2017;17:1729-41.
39. Gelb BE, Diaz-Siso JR, Plana NM, et al. Absence of Rejection in a Facial Allograft Recipient with a Positive Flow Crossmatch 24 Months after Induction with Rabbit Anti-Thymocyte Globulin and Anti-CD20 Monoclonal Antibody. Case Rep Transplant 2018;2018:7691072.
40. Lindsay J, Yong MK, Greenwood M, et al. Epstein-Barr virus related post-transplant lymphoproliferative disorder prevention strategies in allogeneic hematopoietic stem cell transplantation. Rev Med Virol 2020;30:e2108.

CHAPTER 07

A Second Chance at Life

Alexandre G. Lellouch
Laurent A. Lantieri

Adapted from Cambridge Quarterly of Healthcare Ethics (2019), Cambridge University Press 2019. doi:10.1017/S0963180119000380

COMMENTARY

History

Vascularized Composite Allotransplantation (VCA) including Facial Transplantation (FT) emerged as a unique solution for anatomically restoring faces after disfigurement. It became a clinical reality after the first successful face transplantation in 2005.[1] VCA is a rapidly growing new field in plastic surgery; and from November 2005 to the time of this writing in September 2018, 44 transplants have been performed worldwide. It is clear that many more transplantations will be performed in the future. The main concern in VCA remains the risk of chronic rejection[2] and graft loss[3] despite life-long immunosuppression. Scientists are actively working on new strategies to reduce the burden of immunosuppression and prevent immune rejection. The most promising approach is the development of tolerance protocols through the induction of mixed chimerism[4]. Indeed, it is the sole strategy to have demonstrated long-term tolerance in solid organ transplantation in humans.[5] Currently, the gold standard in reconstructive surgery remains the use of autologous tissue. This strategy is still very useful to replace limited missing body parts (like mandible) by tissue transfer (for example, osteomyocutaneous flap). However, the functional sensory organs (mouth, eyes) cannot be restored adequately by these conventional surgical techniques.

The goal of face transplantation is not only to restore the patient's appearance but also, to create a new physiognomy that will allow the facially disfigured person to adapt and reintegrate into life. Before the advent of VCA, the complexity of the face made surgeons unable to reestablish an "acceptable outcome," leaving disfigured patients little chance of social rehabilitation. Nowadays VCA surgery, while offering new hope, represents an important challenge (even for the top facility) for the surgical[6] and medical[7] team. First, there must be a high level of cooperation between the various specialists including surgeons, anesthesiologists and nephrologists. Secondly, the management of immune rejection remains a primary ongoing concern due to the untreatable chronic rejection effect.[8]

Background

Over the last 20 years, our surgical team has been led by Laurent Lantieri. Numerous articles have been written on the ethical issues[9,10,11] pertaining to FT. In our hospital, European George Pompidou in Paris, the anatomical study on face transplantation was performed in 2005[12]. In 2007, we performed our first partial face transplant case[13] (the third in the world); the first successful partial face transplant was performed in 2005 in Amiens, France.[14] We determined that in the light of immunosuppressive regimen improvement, it appeared reasonable to consider VCA as a clinical option, especially for hand and face transplantation.[15]

The scientific community continues to debate the ethical dilemmas regarding FT[16]. Our team already transplanted a patient after shotgun injury.[17] Following the success of our first face transplant, we decided to go further with the elaboration of the first full face transplant.[18] Our first report evaluating the short-term quality of life was encouraging.19 Regarding types of disfigurements, the most frequent situations involve the severely disfiguring facial tumors of neurofibromatosis, shotgun injury, dog bites, and burns; but

in our experience, burn patients are not considered the best candidates for receiving VCA due to the risk of sensitization after care management.[20] Furthermore, there is no benefit to changing the standardized care management of burn patients (using skin allograft, blood transfusion) due to the high risk of medical complications.[21] In our series of VCA/FT patients with more than five years follow-up, we demonstrated positive outcomes.[22] In addition to improvements in immunosuppressive strategies, we described our protocol for management of the facial nerve (one anastomosis at the trunk). The aim was to simplify and improve surgical outcomes in FT by avoiding dyskinesia.[23] Unfortunately, one of our FT patients rejected his face leading to a total graft loss within a few months. We published a letter to the editor in the journal Lancet addressing the risk of face retransplantation.[24] Under extremely urgent conditions we pushed forward in retransplanting the patient.[25]

Comment

In their paper, McQuinn et al. raise the challenge of human FT post Self-Inflicted Gunshot Wound (SIGW). The major ethical concern is the potential risk of recurrence of such a suicide attempt. According to the manuscript, 25% of the FTs performed have been in patients identified as survivors of SIGWs, and one recipient has committed suicide post-transplant.[26] This clinical reality has already been accepted by most VCA surgeons. Interestingly, ballistic trauma patients have eclipsed burn patients who were initially projected to make up the largest number of transplants. This confirms the observation that we made in 2004[27] that ballistic trauma patients can be excellent candidates for FT. Beyond the risk of suicide, patients' intellectual ability and their understanding of long-term treatment is the minimal requisite for suggesting this surgery, and more determinative than the type of injury itself.

In transplants, as in the rest of modern healthcare, the debate also involves the cost for society. In our institution, the transplantation of a face led to higher costs than heart or any other solid organ, and represented twice the costs for a liver transplantation.[28] The question is: how much is society willing to expend resources to help these people? In the case of face transplant (contrary to solid transplantation indications), there are no face shortages so far.

A close follow-up post-face transplant is crucial for detecting the first signs of psychological distress. As reported by the authors, mortality from SIGWs can be as high as 80%. The survivors have important morbidities and lifelong disabilities. A global assessment of the possibilities of motor and sensitive function restoration will help address the risk-benefit ratio in FT. A patient with SIGW suffers significant psychological pain that must be treated. The decision to perform a VCA is multidisciplinary, depending of the psychological state, the surgical feasibility, and the willingness of the patient. We understand and appreciate the importance of the role of psychiatrists and psychologists for identifying "low-risk patients." These professionals need to make the distinction between known psychosocial contraindications (active psychoses, amongst them) and SIGW. Our institution does not exclude patients with SIGW, viewing VCA as a chance to give the patient a "second chance" at life.

The patient's ability to make medical decisions for themselves cannot be overestimated. A prerequisite for the surgery is fully informed consent to all aspects of the procedure,

which becomes a more complex issue as VCA for children is explored,[29] along with understanding and committing to the extensive after-care regimen. This point is particularly important since an inability to adhere to the post-transplant regimen can lead to adverse outcomes, including rejection and graft failure. But even with treatment compliance, we cannot rule out the risk of developing chronic rejection.

The authors stated that the general public in the United States supports solid organ donation but is hesitant about vascularized composite allotransplantation. But looking back at the first heart transplant, when the world was amazed by Christian Barnard's case, support was also not immediate for taking a heart that had only arrested during surgery with cardioplegia. This reluctance may be traced to insufficient communication, and lack of information from the VCA center. Inadequate clarification can lead to confusion and misunderstanding, causing relatives of donors to have misconceptions regarding the procedure, and refusing to donate for VCA. Disfigurement of their diseased loved one is often a major concern. As surgeons, it is imperative that we restore the donor's face to its original structure. The general public remains unaware of this part of the procedure, as does much of the medical community. This is necessary, to show respect to the donor's family, and also the donor.

The ethics of face transplantation go far beyond recipient risk-benefit analysis, and allocation of resources. It involves all the questions regarding the use of body parts after death raised in the literature by Mary Shelley's novel, Frankenstein, written a century ago. For example, are we an amalgamation of organs? If so, is a part of personality is transferred when organs are transferred—even more so when it is related to the face? But a human being is a complex system, and a complex system is not the addition of simple elements. In VCA, we are not transplanting personality through a face transplant, but restoring faces to rehabilitate patients and bring them back to a normal life.

Conclusion

FT goes beyond the restoration of the anatomical subject; it is the retrieval of the self-esteem. The right to have a face goes beyond improving the quality of life; it is at the core of human dignity. The atrocities performed on concentration camp prisoners in World War II, under the guise of "scientific experiments," demonstrated the ravages of a loss of dignity. We agree with the conclusion of the authors regarding the absence of justification for rejecting patients with SIGW. The allocation of health care resources is very important, but the decision to provide VCA lies at the health policy decision level; physicians have the professional duty to provide their patients with the best treatment options available. VCA is the best treatment option available for SIGW, and needs to be considered in the therapeutic arsenal for treating it. The report of long-term follow-up will bring new insight to this topic.

REFERENCES

1. Dubernard J-M, Lengelé B, Morelon E, Testelin S, Badet L, Moure C, et al. Outcomes 18 months after the first human partial face transplantation. New England Journal of Medicine 2007; 357(24):2451–60.
2. Petruzzo P, Kanitakis J, Testelin S, Pialat J-B, Buron F, Badet L, et al. Clinicopathological findings of chronic rejection in a face grafted patient. Transplantation 2015;99(12):1–7.
3. Morelon E, Petruzzo P, Kanitakis J, Dakpé S, Thaunat O, Dubois V, et al. Face transplantation: Partial graft loss of the first case ten years later. American Journal of Transplantation 2017; 17(7):1935–40.
4. Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL. Mixed chimerism-based regimens in VCA. Current Transplantation Reports 2016.
5. Kawai T, Sachs DH, Sykes M, Cosimi AB, Immune Tolerance Network. HLA-mismatched renal transplantation without maintenance immunosuppression. New England Journal of Medicine 2013;368(19):1850–2.
6. Meningaud J-P, Hivelin M, Benjoar M-D, Toure G, Hermeziu O, Lantieri L. The procurement of allotransplants for ballistic trauma: A preclinical study and a report of two clinical cases. Plastic and Reconstructive Surgery 2011;127(5):1892–900.
7. Sedaghati-nia A, Gilton A, Liger C, Binhas M, Cook F, Ait-Mammar B, et al. Anaesthesia and intensive care management of face transplantation. British Journal of Anaesthesia 2013;111(4): 600–6.
8. See note 2, Petruzzo et al. 2015.
9. Petit F, Paraskevas A, Minns AB, Lee WPA, Lantieri LA. Face transplantation: Where do we stand? Plastic and Reconstructive Surgery 2004;113(5):1429–33.
10. Petit F, Paraskevas A, Garrido I, Lantiéri L. [Could (allo)transplantation be the future of microsur- gery?] Annales de Chirurgie Plastique et Esthétique 2005;50(1):76–9.
11. Lantieri LA. Face transplantation: The view from Paris, France. Southern Medical Journal 2006; 99(4):421–3.
12. Paraskevas A, Ingallina F, Meningaud J-P, Lantiéri L. [Face allotransplantation: anatomical study, potential partial and total facial allografts harvesting and clinical application]. Annales de Chirurgie Plastique et Esthétique 2007;52(5):485–93.
13. Lantieri L, Meningaud J-P, Grimbert P, Bellivier F, Lefaucheur J-P, Ortonne N, et al. Repair of the lower and middle parts of the face by composite tissue allotransplantation in a patient with massive plexiform neurofibroma: A 1-year follow-up study. Lancet (London, England) 2008; 372(9639):639–45.
14. See note 1, Dubernard et al. 2007.
15. Lantieri L. [Composite allotransplantation in the upper extremity: From research to clinical reality]. Chirugie de la Main 2009;28(2):67–73.
16. Gordon CR, Siemionow M, Papay F, Pryor L, Gatherwright J, Kodish E, et al. The world's expe- rience with facial transplantation: What have we learned thus far? Annals of Plastic Surgery 2009;63(5):572–8.
17. See note 6, Meningaud et al. 2011.
18. Meningaud J-P, Benjoar M-D, Hivelin M, Hermeziu O, Toure G, Lantieri L. Procurement of total human face graft for allotransplantation: A preclinical study and the first clinical case. Plastic and Reconstructive Surgery 2010;126(4):1181–90.
19. Lantieri L, Hivelin M, Audard V, Benjoar MD, Meningaud JP, Bellivier F, et al. Feasibility, reproduc- ibility, risks and benefits of face transplantation: A prospective study of outcomes. American Journal of Transplantation 2011;11(1):367–78.
20. Duhamel P, Suberbielle C, Grimbert P, Leclerc T, Jacquelinet C, Audry B, et al. Extensively burned patients still need blood transfusions and skin allografts: Unavoidable HLA sensitization requires optimization of VCA access. Transplant International 2015;28(10):1229–30.
21. Ng ZY, Lellouch AG, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL. Vascularized composite Allotransplantation—An emerging concept for burn reconstruction. Journal of Burn Care & Research 2017;38(6):371–8. doi: 10.1097/BCR.0000000000000532.
22. Lantieri L, Grimbert P, Ortonne N, Suberbielle C, Bories D, Gil-Vernet S, et al. Face transplant: Long- term follow-up and results of a prospective open study. Lancet (London, England) 2016; 388(10052):1398–407.
23. Swanson JW, Yu JW, Taylor JA, Kovach S, Kanchwala S, Lantieri L. The retroauricular approach to the facial nerve trunk. Journal of Craniofacial Surgery 2017;28(2):347–51.
24. Lantieri L, Grimbert P, Ortonne N, Lemogne C, Wolkenstein P, Hivelin M. Facial transplantation: Facing the limits, planning the future. Lancet (London, England) 2017;389(10076):1293–4.
25. Breaking news, CNN 19 Apr. Available at https://www.nytimes.com/2018/04/19/world/ europe/jerome-hamon-face-transplant-france.html (last accessed 25 Mar 2019).
26. See note 22, Lantieri et al. 2016.
27. See note 9, Petit et al. 2004.
28. Rüegg EM, Hivelin M, Hemery F, Maciver C, Benjoar MD, Meningaud JP, et al. Face transplantation program in France: A cost analysis of five patients. Transplantation 2012;93(11):1166–72.
29. Marchac A, Kuschner T, Paris J, Picard A, Vazquez MP, Lantieri L. Ethical issues in pediatric face transplantation: Should we perform face transplantation in children? Plastic and Reconstructive Surgery 2016;138(2):449–54.

CHAPTER 08

Vascularized Composite Allotransplantation—An Emerging Concept for Burn Reconstruction

Zhi Yang Ng,
Alexandre G. Lellouch
Tessa Drijkoningen
Irene A. Chang
David H. Sachs
Curtis L. Cetrulo Jr.

Adapted from J Burn Care Res. doi: 10.1097/BCR.0000000000000532. PMID: 28328655

ABSTRACT

Vascularized composite allotransplantation (VCA) has demonstrated utility in the reconstruction of extensive soft-tissue defects following severe burns. However, pre-VCA events such as multiple transfusions, previous transplantation and pregnancies, the use of skin allografts, and mechanical support devices may result in sensitization and ultimately exclude a burn patient, who may benefit most through VCA, from a hand or face transplant. The authors sought to identify the immunologic challenges involved. All reported VCA cases up to July 2016 were reviewed. Relevant data analyzed include patient demographics, burn etiology, type and extent of VCA performed, pretransplant panel reactive antibody (PRA) status, extent of human leukocyte antigen (HLA) mismatch between donor and recipient, and immunologic outcomes. Of the 142 known cases of VCA to date, 30 (mean age = 36 years) were performed for burn reconstruction (mean interval to surgery = 8.3 years). Thermal and electrical burns were most common and performed in 20 and 30% of all reported upper extremity and craniofacial VCA cases, respectively, despite highly variable pretransplant PRA (0–98%). HLA-matching statuses between donors and recipients varied from 2/6 to 6/6. No obvious relationship could be observed between the incidence and severity of acute rejection with the patient's PRA and HLA-matching statuses, although more extensive treatment was required to reverse rejection episodes in sensitized patients (PRA > 0%). Further development and refinement of clinically relevant immunomodulatory protocols is required to achieve immunosuppression minimization and/or successful transplantation tolerance to enable long-term survivalof both the VCA itself and the patient. (J Burn Care Res 2017;XXX:00–00)

INTRODUCTION

Survival after massive burn injuries has improved significantly during the past three decades through better intensive care management.[1] Attention has now shifted toward optimizing functional and aesthetic outcomes as part of the rehabilitative phase in burn treatment.[2] The prevalence of burn patients requiring secondary reconstructive surgery has not been well studied, with recent estimates ranging from 5.3% for facial burns at 2-year follow-up[3] to 13% at up to 10-year follow-up.[4] Outcomes of such reconstructive surgery are oftentimes less than adequate, however, and result in chronic physical and psychosocial sequelae for the patient.[5]

With the advent of vascularized composite allotransplantation (VCA), face and hand transplantation have emerged as attractive reconstructive options for survivors of major burns. The number of burn patients who are candidates for VCA is currently unknown due to variability in selection criteria between centers. Decision modeling analysis has also shown that if life expectancy after VCA is reduced due to the current obligatory requirement for lifelong immunosuppression after transplantation, the overall gain in quality-adjusted life years is similarly decreased.[6] However, this finding is in disconnect with individual psychological assessments of patients who have undergone VCA following burn injury.[7]

Nevertheless, the growing clinical experience in VCA has confirmed technical feasibility of burn reconstruction with face and hand allografts. Much debate has recently been generated with regard to minimizing pre-VCA sensitization events, desensitization techniques, patient selection, and improving access to VCA for burn patients.[8] The purpose of this study was to review all reported cases of VCA to date that have been performed for an underlying burn etiology, including the immunological considerations and events involved, when available.

METHODS

A literature review was performed using PubMed and lay media sources. All reported cases up to July 2016 were verified between publications and news reports (if necessary) before inclusion for analysis. Relevant data extracted included patient demographic profile, burn etiology (thermal, electrical, chemical), the type and extent of VCA performed, pretransplant panel reactive antibody (PRA) status, extent of human leukocyte antigen (HLA) mismatch between donor and recipient, and summary immunologic outcomes at the time of performance of this study (Table 1).

RESULTS

As of August 2015, a total of 110 upper extremity and 32 face transplants have been reported worldwide. Of these 142 known cases of VCA, 30 (21%) were performed for burn reconstruction with the majority of these cases occurring in the United States (n = 8) followed by France and Turkey (n = 5 each).

Patient Demographics

The majority of burn patients who had undergone VCA were male (n = 18, 86%), with an average age of 35.9 years (range, 19–59) at the time of surgery. These numbers did not differ significantly between the upper extremity and facial VCA subgroups (38.2 vs 33.4 years; P = .34). The time interval between the initial burn injury and VCA averaged 8.3 years (range, 1–23.5) and similarly was not significantly different between upper extremity and facial VCA patients (6.4 vs 10.5 years; P = .25). Incidentally, all VCA cases performed in Turkey were for reconstruction of burns suffered in childhood (range, 40 days to 13 years old). There have also been three reported deaths in this cohort of burns VCA patients, and all were recipients of combined VCA procedures (face, upper, or lower extremities; see "Type and Extent of VCA Performed").

Burn Etiology and Severity

Overall, the most common type of burns suffered was thermal (57%), followed by electrical burns (38%). Again, the proportion of thermal and electrical burns was similar in the upper extremity and facial VCA subgroups. There was only one case of chemical burns due to domestic violence, and this patient was reconstructed with a facial VCA.[9] The degree of burn injury was not uniformly reported, but third-degree burns involving 50 to >80% of the total BSA have been described.[9–11]

Type and Extent of Vascularized Composite Allotransplantation Performed

Of the 11 patients who had undergone upper extremity VCA, two were unilateral cases. The remaining nine patients underwent bilateral transplantation at different levels (hand/distal forearm, below elbow, above elbow), and three of them had simultaneous combined procedures (face, bilateral lower limb, unilateral lower limb). Of note, in all three combined VCA cases, there was 100% mortality at postoperative days (PODs) 4, 65, and 100 due to metabolic failure,[12] infection requiring allograft removal, complicated by

Table 1. Overview of VCA performed for burn reconstruction

Patient/ Reference	Date of VCA	VCA Performed	Age/Sex of Patient	Team	Interval to VCA	Type of Burn	PRA Status	HLA Mismatch	Outcome
Face									
1[9]	Apr 09	Partial MC face + bilateral below elbow	37/M	France	1 yr	Thermal (self-harm)	Positive	4/6	VCA removal at POD 31, death on POD 65
2[10]	Apr 09	Partial OMC face	59/M	USA	4 yr	Electrical (accident)	?	3/6	Banff II and III (at POM 34, 56)
3[11]	Nov 09	Partial OMC face	27/M	France	1 yr	Thermal (pyrotechnic)	?	5/6	EBV lymphoma; acute rejection ×8; chronic rejection
4[12]	Mar 11	Full MC face	25/M	USA	3 yr	Electrical (accident)	68%	5/6	Banff III (at POM 22)
5[12]	Apr 11	Full MC face	30/M	USA	10 yr	Electrical (accident)	0%	2/6	Banff I–II (POD 20), II–III (POM 17)
6[13,14]	Jan 12	Full cutaneous face	19/M	Turkey	19 yr	Thermal (accident at 40 d old)	?	?	?
7[13,15]	Feb 12	Full cutaneous face	25/M	Turkey	23 yr	Thermal (accident at 2 yr old)	?	?	?
8[13,15]	May 12	Full cutaneous face	27/M	Turkey	23.5 yr	Thermal (accident at 3.5 yr old)	?	?	?
9[16]	Feb 13	Full MC face	44/F	USA	6 yr	Chemical (domestic)	98%	6/6	Banff III AMR (at POD 5)
10[17]	Aug 15	Full OMC face	41/M	USA	14 yr	Thermal (accident)	?	6/6	?
Upper extremity									
11[18]	Feb 03	Bilateral forearm	41/M	Austria	3 yr	Electrical	0%	4/6	Acute rejection ×8; AMR at 9 yr
12[19]	Feb 07	Bilateral hand	27/F	France	3 yr	Electrical	?	4/6	Banff II, II, III, II, III (at POD 16, 271, 635, 951, 1365)
13[19]	Jul 08	Bilateral hand	28/M	France	5 yr	Thermal	?	3/6	Banff II (POD 65)
14[20]	Nov 08	Bilateral above elbow	29/M	Spain	1 yr	Electrical	<20%	6/6	Banff III (at POM 6, 13, 26)
15[21]	Nov 08	Unilateral hand	43/M	USA	2 yr	Thermal	0%	?	Banff II ×4 (within 1st year post-VCA)
16[21]	Aug 10	Bilateral hand	55/M	USA	4 yr	Thermal	0%	?	Banff II ×2 (by POD 86); AMR at POM 6; chronic rejection
17[22]	Jan 12	Bilateral arm + single lower limb	34/M	Turkey	23 yr	Electrical (accident at 11 yr old)	?	?	Lower limb lost on POD 1, death ~ POD 100
18[23]	Feb 12	Bilateral above elbow + bilateral lower limb	27/M	Turkey	14 yr	Electrical (accident at 13 yr old)	?	6/6	Death on POD 4
19[24]	Oct 12	Unilateral hand	44/M	USA	9 yr	Thermal	0%	4/6	No acute rejection to date
20[25]	Mar 14	Bilateral hand	55/M	Austria	5 yr	Thermal	0%	6/6	Banff I–II (POD 112)

AMR, antibody-mediated rejection; *EBV*, Epstein-Barr virus; *F*, female; *HLA*, human leukocyte antigen; *M*, male; *MC*, myocutaneous; *OMC*, osteomyocutaneous; *POD*, postoperative day; *POM*, postoperative month; *PRA*, panel reactive antibody; *VCA*, vascularized composite allotransplantation.

Vascularized Composite Allotransplantation—An Emerging Concept for Burn Reconstruction

on-table cardiac arrest,[11] and organ failure, respectively.[13] Retrospective analysis of the patient case that developed cardiac arrest suggested that infection and the resulting circulatory insult was due to an indolent and multidrug resistant strain of Pseudomonas aeruginosa identical to that isolated during his initial burn treatment[14]; detailed information about the other two cases have not been reported. Among the patients who received facial VCAs (cutaneous only, myocutaneous, and osteomyocutaneous), both partial (30%) and full facial allografts were transplanted.

Prevascularized Composite Allotransplantation Immunologic Status

Details of individual patient records were not uniformly available or reported. However, from the available data, pretransplant PRA ranged from 0 to 98%, and the extent of HLA mismatch between donors and recipients varied from 2/6 to 6/6. Prior possible sensitization events included more than 50 surgeries,[9] previous pregnancies, and multiple transfusions. All patients were matched for blood group and had negative pretransplant cross matches except for one (with chemical burns).[9]

Immunological Outcomes

Besides a fingertip amputation and chronic rejection of a hand allograft requiring amputation due to severe ischemia,[15] no other allograft loss has been reported in the group of surviving patients, but they have experienced an average of three (range, 0–8) acute skin rejection episodes from as early as POD 5 to first occurrence at 36 months post-VCA, with corresponding histological grades ranging from Banff I to III. While the majority of acute rejection episodes in VCA were treated successfully with steroid pulses and/or by increasing the dose of calcineurin inhibitors,[16] this cohort of VCA burn patients has seen the introduction of various adjuncts including topical tacrolimus,[17] basiliximab and alemtuzumab,[18,19] and rituximab[18] for the treatment of steroid and anti-thymocyte globulin (ATG) refractory acute rejection. Of note, only one living VCA recipient (upper extremity) has been reported to remain rejection free at 3 years posttransplantation,[10] in contrast to the majority who developed acute rejection within the first year.[16]

Antibody-mediated rejection (AMR) was reported in three patients both early (POD 5 after facial VCA)[9] and late (at 6 months[15] and 9 years[18] after upper extremity VCA); one of these cases also occurred in the absence of donor-specific antibodies (DSA; but became DSA positive two days after allograft removal).[15] One patient, who is currently 6 years status after facial VCA, developed Epstein-Barr virus lymphoma, likely due to chronic immunosuppression. Titration of overall immunosuppression as part of the lymphoma treatment has since led to multiple acute rejection episodes that, although adequately treated, have now culminated with the recent development of features suggestive of chronic rejection in VCA.[20]

While the majority of VCA recipients are maintained on conventional triple immunosuppression consisting of tacrolimus, mycophenolate mofetil, and methylprednisolone,[16] various regimen modifications have been reported in order to reduce the overall burden of immunosuppression and associated complications as described earlier. In an effort to induce tolerance, bone marrow cell infusion was

performed on POD 4 in one facial VCA recipient (without preparatory conditioning), but this was unsuccessful and he remains on immunosuppression.20 Other groups have attempted withdrawal of maintenance steroids from 2 to 12 months post VCA[17,21,] but acute rejection episodes persisted and required increasing tacrolimus and mycophenolate mofetil dosages as well as steroid boluses with subsequent taper, and even ATG for resolution.[22] Various other modifications to maintenance regimens have also been reported in this group of VCA burn patients such as the introduction of sirolimus and the substitution of tacrolimus with everolimus.[18,20]

In one patient with preformed DSA, induction therapy required additional plasmapheresis and intravenous immunoglobulin treatment every other day from POD 1 onward; following the development of antibody-mediated acute rejection on POD 5, rescue therapy required further plasmapheresis, intravenous immunoglobulin, ATG, steroid bolus, and additional eculizumab, bortezomib, and alemtuzumab.[9] This observation led us to compare the induction and treatment regimens between sensitized (defined as PRA > 0 in this study) and non-sensitized (PRA = 0%) VCA patients (Table 2). Patients who were sensitized pre-VCA had various adjuncts (eg, extracorporeal photopharesis, plasmapharesis) in addition to standard induction with T-cell depletion and, in contrast to nonsensitized patients, were still more likely to have preformed DSA or produce DSA subsequent to transplantation. Interestingly, one of the nonsensitized, upper extremity VCA patients had erythrocyte concentrate (the origin of which was not reported) given as part of induction and eventually became "sensitized," developing DSA and consequent AMR, 9 years later.[18] While donor-specific transfusions have previously been shown to have an immunomodulatory effect in renal transplantation,[23] this particular patient developed three episodes of steroid and ATG-resistant acute rejection within the first 6 months and required rescue therapy with alemtuzumab.[24] Further observation suggests that current, triple immunosuppression regimens will most likely be required for long-term maintenance to prevent or minimize rejection episodes. This conclusion is based on the fact that, ultimately, most patients who were on steroid-sparing protocols, had to be treated with additional steroids when rejection developed and some also required reintroduction of steroids for maintenance (Table 2).

Opportunistic infections due to chronic immunosuppression have also been reported. In recipients who were serology negative for cytomegalovirus but received allografts from donors who were positive, cytomegalovirus infections developed from POD 21017 up to POD 460.21 Epstein-Barr virus infection has also been reported at POD 60325 and has led to lymphoma as described previously.[20]

Finally, although not a focus of this review, functional and psychosocial outcomes have been reported elsewhere.[26]

Table 2. Comparison of treatment of rejection episodes between sensitized and nonsensitized VCA burn patients

Patient/ Reference	VCA	Induction	Maintenance Immunosuppression	Acute Rejection	DSA	Rescue Therapy
Sensitized						
1[9]	Face	ATG, ECP	FK506, MMF, prednisone	No	Yes	—
4[12]	Face	ATG, methylprednisolone	FK506, MMF, prednisone (steroid withdrawal at POM 2)	Yes (POM 22)	No	FK506 adjustment + ointment, ATG dexamethasone rinse, steroid bolus and taper
9[16]	Face	ATG, IVIG, methylprednisolone, plasmapheresis	FK506, MMF, prednisone (steroid withdrawal at POM 2)	Yes (POD 5)	Yes (pre-VCA)	Steroid bolus, ATG, IVIG, plasmapheresis, eculizumab, bortezomib, alemtuzumab
14[20]	Upper extremity	Alemtuzumab, methylprednisolone	FK506 (switched to sirolimus on POD 332), MMF, prednisone (introduced after POM 6)	Yes (POM 6)	Yes	Steroid bolus and taper to maintenance
Nonsensitized						
5[12]	Face	ATG	FK506, MMF, prednisone (steroid withdrawal at POM 2)	Yes (POD 20, POM 17)	No	FK506 and MMF adjustment, steroid bolus; FK506 and MMF adjustment, steroid bolus and taper
11[18]	Upper extremity	ATG, erythrocyte concentrate	FK506, MMF, prednisone (steroid withdrawal from years 3–5), everolimus (introduced with gradual FK506 reduction)	Yes (6 in first 3 years)	Yes (year 9)	IV and topical steroids, FK506 adjustment, basiliximab, ATG, alemtuzumab; rituximab for year 9 AMR
15[21]	Upper extremity	Alemtuzumab	FK506, MMF (introduced after POM 1; converted to sirolimus from POM 6 due to GV), prednisone (introduced POM 6)	Yes (POM 1)	No	FK506 adjustment, MMF
16[21]	Upper extremity	Alemtuzumab	FK506, MMF (converted to sirolimus from POM 6 due to GV), prednisone	Yes (×2 by POD 86)	No	— (But developed GV requiring IVIG and plasmapheresis)
19[24]	Upper extremity	ATG		FK506, MMF, prednisone	No	No
20[25]	Upper extremity	Alemtuzumab	FK506, MMF, prednisone (withdrawn from POD 21), belatacept (started on POD 100 due to renal insufficiency)	Yes (POD 112)	No	IV steroid bolus ×3

ATG, antithymocyte globulin; *DSA*, donor-specific antibodies; *ECP*, extracorporeal photopharesis; *FK506*, tacrolimus; *GV*, graft vasculopathy; *IVIG*, intravenous immunoglobulin; *MMF*, mycophenolate mofetil; *POM*, postoperative month; *VCA*, vascularized composite allotransplantation.

DISCUSSION

It is clear from this review that reconstructive transplantation of the upper extremity and craniofacial region can be successfully performed in burn patients for whom no other suitable treatment options exist to improve their quality of life.

At present, most VCA centers follow selection criteria that have been well established in solid organ transplantation,[27] including ABO compatibility and negative pretransplant crossmatch. However, difficulty in finding matching donors with similar color and size of allografts translates to VCA being performed between donor–recipient transplant pairs with varying degrees of HLA mismatch, ranging from 2/6 to 6/6 in the current study. Although data suggest that the extent of HLA matching improves transplant outcomes in solid organ transplantation,[26] in this cohort of burn patients who have undergone VCA, the immediate outcomes of patient and allograft survival and the incidence of acute rejection do not seem to correlate with HLA matching (eg, patient 4 had a HLA mismatch of 5/6 but only developed the first episode of rejection 22 months posttransplantation, whereas in patient 5, despite a more "favorable" HLA mismatch at 2/6, he developed rejection by POD 20). A possible confounding factor is the potential for discrepancy between clinical presentation and histological diagnosis of acute rejection based on the current Banff VCA working classification, especially for grade I episodes that are considered "mild."[28] Although histology is the current accepted standard for diagnosis, there remains much interobserver variation in assessment outcomes based on the current Banff grading system.[29] Consequently, treatment dilemmas arise in the event of subclinical rejection, especially when diagnosed through surveillance rather than "for-cause" biopsies. Moreover, the necessity for treating both subclinical and mild–moderate (Banff I–II) rejection episodes remain equivocal and its long-term immunological impact is still poorly understood. Regardless, clinical correlation with histology is paramount and follow-up biopsies to document histological resolution following treatment of rejection is usually performed at most VCA centers.[28] It is clear that, despite initial enthusiasm for the "ease" of monitoring VCAs by visual inspection, these diagnostic and therapeutic challenges have led to ongoing investigations on various combinations of monitoring modalities[30] including sentinel allo-skin grafts (from the same VCA donor), additional protocol biopsies for random surveillance, Duplex ultrasound, MRI, and even novel technologies such as ultrasound biomicroscopy.[15] Therefore, with the concurrent, growing clinical experience in VCA, the Banff VCA classification will continue to evolve and undergo refinement to improve its diagnostic sensitivity and specificity. Until then, the impact of the different extent of HLA mismatches on VCA rejection remains to be determined.

Much debate has been generated with regard to the allocation of resources to highly sensitized burn patients who may be candidates for VCA.[8] However, it appears that immunologic issues related to anti-HLA sensitization are not a major limitation in performing VCA in such patients. In the French experience, an electrical burn survivor was removed from the transplant waiting list for facial VCA after 18 months because of persistently high PRA levels and positive crossmatches with all potential donors; this patient had received prior skin allografts during acute burn care.[11,31] In contrast, a burn patient who had been on the waitlist for 14 months in the United States underwent VCA

despite a PRA of 98%, a positive crossmatch, and a preexisting DSA to the identified donor.[9] In view of the differing experiences, it has been suggested that surgeons and physicians review the acute management of severe burn injuries with regard to the multitude of possible sensitization events, such as the use of cadaveric allograft skin for temporary wound coverage, multiple blood transfusions, and support devices such as extracorporal membrane oxygenation and renal dialysis.[32] Immunologically, the introduction of foreign antigens naturally (via pregnancy) and iatrogenically (eg, blood transfusions, allogeneic skin grafting, extracorporal membrane oxygenation) is probably unavoidable and it would not be realistic to withhold or compromise life-supporting treatment during acute burn care because the patient may, potentially, become a candidate for VCA some 5 to 10 years later. A recent study by Win et al[31] has shown that in major burns patients with an average TBSA of 47.5±13%, treatment with blood transfusions (average, 21.7±17.3 units) and cadaveric skin allografts (from an average of 5.25±4.1 deceased donors) resulted in a PRA of 87.7±27.6% at approximately 4.36±2.06 years following burn injury. Similarly, Duhamel et al[33] showed that the treatment of 54 ± 11% of TBSA burns required an average of 36±13 units of packed red blood cells (PRBCs) for transfusion with 62% of patients developing a resulting PRA of ≥85% (ie, highly sensitized, to be excluded from transplantation) at approximately 3±1 years later. It should be noted that a high pretransplant PRA is associated with a higher risk of developing DSA subsequently and does not necessarily equate to the preexistence of DSA. However, one can certainly have both, as seen in the patient with chemical burns in this series, who subsequently developed even higher levels of the preexisting DSAs, which, not surprisingly, resulted in acute AMR by POD 5.[9] By extension, one would presume that the development of new, de novo DSA and/or increase in pretransplant DSA after transplantation would correlate with increasing severity of immunological outcomes, but threshold levels based on mean fluorescent intensity have not been shown to be predictive. Rather, the likelihood of treatment success cannot be defined or measured simply by resolution of mean fluorescent intensity because DSA are rarely, if ever, removed.[34]

While desensitization can certainly be attempted, perhaps it would be more worthwhile to recognize the potential deleterious role of memory T-cells in these sensitization events and transplantation[35] and to devise therapeutic strategies to overcome the challenges posed. For instance, the use of glycerol-preserved skin grafts has been purported to stimulate less of an antigenic response compared with cryopreserved skin grafts[33] but this change alone will certainly not be adequate in mitigating the risks of sensitization in acute burn care. Moreover, in the actual VCA procedure itself, blood product requirements are highly variable and may range from just two units of PRBCs[17] to 66 units of PRBCs and an additional 9 and 62 units of platelets and fresh frozen plasma.[11] Perhaps most importantly, memory T-cells are resistant to both depletional[36] and costimulatory blockade[37] strategies and portend a highly formidable immunological barrier in VCA.

Yet, preclinical, large animal studies in nonhuman primates have suggested that the high, pretransplant frequencies of memory T-cells may not be insurmountable when ATG is administered in combination with tocilizumab, a monoclonal antibody against IL-6 receptor, as part of induction therapy in lung transplantation.[38] Subsequently, with

standard triple immunosuppression maintenance, rejection and DSA-free allograft survival of >100 days was achieved and attributed to the in vivo upregulation of regulatory T-cells resulting from this protocol.[39] More recently, a similar approach has been reported clinically in small intestinal transplantation, whereby the in vivo upregulation of regulatory T-cells could avoid sensitization, ameliorate acute rejection, and enable prolongation of allograft survival.[40] Donor blood transfusion, the avoidance of high-dose immunosuppression, minimization of ischemia-reperfusion injury in the allograft, and the selection of infection-free donors were key components to the success of this protocol. In turn, fulfillment of these conditions contributed toward an inflammation-contained environment that presumably enabled proliferation of regulatory T-cells with immunosuppressive functions and a decrease in the numbers of effector-type memor y T-cells. Application of such immunomodulatory protocols to burn patients undergoing VCA may therefore be more realistic and of greater utility.

The success of burn care is determined by the extent of social reintegration that can be achieved by the burn survivor. VCA offers an ideal treatment for patients recovering from major burns but at the same time represents an immunological challenge with far-reaching and potentially lethal consequences. Further development and refinement of clinically relevant immunomodulatory protocols are required to achieve immunosuppression–minimization and/or successful transplantation tolerance to enable long-term survival of both the VCA and the patient.

REFERENCES

1. Tompkins RG, Burke JF, Schoenfeld DA, et al. Prompt eschar excision: a treatment system contributing to reduced burn mortality. A statistical evaluation of burn care at the Massachusetts General Hospital (1974-1984). Ann Surg 1986;204:272–81.
2. van Baar ME, Essink-Bot ML, Oen IMMH, Dokter J, Boxma H, van Beeck EF. Functional outcome after burns: a review. Burns 2006;32:1–9.
3. Hoogewerf CJ, van Baar ME, Hop MJ, Bloemen MC, Middelkoop E, Nieuwenhuis MK. Burns to the head and neck: epidemiology and predictors of surgery. Burns 2013;39:1184–92.
4. Hop MJ, Langenberg LC, Hiddingh J, et al. Reconstructive surgery after burns: a 10-year follow-up study. Burns 2014;40:1544–51.
5. Fauerbach JA, Pruzinsky T, Saxe GN. Psychological health and function after burn injury: setting research priorities. J Burn Care Res 2007;28:587–92.
6. Chuback J, Yarascavitch B, Yarascavitch A, Kaur MN, Martin S, Thoma A. Measuring utilities of severe facial disfigurement and composite tissue allotransplantation of the face in patients with severe face and neck burns from the perspectives of the general public, medical experts and patients. Burns 2015;41:1524–31.
7. Chang G, Pomahac B. Psychosocial changes 6 months after face transplantation. Psychosomatics 2013;54:367–71.
8. Duhamel P, Suberbielle C, Grimbert P, et al. Extensively burned patients still need blood transfusions and skin allografts: unavoidable HLA sensitization requires optimization of VCA access. Transpl Int 2015;28:1229–30.
9. Chandraker A, Arscott R, Murphy GF, et al. The management of antibody-mediated rejection in the first presensitized recipient of a full-face allotransplant. Am J Transplant 2014;14:1446–52.
10. Eberlin KR, Leonard DA, Austen WG Jr, et al. The volar forearm fasciocutaneous extension: a strategy to maximize vascular outflow in post-burn injury hand transplantation. Plast Reconstr Surg 2014;134:731–5.
11. Lantieri L, Hivelin M, Audard V, et al. Feasibility, reproducibility, risks and benefits of face transplantation: a prospective study of outcomes. Am J Transplant 2011;11:367–78.
12. Nasir S, Kilic YA, Karaaltin MV, Erdem Y. Lessons learned from the first quadruple extremity transplantation in the world. Ann Plast Surg 2014;73:336–40.
13. Sheets C. Atilla Kavdir dead months after receiving triplelimb transplant in Turkey 2012; available from http://www. ibtimes.com/atilla-kavdir-dead-months-after-receiving-tri- ple-limb-transplant-turkey-694734; accessed December 27, 2015.
14. Leonard DA, Gordon CR, Sachs DH, Cetrulo CL Jr. Immunobiology of face transplantation. J Craniofac Surg 2012;23:268–71.
15. Kaufman CL, Ouseph R, Blair B, et al. Graft vasculopathy in clinical hand transplantation. Am J Transplant 2012;12:1004–16.
16. Petruzzo P, Lanzetta M, Dubernard JM, et al. The International Registry on Hand and Composite Tissue Transplantation. Transplantation 2010;90:1590–4.
17. Pomahac B, Pribaz J, Eriksson E, et al. Three patients with full facial transplantation. N Engl J Med 2012;366:715–22.
18. Weissenbacher A, Hautz T, Zelger B, et al. Antibody- mediated rejection in hand transplantation. Transpl Int 2014;27:e13–7.
19. Cavadas PC, Ibáñez J, Thione A, Alfaro L. Bilateral trans-humeral arm transplantation: result at 2 years. Am J Transplant 2011;11:1085–90.
20. Petruzzo P, Kanitakis J, Testelin S, et al. Clinicopathological findings of chronic rejection in a face grafted patient. Transplantation 2015;99:2644–50.
21. Pomahac B, Pribaz J, Eriksson E, et al. Restoration of facial form and function after severe disfigurement from burn injury by a composite facial allograft. Am J Transplant 2011;11:386–93.
22. Fischer S, Lian CG, Kueckelhaus M, et al. Acute rejection in vascularized composite allotransplantation. Curr Opin Organ Transplant 2014;19:531–44.
23. Marti HP, Henschkowski J, Laux G, et al. Effect of donor-specific transfusions on the outcome of renal allografts in the cyclosporine era. Transpl Int 2006;19:19–26.
24. Schneeberger S, Kreczy A, Brandacher G, Steurer W, Margreiter R. Steroid- and ATG-resistant rejection after double forearm transplantation responds to Campath-1H. Am J Transplant 2004;4:1372–4.
25. Petruzzo P, Dubernard JM. World experience after more than a decade of clinical hand transplantation: update on the French program. Hand Clin 2011;27:411–6, vii.
26. Takemoto S, Port FK, Claas FH, Duquesnoy RJ. HLA matching for kidney transplantation. Hum Immunol 2004;65:1489–505.
27. Ravindra KV, Buell JF, Kaufman CL, et al. Hand transplantation in the United States: experience with 3 patients. Surgery 2008;144:638–43; discussion 643–4.
28. Schneider M, Cardones AR, Selim MA, Cendales LC. Vascularized composite allotransplantation: a closer look at the Banff working classification. Transpl Int 2016;29:663–71.

29. Sarhane KA, Tuffaha SH, Broyles JM, et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. Front Immunol 2013;4:406.
30. Petruzzo P, Kanitakis J, Badet L, et al. Long-term follow-up in composite tissue allotransplantation: in-depth study of five (hand and face) recipients. Am J Transplant 2011;11:808–16.
31. Win TS, Frew Q, Taylor CJ, Peacock S, Pettigrew G, Dziewulski P. Allosensitization following skin allografts in acute burn management: are burns patients suitable face transplant candidates? J Plast Reconstr Aesthet Surg 2015;68:1155–7.
32. Klein HJ, Schanz U, Hivelin M, et al. Sensitization and desensitization of burn patients as potential candidates for vascularized composite allotransplantation. Burns 2016;42:246–57.
33. Duhamel P, Suberbielle C, Grimbert P, et al. Anti-HLA sensitization in extensively burned patients: extent, associated factors, and reduction in potential access to vascularized composite allotransplantation. Transpl Int 2015;28:582–93.
34. Konvalinka A, Tinckam K. Utility of HLA antibody testing in kidney transplantation. J Am Soc Nephrol 2015;26:1489–502.
35. Su CA, Fairchild RL. Memory T cells in transplantation. Curr Transplant Rep 2014;1:137–46.
36. Pearl JP, Parris J, Hale DA, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. Am J Transplant 2005;5:465–74.
37. Riella LV, Sayegh MH. T-cell co-stimulatory blockade in transplantation: two steps forward one step back! Expert Opin Biol Ther 2013;13:1557–68.
38. Tonsho M, Lee S, Aoyama A, et al. Tolerance of lung allografts achieved in nonhuman primates via mixed hematopoietic chimerism. Am J Transplant 2015;15:2231–9.
39. Aoyama A, Tonsho M, Smith RN, et al. Non-human primate lung allograft survival is prolonged by IL-6 inhibition and ATG treatment possibly through expansion of peripheral regulatory T cells. Am J Transplant 2016;16:203–404.
40. Ceulemans LJ, Braza F, Monbaliu D, et al. The Leuven immunomodulatory protocol promotes T-regulatory cells and substantially prolongs survival after first intestinal transplantation. Am J Transplant 2016.

CHAPTER 09

Graft vasculopathy of vascularized composite allografts in humans: a literature review and retrospective study

Zhi Yang Ng,
Alexandre G. Lellouch
Ivy A. Rosales
Luke Geoghegan
Amon-Ra Gama
Robert B. Colvin
Laurent A. Lantieri
Mark A. Randolph
Curtis L. Cetrulo Jr.

Adapted from Transpl Int. 2019 Mar 4. doi: 10.1111/tri.13421

ABSTRACT

Mechanisms of chronic rejection of vascularized composite allografts (VCA) remain poorly understood and likely present along a spectrum of highly varied clinicopathological findings. Across both animal and human VCA however, graft vasculopathy (GV) has been the most consistent pathological finding resulting clinically in irreversible allograft dysfunction and eventual loss. A literature review of all reported clinical VCA cases with documented GV up to December 2018 was thus performed to elucidate the possible mechanisms involved. Relevant data extracted include C4d deposition, donor-specific antibody (DSA) formation, extent of human leukocyte antigen (HLA) mismatch, pretransplant panel reactive antibody levels, induction and maintenance immunosuppression used, the number of preceding acute rejection episodes, and time to histological confirmation of GV. Approximately 6% (13 of 205) of all VCA patients reported to date developed GV at a mean of 6 years post-transplantation. 46% of these patients have either lost or had their VCAs removed. Neither C4d nor DSA alone was predictive of GV development; however, when both are present, VCA loss appears inevitable due to progressive GV. Of utmost concern, GV in VCA does not appear to be abrogated by currently available immunosuppressive treatment and is essentially irreversible by the time of diagnosis with allograft loss a likely eventuality.

INTRODUCTION

Reconstructive transplantation through vascularized composite allografts (VCAs) has revolutionized the treatment of complex soft tissue absence, loss and injuries by providing an anatomically exact tissue unit enabling like-for-like restoration. In recent years, there has been rapid growth in the application of VCA with upper extremity[1], face [2], penis[3], abdominal wall[4], larynx[5], uterus[6] and even knee transplants[7] performed.

Much variation in inclusion/exclusion criteria and induction, maintenance and rescue immunosuppression regimens exists between different VCA centers[8]. As a result, long-term immunological consequences remain poorly understood and are difficult to compare between centers. For instance, the extent of HLA matching has long been known to impact upon allograft rejection and longevity in solid organ transplantation (SOT)[9]; in VCA however, matching of skin color and allograft size between donor and recipient takes priority instead due to the need for optimizing functional and aesthetic outcomes[10].

Despite early enthusiasm in the field, like any transplanted allograft, VCAs are not spared from chronic rejection with recent clinical reports confirming as such[11]. While the mechanisms of chronic rejection in VCA remain to be formally defined, reports have suggested a highly varied range of associated clinical and pathological findings, all of which likely represent manifestation of the underlying disease process along a spectrum[11–13]. Of note, experimental (from small and large animal models) and clinical VCA subjects have both developed a common pathological feature in graft vasculopathy (GV) whereby myointimal proliferation and gradual luminal narrowing lead to progressive graft ischemia, necrosis and eventual loss[11–13]. The roles of antibody-mediated rejection (AMR), donor-specific antibody (DSA) formation and C4d deposition (or not) with consequent GV in the context of VCA remain equivocal, however. Thus, this study aims to review the current clinical evidence on GV as the main component of chronic vascular rejection in VCA and associated outcomes including allograft loss.

Patients and methods

A PubMed search for English language articles on confirmed cases of chronic VCA rejection (based on histological confirmation of GV development) was performed up to December 2018. Search terms include: “graft vasculopathy vascularized composite allotransplantation”, “graft vasculopathy vascularized composite allograft”, “graft vasculopathy composite tissue allotransplantation”, “graft vasculopathy composite tissue allograft”. References of individual articles were reviewed to minimize missing out on potentially relevant reports.

Relevant data extracted include the type of VCA (e.g., osteomyocutaneous, myocutaneous), the extent of HLA matching between donor and recipient, pretransplant panel reactive antibody (PRA) status, induction agent, whether donor bone marrow cells (BMCs) were infused, maintenance immunosuppression regimen, prior nu ber of acute rejection episodes before histological confirmation of GV (including time to diagnosis, site

and type of biopsy performed), DSA and C4d statuses, length of follow-up before development of GV, and final outcome of the VCA after developing GV. Reported cases of chronic rejection without GV development were excluded.

Results

Our literature search yielded a total of 121 articles. After accounting for duplicate data, exclusion of reports on chronic rejection without GV, and initially missed articles located from reference lists, there were a total of 13 individual reports of VCA patients who had developed GV as confirmed by histopathology.

Patient demographics

Overall, approximately 205 cases of all types of VCA have been performed worldwide and at the time of this study, 13 have reported histological confirmation of GV development (overall incidence of 13/205 = 6%). Diagnosis was confirmed by histopathology (through for-cause or terminal biopsies) at a mean of 5.9 years post-transplantation (range, 0.5–13 years). Seven patients have had their allograft removed for an overall VCA survival of 46% from the time of GV diagnosis; of the remaining 6 patients with GV, the mean reported follow-up without subsequent loss of VCA was 5.6 years (range, 0.75–10).

Potential contributory factors to graft vasculopathy development

Due to limited data available, there was no observable association between the extent of HLA mismatch (from 0/6 to 6/6), PRA status, type of VCA performed (with and without vascularized bone marrow as part of the composite unit), immunosuppressive regimen and number of prior acute rejection episodes (mean = 3.5; range 1–8) before GV was diagnosed.

Of note, induction with alemtuzumab does not appear to result in DSA formation (n = 5; 1 was positive only after VCA removal[14]) within the reported follow-up period compared to basiliximab and anti-thymocyte globulin (ATG), where 3 of 5 patients developed DSA (given the available data). In terms of maintenance immunosuppression, steroid-sparing regimens (n = 4) appear to result in earlier development of GV (2.6 vs. 7.4 years in protocols with steroids).

Potential mechanisms of graft vasculopathy in VCA

Evidence for AMR (based on DSA positivity alone) was suggested in five VCA patients who developed GV (Patients #3, 5, 9, 10, 13; Table 1) but corresponding C4d deposition (tested for in all protocol and for-cause biopsies) was not always associated with circulating DSA.

In patient #3, multiple (seven) prior episodes of acute rejection appears to have contributed towards DSA+/ C4d GV at 8 years post-transplantation although the VCA remained viable at 10 years follow-up. Similarly, patient #9 developed DSA+/C4d GV at 6 years post-transplantation in spite of few (three) prior acute rejecstion episodes and eventually required VCA amputation at 11 years post-transplantation due to progressive

ischemia and necrosis of skin lesions. In contrast, patient #5 also had few (two) prior acute rejection episodes but developed ischemia of the VCA that ultimately necessitated amputation at only 9 months post-transplantation; DSA+/C4d GV was detected only on terminal histology analysis.

With regard to C4d deposition, this was seen in five VCA patients who developed GV (Patients #4, 7, 8, 10,13; Table 1). Patients #4 and #7 developed DSA/C4d+ GV after few (two to three) prior episodes of acute rejection and relatively early at only 2 years and 6 months post-transplantation respectively although VCA loss was not reported within the available follow-up data. Patient #8 developed DSA/C4d+ GV at 13 years post-transplantation after five prior episodes of acute rejection and the VCA was eventually removed 1 month later due to gradual, self-discontinuation of immunosuppression over the preceding year. Of note, C4d was detected only in deep vessels of the VCA in these patients.

Patients #10 and #13 both developed DSA+/C4d+ AMR-mediated GV and eventually lost the VCA. In patient #10, there were two episodes of acute rejection prior to detection of high DSA levels from 3 years post-transplantation onwards and two further episodes of acute AMR before GV and C4d were detected after VCA amputation at 7 years post-transplantation due to recalcitrant rejection episodes that were likely due to chronic AMR. Similarly, patient #13 only had two prior episodes of acute rejection before DSA levels became detectable after 7.5 years post-transplantation and eventually, resulted in progressive rejection, necrosis and loss of the VCA at 10 years post-transplantation that was likely also due to underlying, chronic AMR with corresponding detection of GV and C4d.

DISCUSSION

This study has shown that the severity of GV in VCA is highly variable with differing clinical outcomes and temporality from the point of diagnosis. Most concerning, however, is the likelihood for its true incidence to be underestimated, and the pace of GV progression unpredictable due to the inherent challenges of timely detection and accurate diagnosis in clinical VCA[15]. To further compound the problem, diagnostic and treatment options for acute rejection episodes in VCA remain particularly challenging due in part to the ongoing revision and evolution of the Banff 2007 pathological classification of acute VCA rejection[15], and a possible association with eventual GV development[16]. Detection of de novo DSA formation, staining and reporting of C4d status in VCA is also not uniformly performed or reported and unfortunately, their significance in GV remains poorly understood thus far[17]. Furthermore, VCA patients with allograft loss have not always been reported in the literature, which only serves to further compound our already limited understanding of the contribution of GV, if any, towards chronic rejection and loss of VCAs. While the risk factors for GV in VCA remain poorly understood, experimental rodent studies by Unadkat et al.[16] have suggested that the accrual of multiple acute rejection episodes may culminate in GV and similar observations have been made in this study. To illustrate: in patient #12, the development of post-transplant lymphoproliferative disorder[18] necessitated the lowering of maintenance immunosuppression, contributed towards eight acute rejection episodes with eventual sclerosis and dyschromia of the facial allograft at 4 years post-transplantation with GV

seen in the facial arteries on MRI [18,19]; in patient #8 who had experienced five prior episodes of acute rejection, gradual self-tapering of immunosuppression led to upper extremity VCA removal at 13 years post-transplantation with GV found in the radial artery and perineural vessels of the radial nerve on terminal histopathological analysis[20]. Interestingly, patient #3, who was converted from mycophenolate mofetil (MMF) to rapamycin in the early post-transplant period to allow lowering of maintenance tacrolimus levels, developed seven episodes of acute rejection before DSA formation and GV was detected at 7 and 8 years post-transplantation respectively although the VCA remained viable at 10 years follow-up[14]. However, GV may also develop insidiously following episodes of acute rejection that might have been missed altogether or deemed mild and unnecessary for treatment [5,7,11] as demonstrated in nonhuman primate studies by Mundinger et al.[21]. Again, similar observations have been made clinically in patients #5, 9, 10 and 13 with only 2–3 episodes of acute rejection before GV development and eventually, necrosis of the VCA necessitating removal. These findings support our observation that GV in VCA is highly variable in both severity and clinical underestimated, and the pace of GV progression unpredictable due to the inherent challenges of timely detection and accurate diagnosis in clinical VCA[15]. To further compound the problem, diagnostic and treatment options for acute rejection episodes in VCA remain particularly challenging due in part to the ongoing revision and evolution of the Banff 2007 pathological classification of acute VCA rejection [15], and a possible association with eventual GV development[16]. Detection of de novo DSA formation, staining and reporting of C4d status in VCA is also not uniformly performed or reported and unfortunately, their significance in GV remains poorly understood thus far [17]. Furthermore, VCA patients with allograft loss have not always been reported in the literature, which only serves to further compound our already limited understanding of the contribution of GV, if any, towards chronic rejection and loss of VCAs.

While the risk factors for GV in VCA remain poorly understood, experimental rodent studies by Unadkat et al.[16] have suggested that the accrual of multiple acute rejection episodes may culminate in GV and similar observations have been made in this study. To illustrate: in patient #12, the development of post-transplant lymphoproliferative disorder[18] necessitated the lowering of maintenance immunosuppression, contributed towards eight acute rejection episodes with eventual sclerosis and dyschromia of the facial allograft at 4 years post-transplantation with GV seen in the facial arteries on MRI [18,19]; in patient #8 who had experienced five prior episodes of acute rejection, gradual self-tapering of immunosuppression led to upper extremity VCA removal at 13 years post-transplantation with GV found in the radial artery and perineural vessels of the radial nerve on terminal histopathological analysis[20]. Interestingly, patient #3, who was converted from mycophenolate mofetil (MMF) to rapamycin in the early post-transplant period to allow lowering of maintenance tacrolimus levels, developed seven episodes of

Table 1. Reported cases of chronic rejection in VCA based on the development of graft vasculopathy.

Patient	VCA type	HLA mismatch	Pre-VCA PRA	Induction	Maintenance	Donor BMC infusion	Prior TCMR episodes	C4d DSA	GV diagnosis (time, site, biopsy type) final outcome
1 [7]	Knee	N/A	N/A	ATG	Tacrolimus MMF Steroids	No	X 1	N/A N/A	36 months, SSG & VCA synovial and deep tissues, for-cause biopsy Infection, above-knee amputation at 56 months
2 [30]	Upper extremity	N/A	N/A	ATG	Tacrolimus MMF Steroids	No	Yearly	N/A N/A	5 years, VCA skin, for-cause biopsy VCA intact (at 9 years follow-up)
3 [14]	Upper extremity	N/A	0%	Basiliximab	Tacrolimus MMF → Rapamycin (early post-transplant) Steroids (weaned then restarted)	No	X 7	– + (from year 7)	8 years, VCA deep tissue and inter-osseous arteries, for-cause biopsy VCA intact (at 10 years follow-up)
4 [14,31]	Upper extremity	N/A	0%	Alemtuzumab	Tacrolimus Rapamycin Steroids (after GV)	No	X 3	+ (deep vessels) –	2 years, VCA deep tissue and inter-osseous arteries, for-cause biopsy VCA intact (at 6 years follow-up)
5 [14]	Upper extremity	N/A	0%	Alemtuzumab	Tacrolimus MMF	No	X 2	– + (only after amputation)	9 months, VCA arteries & veins, terminal biopsy Ischemia, VCA amputated at 9 months
6 [14]	Upper extremity	N/A	0%	Alemtuzumab	Tacrolimus MMF (after 1st ACR) → rapamycin (after GV) Steroids (after GV)	No	X 3	– –	6 months, VCA deep tissue and inter-osseous arteries, for-cause biopsy VCA intact (at 2 years follow-up)
7 [14]	Upper extremity	N/A	0%	Alemtuzumab	Tacrolimus MMF → rapamycin (after GV) Steroids	No	X 2	+ (adnexae and vessels in adipose, deep vessels in skin) –	6 months, VCA deep tissues, for-cause biopsy VCA intact (at 9 months follow-up)
8 [20]	Upper extremity	6/6	N/A	Basiliximab	Tacrolimus MMF Steroids	No	X 5	+ (perineural vessels only) –	13 years, VCA skin & radial artery, terminal biopsy VCA amputated at 13 years (1 month after stopping Rx)

Table 1. Continued.

Patient	VCA type	HLA mismatch	Pre-VCA PRA	Induction	Maintenance	Donor BMC infusion	Prior TCMR episodes	C4d DSA	GV diagnosis (time, site, biopsy type) final outcome
9 [11]	Upper extremity	4/6	N/A	ATG	Tacrolimus MMF Steroids	No	X 3	N/A + (from year 6)	11 years, VCA skin, for-cause biopsy Amputation of fingers in VCA and eventually whole allograft at 11 years
10 [32]	Upper extremity	6/6	0%	Alemtuzumab	Tacrolimus → Belatacept MMF Steroids (stopped after POD 21)	No	X 4 (had 2 episodes of AMR prior to starting belatacept)	+ (only after amputation) + (from year 3)	7 years, VCA (not specified), terminal biopsy Amputation of affected finger with poor wound healing; VCA amputated at 7 years
11 [5]	Larynx, Pharynx, Trachea, Thyroid, Parathyroid	0/6	N/A	Muromonab-CD3	Cyclosporine → Tacrolimus (after 1st ACR) MMF Steroids	No	X 1	N/A N/A	12 years, VCA (not specified), terminal biopsy VCA explanted at 14 years
12[18]	OMC Face	5/6	0%	ATG	Tacrolimus MMF Steroids	Yes (on day 4)	X 8	– –	4 years, VCA skin, for-cause biopsy VCA intact (at 6 years follow-up)
13 [11,19]	MC Face	1/6	N/A	ATG	Tacrolimus → rapamycin (after 11 months) MMF Steroids	Yes (on days 4 & 11)	X 2	+ (VCA dermal vessels) + (from year 7.5)	10 years, SSG nutrient vessel & VCA facial arteries, for-cause biopsy Necrosis, VCA removed at 10 years; autologous reconstruction

ACR, acute cellular rejection; AMR, antibody-mediated rejection; ATG, anti-thymocyte globulin; BMC, bone marrow cell; DSA, donor-specific antibody; GV, graft vasculopathy; MMF, mycophenolate mofetil; OMC, osteomyocutaneous; PRA, panel reactive antibody; SSG, sentinel skin graft.

acute rejection before DSA formation and GV was detected at 7 and 8 years post-transplantation respectively although the VCA remained viable at 10 years follow-up[14]. However, GV may also develop insidiously following episodes of acute rejection that

might have been missed altogether or deemed mild and unnecessary for treatment [5,7,11] as demonstrated in nonhuman primate studies by Mundinger et al.[21]. Again, similar observations have been made clinically in patients #5, 9, 10 and 13 with only 2–3 episodes of acute rejection before GV development and eventually, necrosis of the VCA necessitating removal. These findings support our observation that GV in VCA is highly variable in both severity and clinical presentation that may potentially be followed by irre- versible allograft loss, regardless of the various rescue and salvage immunosuppression therapies attempted.

Multiple immunomodulatory approaches have thus been attempted in VCA to reduce the incidence of acute rejection episodes. Such modalities include variations in T B cell depletion, steroid-based and steroid-sparing maintenance regimens, switching from calcineurin-based inhibition to rapamycin and/or belatacept, and donor BMC infusion. DSA formation was detected in 3 of 5 VCA patients (#3, 8, 9, 12, 13) who had undergone basiliximab or ATG induction followed by steroid-based maintenance immunosuppression but only in 1 of 5 (patients #4, 5, 6, 7, 10) who had undergone alemtuzumab induction and steroid-sparing maintenance protocols (albeit initially). Perhaps the earlier detection of GV in the latter group and subsequent introduction of additional maintenance steroids halted further progression. If so, it raises doubts about the safety of steroid-sparing regimens that have only had limited follow-up reported so far with further rejection episodes necessitating the re-introduction of, or additional steroid treatment[22,23]. Additionally, it appears that rapamycin may halt the progression of GV (as seen in patients #6 and 7), once detected, with VCA survival of up to 2 years post-transplantation thereafter. The use of novel agents such as belatacept does not appear to have any beneficial effect on DSA+ GV (as seen in patient #10) despite its purported benefits in reducing preexisting DSAs more effectively[24], unlike the anti-proliferative agents rapamycin and MMF. Two patients (#12, 13) in this study underwent donor BMC infusion without recipient conditioning, did not develop detectable levels of mixed chimerism, and had no observable effect on the number of acute rejection episodes or time to development of GV, when compared with the other 11 patients who did not receive BMCs. It appears then that currently, standard triple immunosuppression based on tacrolimus, MMF and steroids would provide the best chance of ensuring longevity of the VCA, with switching to or addition of rapamycin being potentially beneficial in slowing the progression of GV once detected.

Fundamentally, the exact mechanisms behind GV in transplantation are poorly understood although AMR remains the most commonly accepted explanation with corresponding detection of DSA and C4d[25]. However, as with experimental animal data, the detection or absence of C4d in clinical VCA subjects did not portend the subsequent development of GV. Also, similar to AMR in SOT, VCA biopsies that are DSA/C4d+, DSA+/C4d and DSA+/C4d+ have all been reported in this study. It is possible that this phenomenon may be caused by non- HLA antibodies[26] but regardless, GV appears to be the common end point. Of note, in this study, there were 3, 3 and 2 VCA patients that were DSA/C4d+, DSA+/C4d and DSA+/C4d+ respectively. Only one patient (#8) that was DSA/C4d+ lost the VCA but this was due to medication noncompliance whereas two of three patients (#5, #9) that were DSA+/C4d required VCA amputation due to progressive and irreversible ischemia and rejection. Of note, both patients (#10, #13) that were DSA+/C4d+ experienced VCA loss. Based on these observations, C4d may be less accurate

in predicting the development of GV in VCA as it tends to be localized to deep tissues thereby necessitating deep, open biopsies (which is still subject to sampling errors) whereas DSA, which is detected in peripheral blood, may in fact be a better predictor of GV and eventual allograft loss.

Overall, the role of induction therapy, steroid withdrawal and the respective roles of DSA and C4d in the development of GV are difficult to assess due to the retrospective nature of the study and the small number of patients reported. No definitive treatment exists for GV in VCA currently but this study has shown that early diagnosis and management with maintenance steroids and/or anti-proliferative agents such as rapamycin may potentially halt or slow the progression of an essentially irreversible process[14]. Novel, noninvasive monitoring technologies such as ultrasound biomicroscopy have also been described for earlier detection of GV[14], as well as genomic interrogation and immunohistochemistry of VCA biopsy samples[27]. Nevertheless, by the time such changes are detectable or clinically significant, it may be a case of too little too late. Ultimately, we believe that mixed chimerism represents the most likely strategy that will be successful in mitigating GV and chronic VCA loss, as demonstrated in previous swine studies from our laboratory[28,29]. Further studies on reliable methods for the induction of stable, long-term mixed chimerism are required. In the mean time, there is now an urgent need for describing clinical strategies to mitigate the challenges of impending VCA loss due to GV. Such options may include re-transplantation of the face (https://edition.cnn.com/2018/04/17/health/second-face-transplant-bn/ index.html; accessed on 31 October 2018) or involve a combination of autologous reconstruction and/or prosthetic fitting in hand VCA recipients.

REFERENCES

1. Shores JT, Brandacher G, Lee WPA. Hand and upper extremity transplantation: an update of outcomes in the worldwide experience. Plast Reconstr Surg 2015; 135: 351e–360e.
2. Khalifian S, Brazio PS, Mohan R, et al. Facial transplantation: the first 9 years. Lancet (London, England) 2014; 384: 2153–2163.
3. Cetrulo CL, Li K, Salinas HM, et al. Penis transplantation: first US experience. Ann Surg May 2017; 267: 983–988.
4. Giele H, Vaidya A, Reddy S, Vrakas G, Friend P. Current state of abdominal wall transplantation. Curr Opin Organ Transplant 2016; 21: 159–164.
5. Lorenz RR, Strome M. Total laryngeal transplant explanted: 14 years of lessons learned. Otolaryngol Head Neck Surg 2014; 150: 509–511.
6. Br€annstro€m M, Johannesson L, Bokstro€m H, et al. Livebirth after uterus transplantation. Lancet (London, England) 2015; 385: 607–616.
7. Diefenbeck M, Nerlich A, Schneeberger S, Wagner F, Hofmann GO. Allograft vasculopathy after allogeneic vascular- ized knee transplantation. Transpl Int 2011; 24: e1–e5.
8. Petruzzo P, Sardu C, Lanzetta M, Dubernard JM. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). Curr Transplant Rep 2017; 4: 294–303.
9. Zachary AA, Leffell MS. HLA mismatching strategies for solid organ transplantation – a balancing act. Front Immunol 2016; 7: 575.
10. Ashvetiya T, Mundinger GS, Kukuruga D, et al. Donor-recipient human leukocyte antigen matching practices in vascularized composite tissue allotransplantation: a survey of major transplantation centers. Plast Reconstr Surg 2014; 134: 121–129.
11. Kanitakis J, Petruzzo P, Badet L, et al. Chronic rejection in human vascularized composite allotransplantation (hand and face recipients): an update. Transplantation 2016; 100: 2053–2061.
12. Mundinger GS, Drachenberg CB. Chronic rejection in vascularized composite allografts. Curr Opin Organ Transplant 2014; 19: 309–314.
13. Morelon E, Petruzzo P, Kanitakis J. Chronic rejection in vascularized composite allotransplantation. Curr Opin Organ Transplant 2018; 23: 582–591.
14. Kaufman CL, Ouseph R, Blair B, et al. Graft vasculopathy in clinical hand transplantation. Am J Transplant 2012; 12: 1004–1016.
15. Schneider M, Cardones ARG, Selim MA, Cendales LC. Vascularized composite allotransplantation: a closer look at the banff working classification. Transpl Int 2016; 29: 663–671.
16. Unadkat JV, Schneeberger S, Horibe EH, et al. Composite tissue vasculopathy and degeneration following multiple episodes of acute rejection in reconstructive transplantation. Am J Transplant 2010; 10: 251–261.
17. Sarhane KA, Khalifian S, Ibrahim Z, et al. Diagnosing skin rejection in vascularized composite allotrans- plantation: advances and challenges. Clin Transplant 2014; 28: 277–285.
18. Petruzzo P, Kanitakis J, Testelin S, et al. Clinicopathological findings of chronic rejection in a face grafted patient. Transplantation 2015; 99: 2644–2650.
19. Morelon E, Petruzzo P, Kanitakis J, et al. Face transplantation: partial graft loss of the first case 10 years later. Am J Transplant 2017; 17: 1935–1940.
20. Kanitakis J, Karayannopoulou G, Lanzetta M, Petruzzo P. Graft vasculopathy in the skin of a human hand allograft: implications for diagnosis of rejection of vascularized composite allografts. Transpl Int 2014; 27: e118– e123.
21. Mundinger GS, Munivenkatappa R, Drachenberg CB, et al. Histopathology of chronic rejection in a nonhuman primate model of vascularized composite allotransplantation. Transplantation 2013; 95: 1204–1210.
22. Schneeberger S, Gorantla VS, Brandacher G, et al. Upper-extremity transplantation using a cell-based protocol to minimize immunosuppression. Ann Surg 2013; 257: 345–351.
23. Diaz-Siso JR, Fischer S, Sisk GC, et al. Initial experience of dual maintenance immunosuppression with steroid withdrawal in vascular composite tissue allotransplantation. Am J Transplant 2015; 15: 1421–1431.
24. Bray RA, Gebel HM, Townsend R, et al. Posttransplant reduction in preexisting donor-specific antibody levels after belatacept- versus cyclosporine-based immunosuppression: post hoc analyses of BENEFIT and BENEFIT-EXT. Am J Transplant 2018; 18: 1774–1782.
25. Abrahimi P, Liu R, Pober JS. Blood vessels in allotransplantation. Am J Transplant 2015; 15: 1748–1754.
26. Shinoda K, Akiyoshi T, Chase CM, et al. Depletion of foxp3(+) T cells abrogates tolerance of skin and heart allografts in murine mixed chimeras without the loss of mixed chimerism. Am J Transplant 2014; 14: 2263–2274.
27. Krezdorn N, Lian CG, Wells M, et al. Chronic rejection of human face allografts. Am J Transplant 2018; https://doi.org/10. 1111/ajt.15143.
28. Leonard DA, Kurtz JM, Mallard C, et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant 2014; 14: 343–355.

Graft vasculopathy of vascularized composite allografts in humans: a literature review and retrospective study

29. Shanmugarajah K, Powell H, Leonard DA, et al. The effect of MHC antigen matching between donors and recipients on skin tolerance of vascularized composite allografts. Am J Transplant 2017; 17: 1729–1741.
30. PeiG,XiangD,GuL,etal.Areportof 15 hand allotransplantations in 12 patients and their outcomes in China. Transplant J 2012; 94: 1052–1059.
31. Kaufman C, Marvin M, Ouseph R, Manon-Matos Y, Blair BKJ. Evidence for chronic rejection of the skin and adnexal structures in a hand transplant recipient at six years posttransplant without significant graft vasculopathy. Am J Transplant 2013; 13(Suppl 5): 286.
32. Grahammer J, Weissenbacher A, Zelger BG, et al. Benefits and limitations of belatacept in 4 hand-transplanted patients. Am J Transplant 2017; 17: 3228–3235.

CHAPTER 10

First human facial retransplantation: 30-month follow-up

Laurent A. Lantieri
Bernard Cholley
Cedric Lemogne
Romain Guillemain
Nicolas Ortonne
Philippe Grimbert
Eric Thervet
Alexandre G. Lellouch

Adapted from Lancet. 2020 Nov 28;396(10264):1758-1765. doi: 10.1016/S0140-6736(20)32438-7. PMID: 33248497

SUMMARY

Background

Since the first successful facial transplantation in 2005, the benefits of this procedure in terms of aesthetics, functionality, and quality of life have been firmly established. However, despite immunosuppressive treatment, long- term survival of the allograft might be compromised by chronic antibody-mediated rejection (CAMR), leading to irreversible necrosis of the tissue. In the absence of therapeutic options, this complication is inevitably life-threatening.

Methods

We report facial retransplantation in a man, 8 years after his first facial transplantation because of extensive disfigurement from type 1 neurofibromatosis and 6 weeks after complete loss of his allograft due to severe CAMR. We describe the chronology of immune-related problems that culminated in allograft necrosis and the eventual loss of the facial transplant, the desensitisation protocol used for this highly immunosensitised recipient, the surgical technicalities of the procedure, the specific psychological management of this patient, and the results from follow-up at 30 months.

Findings

Although the patient had a complicated postoperative course with numerous immunological, infectious, cardiorespiratory, and psychological events, he was discharged after a hospital stay of almost 1 year. He has since been able to re-integrate into his community with acceptable restoration of his quality of life.

Interpretation

This clinical report of the first documented human facial retransplantation is proof-of-concept that the loss of a facial transplant after CAMR can be mitigated successfully by retransplantation combined with an aggressive desensitisation process.

Funding

Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris.

INTRODUCTION

Vascularised composite allotransplantation (VCA) has emerged as the most advanced reconstruction technique for complex defects such as total hand amputation or massive destruction of the face, where no conventional reconstruction options are available. More than 20 years after the first hand transplantation showed long-term success,[1] VCA surgical outcomes are now more predictable and reproducible, and have improved drastically with technological advancements.[2]

However, despite these improvements, the number of complications (metabolic, infectious, and neoplastic) resulting from long-term immunosuppression[3] presents many ethical issues[4] and remains the source of much controversy.[5,6]

Patients who undergo VCA must comply with lifelong maintenance immunosuppression, typically using a standard triple drug regimen (ie, a calcineurin inhibitor, mycophenolate mofetil, and corticosteroids). Strict adherence to therapy helps prevent allograft rejection.[7] Compared with solid organ transplantation, the detection of acute rejection in VCA is arguably easier because skin inflammation represents an early warning sign of rejection, and can trigger prompt therapeutic response.[8] Surveillance biopsies of VCA skin also allow for the detection of subclinical episodes of acute rejection.

Chronic antibody-mediated rejection (CAMR) resulting in late allograft loss can occur in all types of solid organ transplantation. In facial transplantation, chronic rejection can result in cellular lichenoid reactions as well as severe cutaneous fibrosis and chronic scleroderma-like graft-versus-host disease.[9] In hand-transplant recipients, severe chronic rejection resulted in amputation, and patients returned to pretransplantation functional status. However, in facial-transplant recipients, allograft loss can result in a life-threatening defect.[10]

We present our experience with a patient who suffered complete loss of his facial transplant due to CAMR, 8 years after transplantation, and who then underwent facial retransplantation. To our knowledge, there has been no other case of facial retransplantation reported to date.

METHODS

Case Report

A male patient underwent facial transplantation in 2010 at the age of 35 years after excision of a massive plexiform neurofibroma caused by type 1 neurofibromatosis (figure 1A, B). We previously reported the initial 6-year follow-up for this patient (patient number five), among other facial-transplant recipients.[11,12] A progressive humoral rejectionwassuspectedwhenanincreaseinplasmalevels of donor-specific antibody (HLA-B51) was detected 6 years after the transplantation. This immunisation against the allograft persisted over the next 2 years despite repeated corticosteroid boli and antithymocyte globulin infusions (figure 2). Clinically, the facial transplant became progressively more fibrotic (figure 1C) while the patient experienced pain and loss of

Research in context

Evidence before this study

Facial vascularised composite allotransplantation (VCA) has been a therapeutic option for extensive facial injuries that are not amenable to conventional, autologous, reconstructive surgery following the first successful case in 2005. Since then, 48 facial transplantations have been done worldwide, but long-term follow-up and complications have scarcely been reported. The development of chronic antibody-mediated rejection (CAMR) leading to the irreversible loss of the facial transplant is a potentially life-threatening complication that was, up until now, beyond therapeutic resources.

Added value of this study

We report, to our knowledge, the first facial retransplantation in the world, in a patient who developed CAMR resulting in the complete loss of his facial transplant. We describe the surgical technicalities of the procedure as well as the immunological and psychological management of this patient, who remains the only person thus far to have received two facial transplants.

Implications of all the available evidence

The rate-limiting step to a wider adoption of facial VCA was the potential development of CAMR and eventual allograft necrosis, with no recourse available. We have shown that facial retransplantation is possible, thereby fundamentally altering the risk-benefit ratio of facial VCA.

facial motion. These symptoms prompted the patient to be admitted to hospital on Oct 25, 2017. Skin biopsies showed diffuse dermal fibrosis, associated with foci of lichenoid tissue reaction or interface dermatitis and postinflammatory pigmentation. The patient was registered on the waiting list for facial retransplantation on Oct 31, 2017. Over the next 2 months, the facial transplant showed progressive and irreversible necrosis (figure 1D), and could no longer be left in place due to pain and local infection. Eventually, it was excised completely on Dec 1, 2017. Histological examination of the graft confirmed scleroderma-like chronic rejection (figure 3). In order to cover the facial skin defect, we first used a dermal matrix (Integra; Integra LifeSciences, Princeton, NJ, USA), but a Staphylococcus aureus sepsis forced us to remove the matrix 1 week later. Facial coverage was subsequently achieved using a split- thickness skin graft from the thigh (figure 1E). The immunosuppressive treatment was then discontinued because the allograft was no longer present and septic complications represented a major threat. A tracheostomy and a gastrostomy were done, and the patient was transferred to the intensive-care unit (ICU).

A preoperative CT angiogram was done to evaluate the quality of the recipient's blood vessels, which revealed that the distal external carotid artery was stenotic and that no veins other than the internal jugular vein could be visualised. We planned to have arterial graft inflow provided by the external carotid artery and venous outflow via the internal jugular vein in continuation with the thyrolingual-facial trunk. We organised cadaveric rehearsals of a modified full-face transplantation technique[13] for the surgeons to practise the various technical steps of the procedure. The rehearsal was done mainly for the face procurement (a reproducible procedure) but not for the retransplantation itself, due to the difficulty in imitating the dense fibrotic tissue resulting from CAMR.

After 1 month without any donor proposal, the call for a potential donor was extended from the Greater Paris area to the entire country of France, to increase the chances of obtaining a graft. The patient provided written consent for the publication of this report.

Procedures

A facial allograft from a brain-dead donor (age 22 years) became available on Jan 15, 2018. The donor and the recipient were matched for blood group (O positive) and both

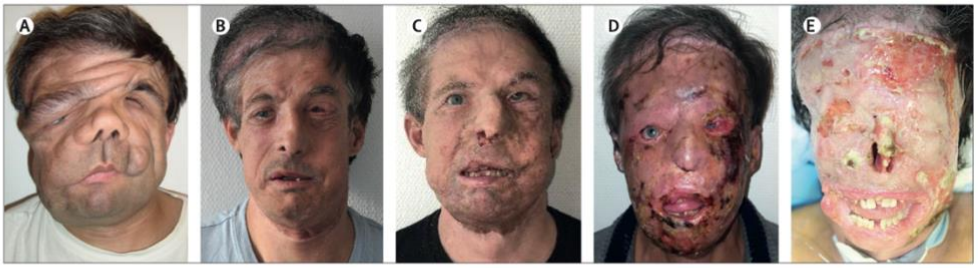


Figure 1: **Timecourse of the morphological changes in the patient's face**
Aspect of the patient's face before the first facial transplantation (A); 1 year after the first transplantation (B); 6 years after the first transplantation, showing evidence of advanced chronic antibody-mediated rejection (C); 7 years after the first transplantation, showing end-stage deterioration of the facial transplant (D); and after removal of the necrotic facial allograft and replacement by a split-thickness skin graft (E).

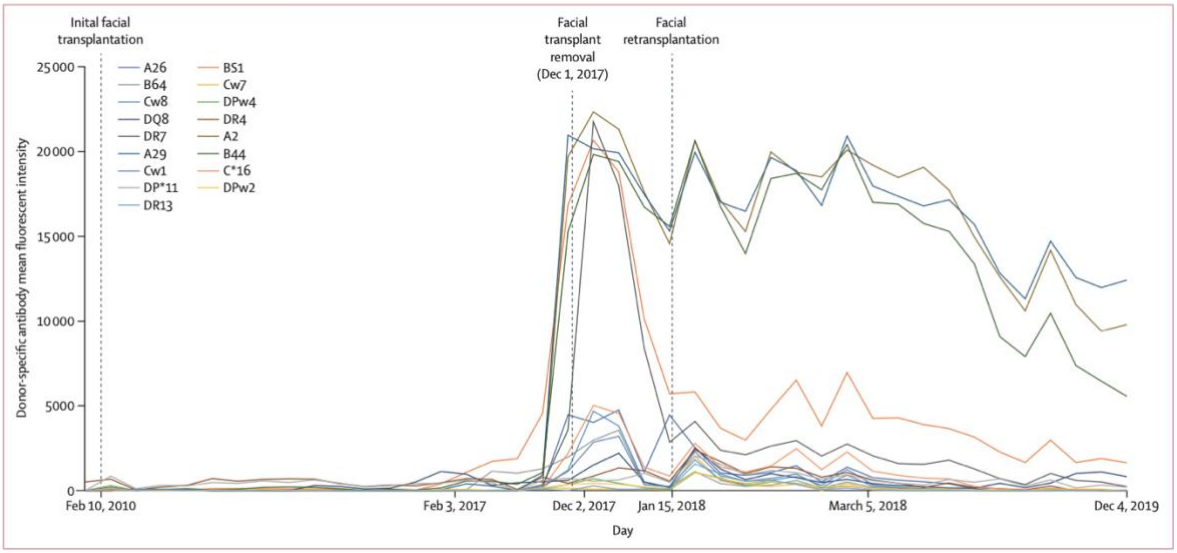


Figure 2: **Timecourse of donor-specific anti-HLA antibody plasma levels before and after facial retransplantation**

tested negative for cytomegalovirus, Epstein-Barr virus, and toxoplasmosis. However, there were eight full HLA mismatches between recipient and donor, and the pretransplant panel-reactive antibody levels were more than 80%, attesting to a high level of immunosensitisation. The crossmatch was positive, but we decided to proceed with the surgery because the patient could not remain any longer with this complex maxillofacial defect.

The donor and the recipient were located 390 km apart and two surgical teams worked simultaneously: one team prepared the recipient while the other team harvested the facial graft from the donor. On the recipient, we first removed the skin graft that had been placed as a temporary cover. The facial nerve was tagged at the stylomastoid foramen. The recipient's blood vessels (external carotid artery and internal jugular vein) on both sides were identified and prepared. All remaining scar tissue was excised down to the facial bones and removed. The facial allograft was harvested from the donor before the other transplantable organs. The face procurement was done using the same surgical model as for the initial face transplantation, using a pre-established checklist (appendix p 2). In brief, the facial nerve was approached via the transection of the external auditory meatus to reach the nerve trunk, followed by a coronal incision behind the ear, and

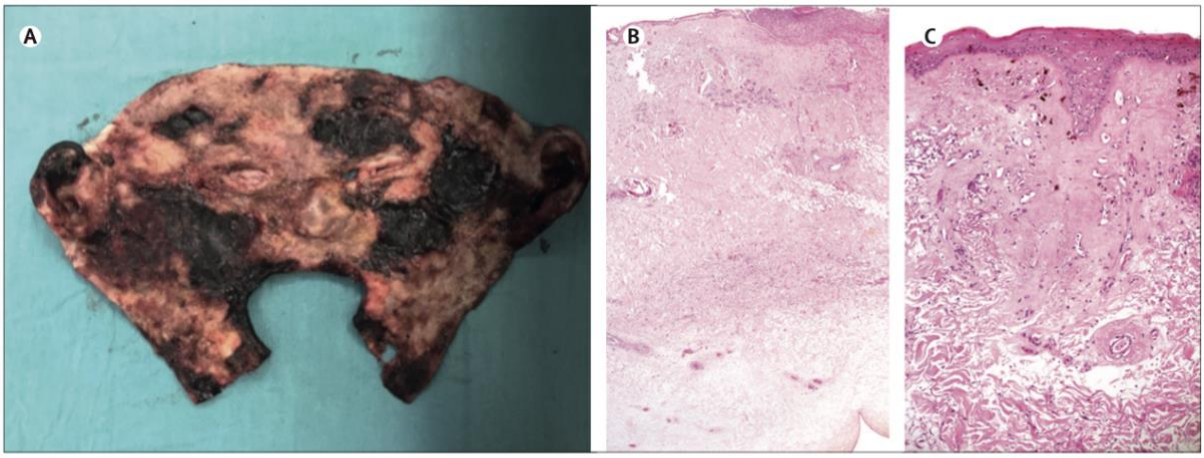


Figure 3: **Explanted facial allograft necrosis**
(A) Macroscopic, explanted facial allograft with areas of advanced necrosis. (B) Skin sample from the explanted facial allograft showing diffuse dermal fibrosis expanding into the hypodermis, replacing normal adipose tissue with some residual fat cells and atrophic skeletal muscle fibres (haematoxylin and eosin; magnification ×25). (C) Hyaline fibrosis involving the superficial dermis with numerous melanophages suggestive of postinflammatory pigmentation (haematoxylin and eosin; magnification ×100).

ending at the posterior neck, as reported previously.[14,15] The facial allograft was then raised along the masseter plane up to the oral mucosa. The graft included the skin; facial, mental, and infraorbital nerves; parotid glands; nose; and eyelids. Both external carotid arteries were isolated at their trunk. The veins included the external jugular vein and the thyrolingual-facial trunk, as well as the internal jugular vein on both sides. A sural nerve was also harvested from the donor and stored in preservation solution to allow grafting of the facial nerve if necessary. At the end of the procurement procedure, the donor's body was returned to the family with a dedicated tailored resin mask restoring the facial defect.[15]

To mitigate ischaemia-reperfusion injury to the graft, we used a new oxygen carrier (HEMO2life; Hemarina, Morlaix, France) as an additive to standard heparinised Institut Georges Lopez-1 storage solution,[16] after obtaining special permission from the national regulatory health authority.[17] The facial allograft was flushed through both external carotid arteries with this solution until blood- free, clear outflow was obtained. The graft was then placed in a conventional portable container at 4°C and transported to the recipient. We anastomosed the donor's face to the corresponding recipient vessels (external carotid artery and internal jugular vein) more proximally than for the previous facial transplantation. The first end-to-end anastomosis between the recipient's left external carotid artery (at its root) and the donor's left external carotid artery was able to perfuse the entire facial allograft. This was followed by an end-to-side anastomosis of the recipient's and donor's left internal jugular veins. The facial nerve was then anastomosed at the level of the nerve trunk. The same procedure was repeated on the right side (figure 4). The trigeminal nerves (V1, V2, and V3) were juxtaposed but not anastomosed, as previously reported.[18] There was massive intraoperative blood loss requiring multiple blood products; the recipient received 32 units of packed red blood cells, 30 units of fresh frozen plasma, and nine concentrated suspensions of blood platelets. Overall, the duration for recipient preparation was 9 h, for donor harvest was 6 h, and for retransplantation was 5 h. Cold ischaemia time was 5 h 55 min, and warm ischaemia time was 1 h 55 min.

Desensitisation regimen

The patient was highly immunosensitised as a result of his previous facial transplantation and multiple blood transfusions. The desensitisation protocol was initiated as soon as the patient's condition was stable (ie, no uncontrolled infection) and included five doses of intravenous antithymocyte globulins (75 mg once per day, on day 3, 4, 5, 6, and 7 after the retransplantation); 25 doses of intravenous methylprednisolone (on day 1 [250 mg], 3 [125 mg], 4 [125 mg], 7–24 [20 mg], and 25–28 [1 g]); four doses of intravenous anti-CD20 monoclonal antibodies (rituximab; on day –20 [600 mg], 7 [600 mg], 14 [500 mg], and 21 [500 mg]); four doses of intravenous anti-B-cell activating factor monoclonal antibodies (belimumab; on day 1 [520 mg], 8 [750 mg], 15 [750 mg], and 22 [800 mg]); 17 sessions of plasma exchange (on day –8, –4, –1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 16, 17, 19, and 20); three doses of intravenous immunoglobulins (150 mg/kg;19 on day 6, 23, and 24); and one dose of intravenous eculizumab (on day 6 [900 mg]; figure 5). This aggressive desensitisation was of paramount importance to reduce the risk of acute rejection of the allograft. A donor became available 2 weeks later and desensitisation was continued for another 2 weeks following the facial retransplantation.

Psychological preparation for retransplantation

After his first facial transplantation, the patient was followed-up by a psychiatrist (CL) with a systematic appointment at each visit to the Department of Plastic, Reconstructive,and AestheticSurgery, Hôpital Européen Georges Pompidou, Paris, France. Although the first facial transplantation had a positive impact on the patient's mental health,[12] the diagnosis of chronic rejection in 2015 resulted in the patient experiencing increased anxiety. At that time, he was encouraged to familiarise himself with the practice of mindfulness, to strengthen his ability to regulate his emotions in antici- pation of the challenges ahead. In September, 2017, he experienced recurrent panic attacks and had increased concerns about other people staring at him. He started referring to his facial transplant not as "my face" but as "the graft". He strengthened his practise of mindfulness by attending a group programme of mindfulness-based stress reduction supervised by a certified therapist. The possibility of introducing a selective serotonin reuptake inhibitor as prophylaxis against panic attacks was explained to the patient, as was the likelihood of expe- riencing visual hallucinations in the event of prolonged sensory deprivation (appendix pp 4–5).

Immunosuppressive regimen and infection prophylaxis

Induction of immunosuppression was achieved using intravenous antithymocyte globulins once per day for 5 days (figure 5). The immunosuppression maintenance regimen after retransplantation included tacrolimus, mycophenolate mofetil, and corticosteroids. We aimed to maintain tacrolimus plasma levels at 10–15 ng/mL and mycophenolate mofetil area under the curve at 40–60 ng/mL, and methylprednisolone was progressively tapered (250 mg on day 1, 125 mg on days 2 and 4, 20 mg per day on days 7–24, and then 10 mg per day to continue thereafter; on day 29 this was switched

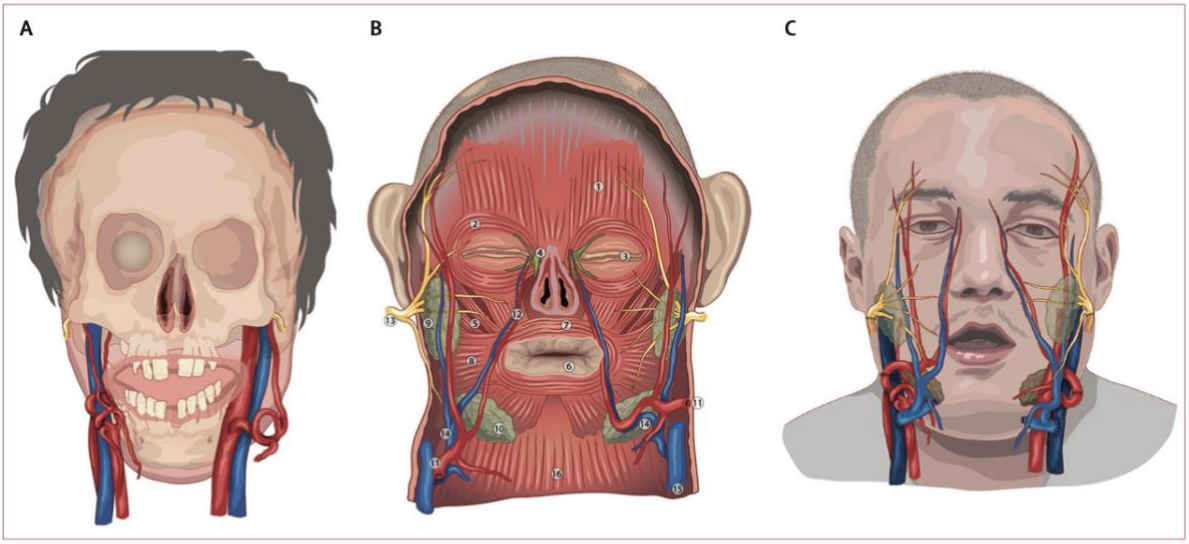


Figure 4: **Schematic drawings of the facial anatomy**
(A) Recipient's face and blood vessel anatomy before retransplantation. (B) Internal view of the harvested donor face; numbers indicate the main anatomical structures: 1=frontalis musculus. 2=musculus orbicularis oculi. 3=eyelid. 4=tear duct. 5=musculus zygomaticus minor and major. 6=buccal mucosa. 7=musculus orbicularis oris. 8=musculus risorius. 9=parotid gland. 10=submaxillary gland. 11=external carotid artery. 12=facial artery. 13=facial nerve. 14=common facial nerve. 15=internal jugular vein. 16=musculus platysma. (C) The main vascular and nervous anastomoses after retransplantation.

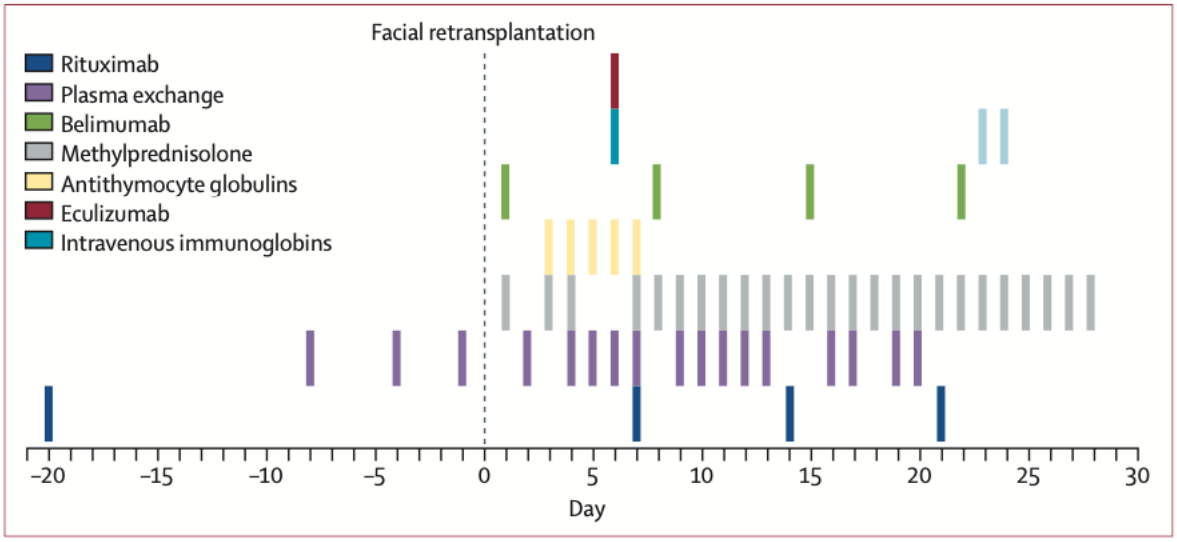


Figure 5: **Timeline of immunological management and immunosuppressive regimens used for the desensitisation protocol**
Doses were as follows: intravenous immunoglobulins (150 mg/kg); belimumab day 1 (520 mg), day 8 (750 mg), day 16 (750 mg), and day 22 (800 mg); rituximab day −20 (600 mg), day 7 (600 mg), day 14 (500 mg), and day 21 (500 mg); eculizumab (900 mg); methylprednisolone day 1 (250 mg), day 3 (125 mg), day 4 (125 mg), day 7–24 (20 mg), and day 25–28 (1 g); and antithymocyte globulins (75 mg).

to oral prednisolone). This triple immunosuppression therapy was never interrupted during the entire follow-up.

Despite cytomegalovirus matching, valganciclovir was administered prophylactically (900 mg per day for 6 months). The patient also received trimethoprimsulfamethoxazole (800 mg three times per week for 6 months) for prophylaxis against Pneumocystis jirovecii.

Skin rejection monitoring

Sequential biopsies of VCA skin and mucosa were performed on postoperative day 2 and were repeated once per week for the first 2 months, twice per month for the next 2 months, and then at month 6, 9, 10, 14, 21, and 23. Although skin biopsies were requested when there was a clinical suspicion of acute rejection, we mostly relied on systematic scheduled biopsies because the donor was a 22-year-old patient with severe facial acne lesions, which could be confused with early signs of graft rejection. The grade of rejection was assessed according to the 2007 international Banff classification.[20] Donor-specific-antibody plasma levels were monitored once per week for 2 months, and once per month thereafter.

Post-transplantation skin (right cervical area) and endobuccal mucosal biopsies on postoperative day 2 and 7 showed mild lymphocyte infiltration (Banff grade 1). Skin biopsies (from the right cervical area) performed on postoperative day 14 showed grade 2 or 3 rejection with dermal CD3-positive lymphocyte infiltrates and interface dermatitis characterised by basal vacuolisation and apoptotic keratinocytes. Similar alterations were observed on the mucosal biopsies (from the right internal cheek; appendix p 6). The development of skin erythema combined with grade 2 or 3 rejection on skin biopsies despite corticosteroid boli was attributed to antibody-mediated rejection and was treated using one intravenous dose of 900 mg eculizumab on postoperative day 6. As immunohistochemistry showed no evidence of C4d depositiononskinbiopsies ,nofurtheradministrationof eculizumab was required. Due to persistent grade 2 or 3 rejection, methylprednisone boli (1 g per day) were administered for 4 consecutive days (days 25–28). This management led to complete resolution of the erythema and subsequent skin biopsies did not show any sign of rejection (Banff grade 0 or 1). Some lymphocyte infiltrates persisted in the mucosa, but no clinical rejection episode has been observed since. The donor-specific antibodies directed against the initial donor decreased abruptly with the desensitisation protocol following retransplantation, and the donor-specific antibodies directed against the second donor decreased progressively during the first 6 months after retransplantation (figure 2).

Psychological aspects of retransplantation

During the 2 months before retransplantation, the patient developed transient symptoms of delirium, which required low doses of haloperidol (1 mg once to three times per day as required). These symptoms of delirium were likely multifactorial, involving drug side-effects and transient electrolyte imbalances. The first psychological interview (after removal of the first facial transplant) revealed severe anxiety linked to his loss of autonomy, disorientation episodes, uncertainty regarding his medical condition, and impaired ability to communicate following the loss of his first facial transplant. The patient also reported visual hallucinations that he spontaneously attributed to sensory deprivation after removal of the necrotic facial transplant. This hypothesis was supported because the visual hallu- cinations persisted for days after the other symptoms of delirium (eg, disorientation and disruption of attention) had resolved and the visual hallucinations were not associated with hallucinations in other sensory modalities or delusional adherence to their contents. Haloperidol was switched to olanzapine 5 mg once per day and sertraline was introduced at 25 mg once per day then at 50 mg once per

day to alleviate anxiety without increasing the risk of delirium relapses. At that time, the patient actively used mindfulness to maintain his emotional state.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Following facial retransplantation, the patient saw his new face for the first time on postoperative day 10. Just like after his first facial transplantation, this experience was characterised by an immediate sense of ownership (ie, the patient's feeling that the new face was his own), despite the absence of sensory feedback at this time. The patient's body image (ie, his perception of his appearance), which had been severely damaged during the progressive necrosis of the previous transplant, also immediately improved. His sense of identity remained unaffected. Subsequently, a team of psychiatrists and psychologists provided close psychological follow-up once per day. Although the patient experienced transient episodes of discouragement, he never met the criteria (according to the Diagnostic and Statistical Manual of Mental Disorders) for a major depressive episode and has not had issues with compliance thus far. He did experience several panic attacks requiring continued treatment with clorazepate 5 mg and increased sertra-line doses. A systematic screening for post-traumatic stress disorder 1 month after discharge from the ICU was negative. A dedicated clinical interview (with CL) at the same time did not find diagnostic criteria for

post-traumatic stress disorder. Discharge from the ICU to the plastic surgery ward was accompanied by a paradoxical increase in anxiety in the patient; however, his mental state gradually improved over the next 4 months with functional improvement and more frequent home leave (appendix pp 4–5).

During the postoperative period, the patient developed several infections. First, at 2 months after the retrans- plantation, he contracted a norovirus infection, which led to severe diarrhoea and resulted in massive weight loss (–15 kg). A cytomegalovirus oesophagitis resulted in functional achalasia and impaired oesophageal emptying. Two episodes of aspiration pneumonia complicated by brief hypoxic cardiac arrest requiring tracheal intubation occurred (at months 3 and 8), which were treated using systemic intravenous antibiotics (meropenem 5 g per day). Normal oral feeding could be resumed only 9 months after retransplantation.

The overall length of hospital stay for the episode of care surrounding retransplantation was 1 year. Before the facial retransplantation procedure, the patient had spent 3 months in the hospital, including 6 weeks in the ICU with no face and with very limited ability to communicate. Postoperatively, the patient remained in the ICU for 2 months and spent the next 6 months in the plastic surgery ward. He was able to resume walking on postoperative day 12 and started to talk and drink by day 30. The tracheostomy cannula

was removed on day 50. The timeline of postoperative events is depicted in the appendix (p 7).

The patient was eventually discharged home on Oct 18, 2018, and was followed-up once every 2 months via outpatient appointments. Benzodiazepines, and then sertraline, were gradually withdrawn between postoperative months 18 and 24 without any clinical relapse of anxiety. 2 years after the retransplantation surgery, the patient was considered to be in good mental health and was able to do his activities of daily living unassisted. From a functional perspective, the motor function of the facial nerve allows good eyelid closing and some labial motion on the left side, but there is a complete right-sided facial palsy. Face sensitivity is present on both sides. The facial retransplantation also reduced the patient's concerns about his appearance and restored his quality of life (figure 6). He was able to resume part-time work as a librarian. No other com- plications have been reported thus far. A 4-min video, showing the various phases of the patient's clinical course and of the surgical management, including interviews with the patient, which summarises this unique facial retransplantation and provides insight into the patient's own appreciation of the result.

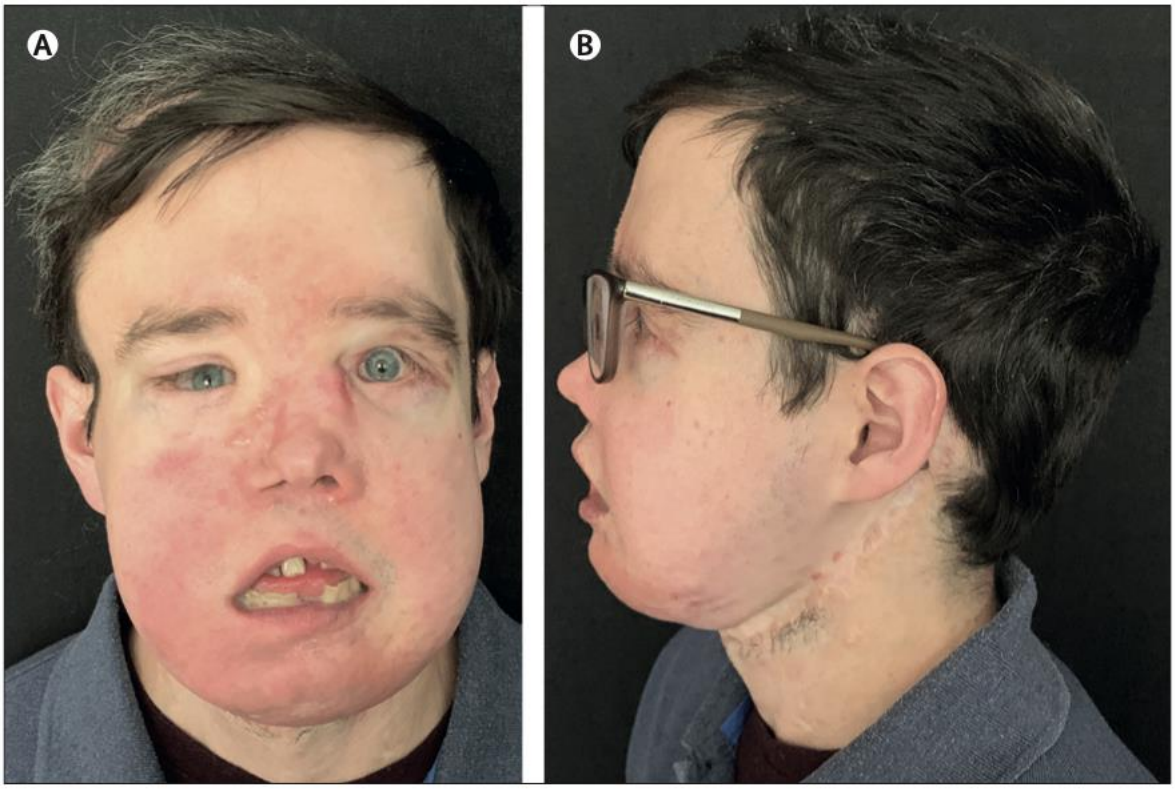


Figure 6: **The patient 30 months after facial retransplantation**

DISCUSSION

Since the first successful facial transplantation in 2005,[21] more than 40 of these operations have been reported worldwide.[22] Although initial successes have been published in the literature, long-term follow-up reports have been few. Little is known about the prevalence of CAMR and its consequences among VCA recipients.10 Although CAMR was the underlying cause of facial transplant deterioration in this patient, many

questions remain unanswered regarding the pathophysiology of this complication. Although the exact trigger of the rejection process is still largely hypothetical, several factors might have had a role. Transient insufficient plasma level of calcineurin inhibitors,[11] graft ischaemic-reperfusion injury, inflammation triggered by regional lipofilling,[23] intercurrent infectious diseases, or skin mechanical trauma[24] are all potential contributors to the CAMR observed in this patient. Early detection of this complication might be facilitated in the future by using innovative monitoring techniques which assess the graft pedicle. Possible underlying CAMR can be detected early by using high-resolution ultrasound to measure arterial intima or media thickening,[25] or MRI to measure graft flow reductions.[26] CAMR symptoms include progressive skin sclerosis and ultimately necrosis, leading to complete loss of the transplant within 12 months.[27] At this stage, the patient experiences a decrease in their quality of life and the allograft becomes non-functional, which defines end-stage transplant failure in VCA recipients.

During the 6-week period following explantation of this patient's first facial transplant, the immunosuppressive treatment was withdrawn because we estimated that its risks could exceed the benefits. However, this resulted in a rise in plasma levels of donor-specific antibodies from the first transplant in the period before retransplantation. We retrospectively hypothesised that continuing the immunosuppressive treatment could have mitigated the intensity of immunisation as measured by elevated donor-specific antibody plasma levels from the second facial transplant, although we do not know whether life-threatening septic complications might have been facilitated at a time when the patient had no proper cutaneous barrier. The desensitisation protocol that was initiated 20 days before retransplantation reduced donor-specific antibody plasma levels and no recurrent increase was observed. We cannot overemphasise the importance of meticulous planning and coordination of all technical, immunological, and psychological aspects of the management of this patient. Because this was a second facial transplantation, vascular and nervous anastomoses had to be done more proximally and the dissection for exposure of the recipient's neck blood vessels was technically more challenging. This technical difficulty was responsible in part for the massive intraoperative blood loss. Allograft ischaemia and its potential sequelae were a major concern in this case due to the geographical distance separating the donor and the recipient. The addition of the oxygen carrier HEMO2life in the preservation solution was used because it had been reported to reduce initial graft dysfunction in patients who underwent kidney transplantation.[28] In all our previous facial transplantation cases, the allografts showed some late revascularisation areas (video), but this phenomenon was not observed in this patient's case. Whether the oxygen carrier helped prevent ischaemia-reperfusion injury to the graft remains to be determined. This question should be addressed in future studies, as ischaemia-reperfusion injuries have been suggested to have a role in the development of CAMR.[29] Finally, preparing the patient psychologicallytolivewithoutafaceforanumberofweeks was of paramount importance to help him overcome this long and painful process. Preoperative and postoperative psychological assessments and management, including handling early psychiatric complications in the ICU (eg, delirium) and fostering long-term medical adherence through the prevention of depressive episodes certainly helped the patient accept his unprecedented situation. This psychological management might also have indirectly fostered long-term adherence to his immunosuppressive regimen, although longer-term follow-up would be required to fully evaluate the psychological consequences of this unique experience.

In conclusion, we have shown that retransplantation can be feasible for patients with irreversible necrosis and loss of their facial transplants due to CAMR. Although still exceptional for VCA, retransplantation appears to be a possible valid treatment option in selected suitable patients. This finding is especially important for patients with facial transplants in whom there is no alternative in the case of graft loss. We hope that this report of a possible rescue therapy in the case of graft failure will help patients who are in need of a facial transplant, and their caregivers, in their decision making regarding transplantation in the future.

REFERENCES

1. Dubernard JM, Owen E, Lefrançois N, et al. First human hand transplantation. Case report. Transpl Int 2000; 13 (suppl 1): S521–24.
2. Kantar RS, Ceradini DJ, Gelb BE, et al. Facial transplantation for an irreparable central and lower face injury: a modernized approach to a classic challenge. Plast Reconstr Surg 2019; 144: 264–83e.
3. Uluer MC, Brazio PS, Woodall JD, Nam AJ, Bartlett ST, Barth RN. Vascularized composite allotransplantation: medical complications. Curr Transplant Rep 2016; 3: 395–403.
4. Cooney CM, Siotos C, Aston JW, et al. The ethics of hand transplantation: a systematic review. J Hand Surg Am 2018; 43: 84.e1–15.
5. Weissenbacher A, Cendales L, Morelon E, et al. Meeting report of the 13th congress of the International Society of Vascularized Composite Allotransplantation. Transplantation 2018; 102: 1250–52.
6. Lellouch AG, Lantieri LA. A second chance at life. Camb Q Healthc Ethics 2019; 28: 463–67.
7. Shores JT, Brandacher G, Lee WPA. Hand and upper extremity transplantation: an update of outcomes in the worldwide experience. Plast Reconstr Surg 2015; 135: 351e–60e.
8. Kueckelhaus M, Fischer S, Lian CG, et al. Utility of sentinel flaps in assessing facial allograft rejection. Plast Reconstr Surg 2015; 135: 250–58.
9. Krezdorn N, Lian CG, Wells M, et al. Chronic rejection of human face allografts. Am J Transplant 2019; 19: 1168–77.
10. Morelon E, Petruzzo P, Kanitakis J, et al. Face transplantation: partial graft loss of the first case 10 years later. Am J Transplant 2017; 17: 1935–40.
11. Lantieri L, Grimbert P, Ortonne N, et al. Face transplant: long-term follow-up and results of a prospective open study. Lancet 2016; 388: 1398–407.
12. Lemogne C, Bellivier F, Fakra E, et al. Psychological and psychiatric aspects of face transplantation: lessons learned from the long-term follow-up of six patients. J Psychosom Res 2019; 119: 42–49.
13. Meningaud J-P, Hivelin M, Benjoar M-D, Toure G, Hermeziu O, Lantieri L. The procurement of allotransplants for ballistic trauma: a preclinical study and a report of two clinical cases. Plast Reconstr Surg 2011; 127: 1892–900.
14. Swanson JW, Yu JW, Taylor JA, Kovach S, Kanchwala S, Lantieri L. The retroauricular approach to the facial nerve trunk. J Craniofac Surg 2017; 28: 347–51.
15. Meningaud J-P, Benjoar M-D, Hivelin M, Hermeziu O, Toure G, Lantieri L. Procurement of total human face graft for allotransplantation: a preclinical study and the first clinical case. Plast Reconstr Surg 2010; 126: 1181–90.
16. Maathuis M-HJ, Ottens PJ, van Goor H, et al. Static cold storage preservation of ischemically damaged kidneys. A comparison between IGL-1 and UW solution. Transpl Int 2008; 21: 473–82.
17. Le Meur Y, Badet L, Essig M, et al. First-in-human use of a marine oxygen carrier (M101) for organ preservation: a safety and proof-of- principle study. Am J Transplant 2020; 20: 1729–38.
18. Lantieri L, Meningaud J-P, Grimbert P, et al. Repair of the lower and middle parts of the face by composite tissue allotransplantation in a patient with massive plexiform neurofibroma: a 1-year follow-up study. Lancet 2008; 372: 639–45.
19. Kumlien G, Ullström L, Losvall A, Persson L-G, Tydén G. Clinical experience with a new apheresis filter that specifically depletes ABO blood group antibodies. Transfusion 2006; 46: 1568–75.
20. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. Am J Transplant 2008; 8: 1396–400.
21. Devauchelle B, Badet L, Lengelé B, et al. First human face allograft: early report. Lancet 2006; 15: 203–09.
22. Khalifian S, Brazio PS, Mohan R, et al. Facial transplantation: the first 9 years. Lancet 2014; 384: 2153–63.
23. Brandacher G, Lee WPA, Schneeberger S. Minimizing immunosuppression in hand transplantation. Expert Rev Clin Immunol 2012; 8: 673–83, quiz 684.
24. Cetrulo CL Jr. Mechanical Trauma and the skin immune system in hand transplant rejection. Ann Surg 2020; 271: e115.
25. Kaufman CL, Ouseph R, Blair B, et al. Graft vasculopathy in clinical hand transplantation. Am J Transplant 2012; 12: 1004–16.
26. Bettoni J, Balédent O, Petruzzo P, et al. Role of flow magnetic resonance imaging in the monitoring of facial allotransplantations: preliminary results on graft vasculopathy. Int J Oral Maxillofac Surg 2020; 49: 169–75.
27. Morelon E, Petruzzo P, Kanitakis J. Chronic rejection in vascularized composite allotransplantation. Curr Opin Organ Transplant 2018; 23: 582–91.
28. Thuillier R, Delpy E, Matillon X, et al. Preventing acute kidney injury during transplantation: the application of novel oxygen carriers. Expert Opin Investig Drugs 2019; 28: 643–57.
29. Nankivell BJ, Chapman JR. Chronic allograft nephropathy: current concepts and future directions. Transplantation 2006; 81: 643–54.

CHAPTER 11

Facial Trauma 8 years after a Face Transplantation

Marion Goutard
Alexandre G. Lellouch
Bertrand Dussol
Laurent A. Lantieri

Adapted from Plast Reconstr Surg. Global Open Vol. 9, No. 5, May 2021 DOI: 10.1097/GOX.0000000000003575

SUMMARY

Over the past 20 years, vascularized composite allografts (VCAs) have emerged as a realistic option in reconstructive surgery. Long-term follow-up reports indicate that face transplant patients have gained in quality of life and social integration. However, they require close monitoring of their immunosuppressive therapy because they are at high-risk for acute rejection episodes, leading eventually to chronic rejection and allograft loss. Reported acute rejection episodes in VCA recipients occur due to low immunosuppressive therapy (mainly due to lack of patient compliance or decreased doses of immunosuppressants to counter side-effects). Repeated mechanical traumas have recently been shown to trigger acute rejection episodes, especially in hand transplant patients. This article reports our experience of a 10-year follow-up of a 57-year-old face transplant patient and the management of his accidental facial trauma. To our knowledge, our patient is the first to undergo a major trauma on his VCA endangering his graft function and vitality. This report discusses the management of an acute surgical situation in those particular patients, and the challenges that arise to avoid acute rejection of the allograft. Ten years into his face transplant and at 18 months follow-up after his facial trauma, our patient shows great aesthetic and functional outcomes and remains rejection-free; a very encouraging result for all VCA candidates.

ABSTRACT

Face transplantation is limited to severely disfigured patients whose disfigurement cannot be addressed by autologous surgery.[1] To this day, 44 face transplants have been performed worldwide to treat large facial defects due to burns, ballistic injuries, or deforming diseases such as neurofibromatosis.[2] Follow-up reports indicate a true benefit in terms of quality of life for patients who get a second face.[3] We report here an uncommon surgical situation in a face transplant patient—a 57-year-old man who presented with a bifocal mandibular fracture and a nasal bone fracture due to a domestic accident 8 years after his partial face transplantation. Fifteen years after the first face transplant ever performed, it remains crucial to report the outcomes in the mid- and long-term follow-up for each patient. Repeated traumas have recently been discovered to trigger a cell-mediated graft rejection.[4] However, no major trauma necessitating surgical treatment has been reported in VCA recipients, and the specific immunological challenges involved are yet to be determined.

CASE REPORT

The patient, Mr. S, is a 57-year-old man who suffered a ballistic trauma of the mid lower face in 2009 during a hunting accident.[2] He had a history of ischemic cardiopathy and hypercholesterolemia for which he was prescribed Kardegic and statin. He benefited from a mid-lower face allograft in April 2011 at Henri Mondor Hospital, France. During follow-up, there was no major complication. Mr. S underwent several corrective surgeries until 2014. Since then, he has shown no sign of chronic rejection and has physically and socially adjusted to his new face.

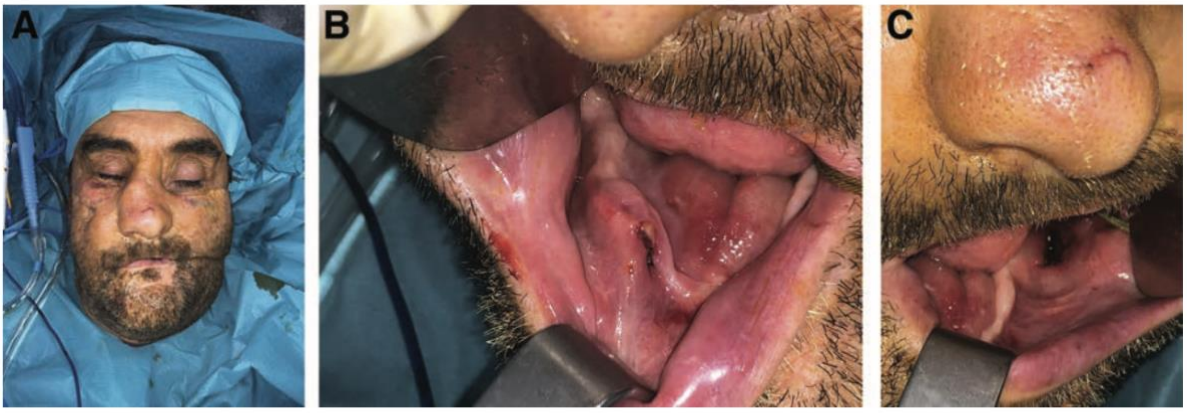


Fig. 1. Preoperative pictures. A, Facial deformities: bilateral palpebral ecchymosis, right deviation of nasal bones, and edema of the lower part of his face. B, Right parasymphyseal open wound. C, Left vestibular open wound.

Almost 8 years after his face transplant, Mr. S had a domestic accident falling down a staircase. On physical examination, he showed bilateral palpebral ecchymosis, a right deviation of his nasal bones, and edema of the lower part of his face. He had 2 open wounds in the inferior vestibules laying bare mandibular osteosynthesis material (Fig. 1). He had no other sign of associated trauma, and the rest of the examination was normal. A craniofacial CT was performed: it showed a bifocal fracture of the mandible and a nasal bone fracture (Fig. 2).

His immunosuppressive therapy included tacrolimus 2 mg twice daily, mycophenolate mofetil 750 mg twice daily, and corticosteroid 10 mg daily. Surgical treatment of his fractures was planned within a week after the trauma. Preoperative serum level of tacrolimus was 6.1ng/ml (N 5: 10 ng/ml). Whole blood count and kidney function were normal.

The mandible left horizontal branch fracture was treated with a left cervical approach on the existing cervical scar at the junction between his own skin and the face transplant and was internally fixed using a 1.5-mm-thick pure titanium plate 3+3 holes (DePuy Synthes, MatrixMandible); the parasymphyseal fracture was treated with a vestibular approach using a 1.5-mm-thick pure titanium plate 2+2 holes (DePuy Synthes, MatrixMandible). The nasal fracture was reduced with external maneuvers. Skin biopsies were taken pre and postoperatively.

Mr. S recovered quickly in the postoperative imme- diate follow-up. The skin biopsies showed no sign of rejection with minimal dermal lymphocyte infiltrate. His

immunosuppressive treatment was unchanged. Pain was controlled with a morphine pump until day 2, and he recovered his abilities to talk and eat solid food by that time. Postoperative craniofacial CT was performed at day 3 and showed a good reduction and osteosynthesis of both mandibular sites of fracture (Fig. 3). Mr. S was discharged from the hospital at postoperative day 6. After 18 months, Mr. S's facial function has returned to baseline. During this time, he maintained the immunosuppressive tri-therapy and showed no sign of rejection.

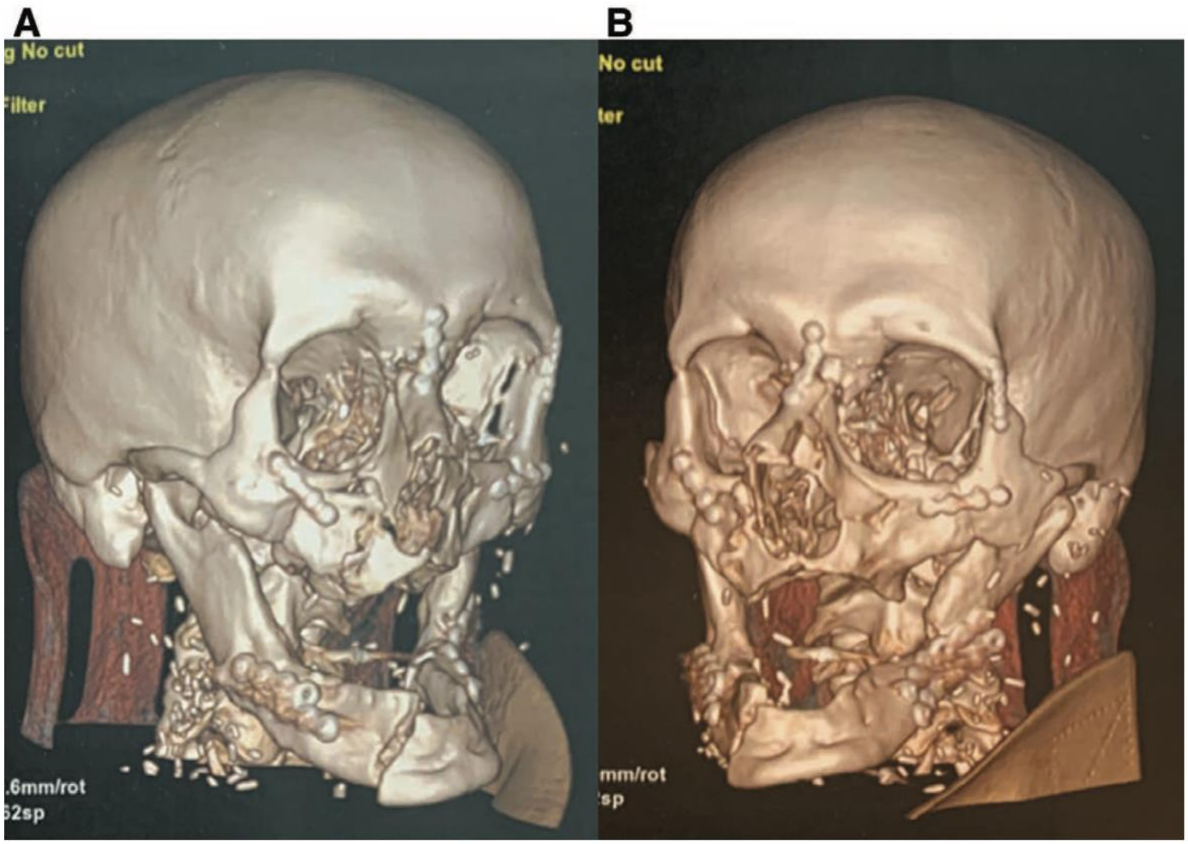


Fig. 2. 3D scanner reconstruction images showing the bifocal mandibular fracture with a right parasymphyseal fracture (A), a fracture on the left horizontal branch with torn osteosynthesis plates (B), and nasal bone fracture (A, B).

DISCUSSION

This case reports an unusual surgical situation with a face transplanted patient. To our knowledge, this is the first described case of severe facial trauma in a face transplant recipient. The biggest challenge in the postoperative care of transplanted patients is to avoid rejection and metabolic complications by aiming for an optimal balance in immunosuppressive medications. Another challenge has risen in the past 2 decades of the VCA field: management of postoperative trauma and its immediate and long-term consequences. Mechanical trauma has recently been reported as a cause of atypical VCA rejection in hand transplanted patients.[5] Repeated mechanical micro-traumas are thought to induce recipient cell infiltration to the allograft, and therefore increase the risk of donor antigen recognition.[6] No studies have yet reported this issue in face transplant recipients. Fortunately, although the risk was high, our patient did not suffer any rejection episode following surgical treatment of his fractures . Episodes of stress are well known to be responsible for graft endangerment through an immunological vascular

aggression to the graft.[7] We hypothesize that our patient remained rejection-free because he was not sensitized before his allograft and remained immunosuppression compliant through his entire follow-up period. The mandibular fracture on the left horizontal branch appeared to be facilitated by a fibrous bony union between the allograft and the recipient's bone. This suboptimal bone healing, described in another face transplant

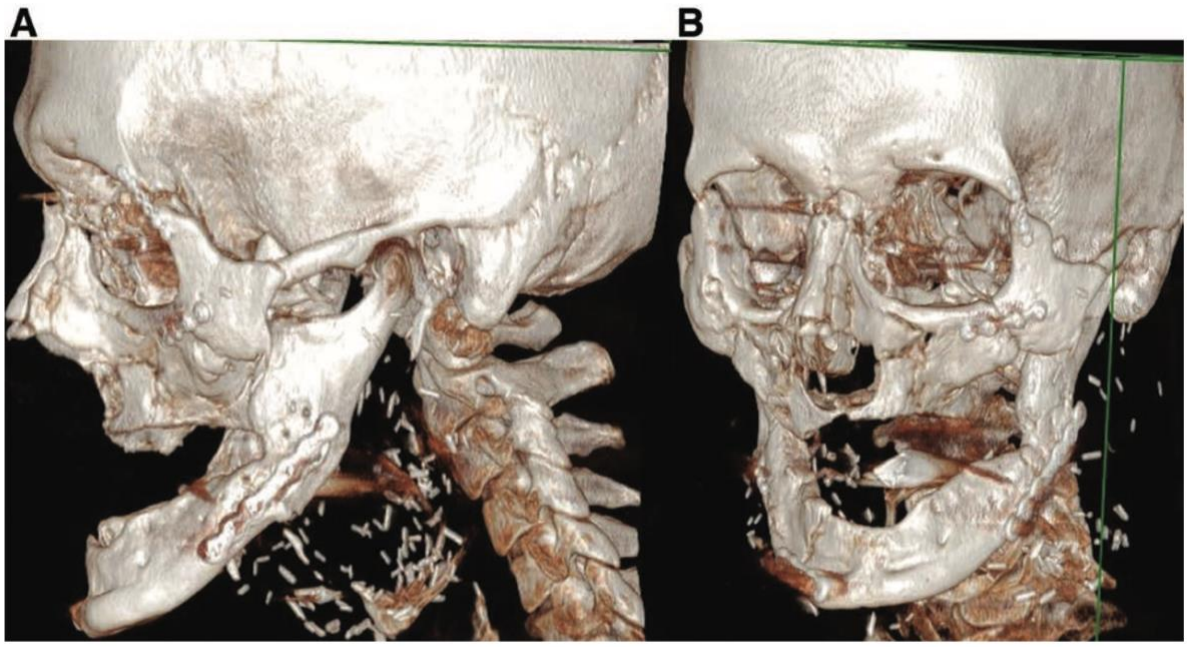


Fig. 3. T images at postoperative day 3 showing return to physiological anatomy of the bone fragments and stable osteosynthesis of both mandibular fracture sites. The mandible left horizontal branch fracture was fixed using a titanium plate (A); the parasymphyseal fracture was reduced and treated with a titanium plate (B). The nasal bones fracture was reduced (A, B).

patient, remains unexplained.[8] Whether it is due to a failure of skeletal integration, hypoperfusion of the bone, or linked to the use of osteopenic medications is still to be investigated. Long-term usage of steroids and calcineurin inhibitor is known to impact bone metabolism and increase the risk of fractures.[9] In our case, this is the first fracture the patient presented since the beginning of his treatment.

Steroid withdrawal has been reported in some patients during the long-term postoperative period without increasing the risk of rejection. This strategy, alongside bisphosphonates medication, could be discussed in patients presenting with pathological fractures.

Fifteen years after the first successful face transplant,[10] our overview on long-term management and follow-up of these particular patients has demonstrated many challenges in the first 2 postoperative years, balancing social acceptance and intensive motor and sensitive rehabilitation with ideal immunosuppression dosing. Our patient is now almost 10 years past his face transplantation surgery; he shows no major metabolic complication and is socially reinstated.

REFERENCES

1. Lantieri L, Hivelin M, Audard V, et al. Feasibility, reproducibility, risks and benefits of face transplantation: a prospective study of outcomes. Am J Transplant. 2011;11:367–378.
2. Lantieri L, Grimbert P, Ortonne N, et al. Face transplant: long- term follow-up and results of a prospective open study. Lancet. 2016;388:1398–1407.
3. Lellouch AG, Lantieri LA. A second chance at life. Camb Q Healthc Ethics. 2019;28:463–467.
4. Etra JW, Raimondi G, Brandacher G. Mechanisms of rejection in vascular composite allotransplantation. Curr Opin Organ Transplant. 2018;23:28–33.
5. Etra JW, Shores JT, Sander IB, et al. Trauma-induced rejection in vascularized composite allotransplantation. Ann Surg. 2020;271:e113–e114.
6. Cetrulo CL Jr. Mechanical trauma and the skin immune system in hand transplant rejection. Ann Surg. 2020;271:e115.
7. Ng ZY, Lellouch AG, Rosales IA, et al. Graft vasculopathy of vascularized composite allografts in humans: a literature review and retrospective study. Transpl Int. 2019;32:831–838.
8. Mohan R, Fisher M, Dorafshar A, et al. Principles of face transplant revision: beyond primary repair. Plast Reconstr Surg. 2014;134:1295–1304.
9. Löfdahl E, Rådegran G. Osteoporosis following heart transplantation and immunosuppressive therapy. Transplant Rev (Orlando). 2017;31:232–239.
10. Devauchelle B, Badet L, Lengelé B, et al. First human face allograft: early report. Lancet. 2006;368:203–209.

CHAPTER 12

Partial Heterotopic Hindlimb Transplantation Model in Rats

Marion Goutard
Mark A. Randolph
Corentin B. Taveau
Elise Lupon
Laurent A. Lantieri
Korkut Uygun
Curtis L. Cetrulo Jr.
Alexandre G. Lellouch

Adapted from* J. *Vis. Exp. 2021, e62586, doi:10.3791/62586 (2021).

ABSTRACT

Vascularized composite allotransplantations (VCA) represent the most advanced reconstruction option for patients without autologous surgical possibilities after a complex tissue defect. Face and hand transplantations have changed disfigured patients' lives, giving them a new aesthetic and functional social organ. Despite promising outcomes, VCA is still underperformed due to life-long immunosuppression comorbidities and infectious complications. The rat is an ideal animal model for in vivo studies investigating immunological pathways and graft rejection mechanisms. Rats are also widely used in novel composite tissue graft preservation techniques, including perfusion and cryopreservation studies. Models used for VCA in rats must be reproducible, reliable, and efficient with low postoperative morbidity and mortality. Heterotopic limb transplantation procedures fulfill these criteria and are easier to perform than orthotopic limb transplants. Mastering rodent microsurgical models requires solid experience in microsurgery and animal care. Herein is reported a reliable and reproducible model of partial heterotopic osteomyocutaneous flap transplantation in rats, the postoperative outcomes, and the means of prevention of potential complications.

INTRODUCTION

Over the past two decades, VCA has evolved as a revolutionary treatment for patients who suffer severe disfigurement including face[1] , upper limb amputations[2] , penile[3] , and other complex tissue defects[4 , 5] . However, the consequences of life-long immunosuppression still hinder a broader application of these complex reconstructive surgeries. Basic research is crucial to improve anti-rejection strategies. Increasing VCA preservation time is also essential to improve transplantation logistics and increase the donor pool (as VCA donors must fulfill more criteria than solid organ donors, including skin tone, anatomic size, gender). In this context, rat limb transplantations are widely used in studies on the immune rejection of allografts[6 , 7] , novel tolerance induction protocols8 , and preservation studies[9 , 10 , 11] . Hence, these VCA models are a key element to master for VCA translational research. Osteomyocutaneous flaps have been described in the literature as reliable models to study VCA in rats[8 , 12 , 13 , 14] . Although orthotopic whole-limb transplantations allow for long-term evaluation of graft function, it is a time-consuming procedure associated with higher postoperative morbidity and mortality rates[14] . In contrast, heterotopic limb transplantation models are non-functional, but enable reproducible studies on VCA. Postoperative outcomes can be reliably anticipated before the start of a rat VCA transplantation study. This study reports a partial heterotopic osteomyocutaneous flap transplantation model in the rat that includes frequent possible outcomes and complications that can arise intra-operatively and postoperatively during a follow-up period of three weeks.

PROTOCOL

All animals received humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC-protocol 2017N000184) and Animal Care and Use Review Office (ACURO) approved all animal protocols. Inbred male Lewis rats (250-400 g) were used for all experiments.

1. Surgery

1. Anesthetize the Lewis rats using isoflurane inhalation. Induce anesthesia with 5% isoflurane in the induction chamber, and maintain anesthesia with 1.5-3% isoflurane inhalation through a breathing cone.

2. Apply eye lubricant before surgery in survival procedures. Shave the surgical site, treat with depilatory cream, scrub, and drape with sterile drapes.

3. Confirm total anesthesia with a toe pinch test before incision and regularly during the procedure. Monitor heart and respiratory rates throughout the entire procedure. For all surgeries, maintain sterile conditions by using sterile instruments, supplies, drapes, and gloves. See the Table of Materials for the list of instruments used for the procedures.

2. Donor right partial hindlimb procurement

1. Make a circumferential incision of the skin above the ankle at the distal third of the leg.

2. Skeletonize and cauterize the saphenous artery and the terminal branch of the popliteal artery using bipolar forceps. Cauterize and cut off the gastrocnemius, soleus, tibialis anterior, and biceps femoris muscles until the tibial bone is exposed.

3. Make a 2.5 cm incision at the right inguinal crease. Dissect out the inguinal fat pad and retract it distally to expose the femoral vessels. Use a fishhook retractor to grasp the inguinal ligament and clamping forceps to hold the inguinal fat pad distally.

NOTE: The inguinal fat pad is included in the harvest of the partial limb.

4. Dissect the femoral vessels, individualize Murphy branches (deep muscular collateral branches usually located halfway between the inguinal ligament and the epigastric branch), and ligate with 8/0 nylon ties.

5. Heparinize the donor rat with 100 IU/kg heparin, injected in the penile dorsal vein using a 27.5 G needle.

6. Complete the skin incision around the hip.

7. Cauterize the biceps femoris and gluteus superficialis muscles using bipolar forceps. Cauterize and cut the sciatic nerve at mid femur length. Expose the femur proximally at the level of the posterior femoral crest. **NOTE:** Adductor and quadriceps muscles are left out of the procurement. The innominate pedicle is preserved.

8. Ligate femoral vessels with 8/0 nylon ties at the level of the inguinal ligament. Perform an arteriotomy on the femoral artery just below the ligature and dilate to allow for the insertion of a 24 G angio-catheter.

9. Cauterize and cut remaining muscle underneath the pedicle, exposing the anterior side of the femur.

10. Cutthetibiaandfemurusingabonecutterasproximally and distally as possible, respectively (mid-length).

11. Flush the partial hindlimb with 2 mL of heparin saline (100 IU/mL) to obtain a clear venous outflow. Store on ice in a sterile gauze until microvascular transfer (Figure 1).

12. While the animal is under general anesthesia, perform euthanasia by exsanguination until the animal shows no sign of life (no respiratory movement and no heartbeat).

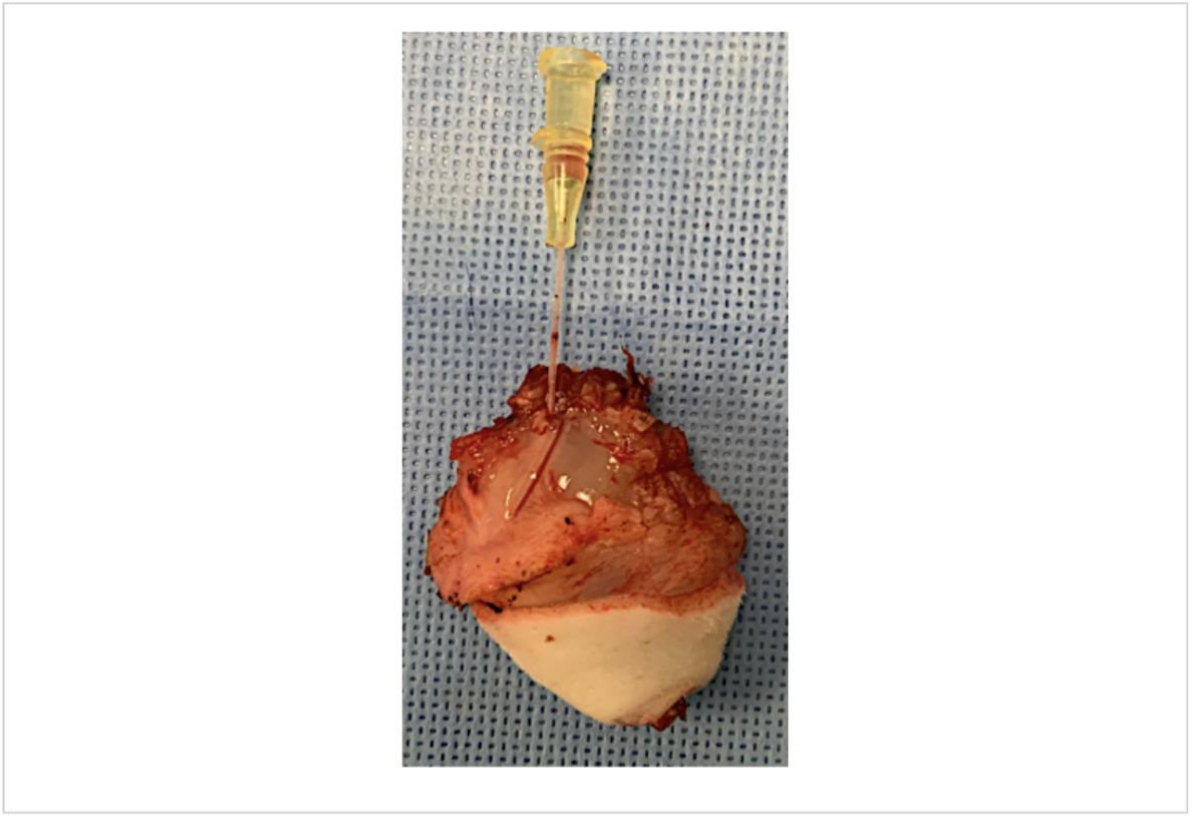


Figure 1: Rat partial hindlimb harvested. A 24 G angiocath is inserted in the femoral artery, ready for heterotopic microvascular transfer.

3. Recipient surgery

1. Before the incision, shave the back of the neck, and administer buprenorphine 0.01-0.05 mg/kg subcutaneously. Place the rat in a supine position on a heating pad.

2. Make a 2.5 cm incision in the right inguinal crease. Dissect the inguinal fat pad and recline it distally to expose the femoral vessels. Use a hook to retract the inguinal ligament and clamping forceps to hold the inguinal fat pad distally.

3. Dissect the femoral vessels, individualize the Murphy branches, and ligate with 8/0 nylon ties.

4. Ligate both vessels above the epigastric vessels using 8/0 nylon ties. Place approximator clamps proximally and dilate vessel ends; rinse with heparin saline.

5. Make an incision on the left flank above the hip, and create a subcutaneous pocket with a subcutaneous tunnel to the inguinal crease. NOTE: The inset incision is made above the range of motion of the hip to ensure that the animal maintains a normal hindlimb motion. Additionally, keeping a cutaneous bridge between the graft inset and the microvascular transfer site allows for better fixation of the graft (Figure 2).

6. Place the proximal part of the partial limb and the inguinal fat pad through the subcutaneous tunnel for microvascular transfer. Perform venous and arterial anastomoses using 10/0 nylon sutures. Remove both approximator clamps, and observe revascularization of the limb. Perform a "milking test" on both vessels to assess the patency of each anastomosis. **NOTE:** Eight to nine sutures are usually necessary for venous anastomosis, 6 sutures on average for arterial anastomosis.

7. Make a longitudinal skin incision on the medial side of the transplanted limb, and insert the graft. Remove excess skin of the graft, and close the wound with separate sutures and a running suture using absorbable 4/0 sutures.

8. Suture together the inguinal fat pads of the transplanted limb and the recipient using two separate absorbable sutures, and close the inguinal crease at the very end after a last checkup of the microvascular anastomoses. **NOTE:** Inguinal fat pads are sutured tightly to add a protective layer of fat above the anastomoses and ensure a secured position of the graft and its pedicle. A meticulous closure is better for wound healing; it also prevents residual bleeding from the wound and decreases the risk of self-mutilation.

9. Compensate fluid loss subcutaneously with 1-3 mL of saline according to the amount of perioperative bleeding.

10. Place an Elizabethan collar around the neck of the animal, and apply 2 loose sutures to the skin to maintain it in the correct position.

11. Stop isoflurane inhalation, and monitor the animal continuously on a warming pad until fully conscious and ambulatory.

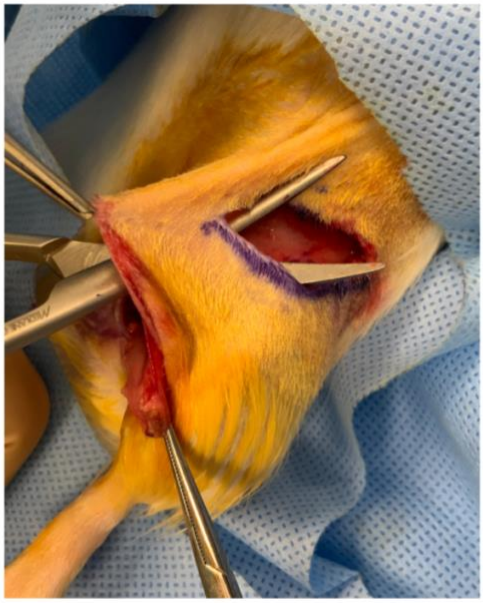


Figure 2: Perioperative image before inset of the osteomyocutaneous limb. A cutaneous bridge of approximately 1 cm is preserved between the inguinal crease incision and the inset of the graft above the hip. The graft is placed under the bridge, maintaining it steady for microvascular transfer.

4. Postoperative care

1. Monitor the animal twice daily for 72 h, then once daily 2. until postoperative day (POD) 7, and then twice per week. **NOTE:** Monitoring must be adjusted to the animal andgraft condition (pale eyes might require supplementary fluids, porphyrin staining as an indicator of animal pain, abnormal graft color/temperature), and further care should be discussed with the veterinarian. Single housing is required for the recipient rats during the entire study period to avoid any damage to the graft.

2. Perform analgesia with subcutaneous injection of buprenorphine and/or non-steroid anti-inflammatory drug according to IACUC guidelines.

3. Evaluate the graft, and perform physical examination daily with pictures using the same device.
NOTE: Using hair removal cream on the graft's skin is helpful to better assess the transplant skin color.

Representative Results

In this single-operator study, 30 syngeneic heterotopic partial limb transplants were performed. Success was defined at postoperative day 21 as the absence of VCA failure or complications requiring euthanasia. The normal evolution of the graft is represented in Figure 3. The mean duration for partial limb procurement and graft inset in the

recipient were 35 and 105 min, respectively; the mean ischemia time was 105 min. During follow-up, two types of complications occurred (Table 1)-early or late. Some required euthanasia, others were salvaged, all were discussed with staff veterinarians (Table 2). This study reports the authors' experience and advice for beginners in rodent microsurgery (Supplementary Table 1).

Figure 3: Normal evolution of the heterotopic hindlimb model until the end of the study. Hair regrowth is observed during the first postoperative week; cutaneous retraction appears after 2 weeks. Abbreviation: POD = postoperative day.

Management of perioperative complications

Arterial or venous thrombosis is the most common perioperative complication. The most important tip to overcome this and turn the surgery into a success is to make an early diagnosis, i.e., continuous monitoring of the flap color/bleeding and recurrent patency test are fundamental before closing the anastomoses surgical site. The prevention of this complication should be in the surgeon's mind as soon as an incision is made. The donor

should be heparinized with IV injection of heparin 100 IU/kg 5 min prior to graft arterial ischemia. Once harvested, the graft should be flushed with heparin saline (100 IU/mL) until venous outflow is clear. In this study, perioperative arterial thrombosis happened in 10% of cases.

Bleeding is a less frequent occurrence and is easily mitigated with a cautious cauterization of the graft muscles and a thorough dissection of the recipient site. The primary cause of bleeding is the anastomotic leak in the artery. This should resolve by itself within 3 min, or if not, re-clamping and revision of the leaking anastomosis are necessary. Anesthesia-related complications, which occurred in 6.7% of cases, are more frequent with untrained microsurgeons. Using isoflurane is a reliable way to anesthetize rats, and the length of anesthesia can be adjusted in real time. The surgeon must be trained to use the machine correctly, follow the guidelines for induction and maintenance regimen, and closely monitor the heart and respiratory rates throughout the operation. Depending on the species, age, or weight of the animal, the amount of isoflurane needed may vary.

Unpredictable animal loss is a rare event and is usually not explained by an anesthetic or surgical mistakes.

Early complications (<POD7)

VCA failure can occur during the first postoperative week due to microvascular thrombosis (venous thrombosis more frequent than arterial). Perioperative pedicle inset at the groin level is a very important step. Moving the rat's hindlimb to mimic the movement's effect on the pedicle is crucial; the pedicle should never be too loose nor too tight. Intensive monitoring is a fundamental requirement, as necropsy must

be performed as soon as a VCA failure diagnosis is made. During necropsy, an analysis of the position of the pedicle (kinking or tension) and quality of the thrombosed anastomosis (back wall suture, intraluminal flap) provides much information on what can be improved during the next procedure and thus, should be performed by the operating surgeon. Venous thrombosis was a cause of early euthanasia in 20% of cases, all of which occurred before POD5 (Figure 4).

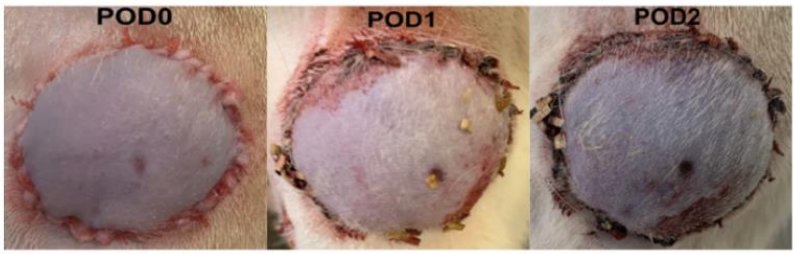


Figure 4: Postoperative venous thrombosis. The skin appears blue and becomes darker each day. Abbreviation: POD = postoperative day.

Self-mutilation (or autophagia) is a serious concern in non- sensate grafts; it often occurs between POD2 and POD7. If limited to less than a third of the graft surface and concerning only skin, surgical debridement and suture using non-absorbable sutures can be discussed with the staff

veterinarian (Figure 5A-C). Prevention relies on the use of an E-collar stitched to the neck until POD7 and the cleaning of any blood or crust on the animal's surgical wounds. Repeated autophagia or deep mutilation requires euthanasia (Figure 5D).

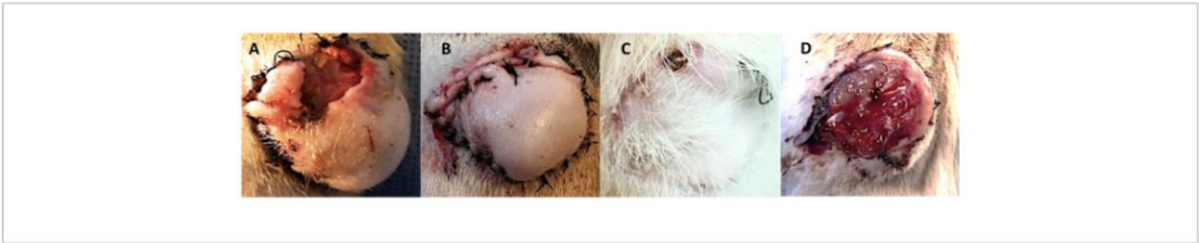


Figure 5: Postoperative self-mutilation of the non-sensate graft. (**A-C**) The limited surface of auto-mutilation at (**A**) POD2. (**B**) Surgically debrided and re-sutured; (**C**) aspect at POD21. (**D**) Severe autophagia of multiple layers of the graft leading to euthanasia of the animal. Abbreviation: POD = postoperative day.

Late complications (>POD7)

Less frequent and less lethal, these complications demand veterinarian consultation to provide adequate treatment. First, bone exposure can be observed in this model, usually after initial healing on the third week post-operation. Prevention is based on careful bone-cutting (use of bone cutter creating smooth edges); covering the bone edges with surrounding muscles while accounting for later muscle atrophy is helpful. If detected early and the animal is in good condition, surgical

revision can be discussed with the veterinarian. Second, dermal cysts can occur around the surgical site after two weeks (Figure 6). They usually do not interfere with the rat's or the graft's condition, but can fistulize to the skin and get infected. Washing the surgical site of the graft inset to avoid any residual hair in the wound prevents the creation of the cyst. The indication for surgical drainage can be evaluated with the veterinarian.

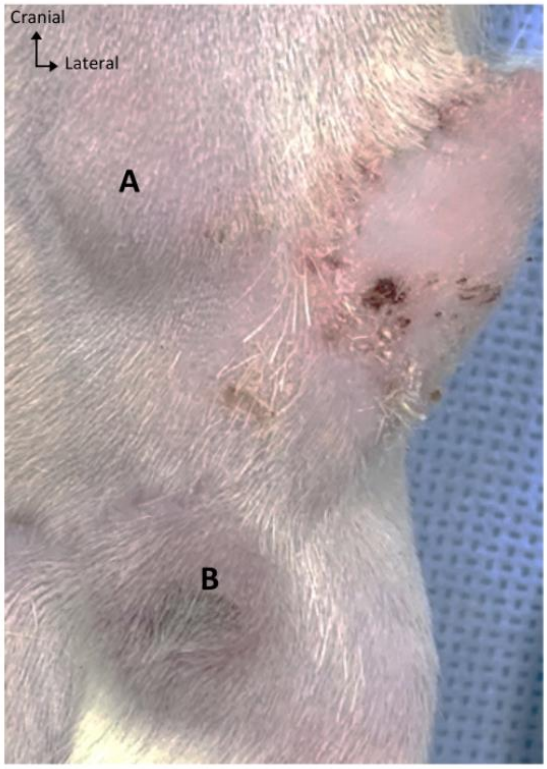


Figure 6: Dermal cysts. Dermal cysts appearing(**A**) after POD14, (**B**) sometimes with a cutaneous necrotic center prior to the fistula. Abbreviation: POD = postoperative day.

	Complication	Solution	Prevention
Peri-operative	Microvascular thrombosis	Re-do the anastomosis rapidly after rinsing the vessel ends with heparin saline and attesting good flow.	Heparinize the donor, flush the flap, master the anastomosis technique, and use adequate instruments and suture.
	Bleeding	Cauterize if from muscles, reclamp if from the anastomosis and not stopping spontaneously within 3 min.	Thorough cauterization during graft harvest
	Anesthesia-related death	Discuss the encountered problem with vet staff.	Training in use of anesthesia machine and perioperative rat monitoring
Early (<POD7)	Microvascular thrombosis	Early euthanasia and necropsy to reveal the cause of thrombosis	Depends on the cause
	Auto-mutilation	Discuss surgical repair with the vet if first event and superficial damage to the graft.	E-collar from POD-2 to POD7
Late (>POD7)	Bone exposure	Discuss surgical revision with the vet.	Cut the bone with bone cutter, make sure the edge is smooth, recover with surrounding muscles, account for later muscle atrophy (cut short).
	Dermal cyst	Discuss surgical drainage and antibiotic treatment with the vet	Wash the inset site with water; avoid leaving any hair in surgical site.

Table 1: Potential outcomes. Prevention and solutions.

End of Study

The end of the study in this model was set at POD21; animals were euthanized using CO2 asphyxiation or by exsanguination. As a non-functional graft, muscle atrophy and fatty degeneration were observed as consequences of the lack of reinnervation. Biopsies of the skin and muscle are saved for histological analysis. The inguinal crease is reopened for the evaluation of the vascular pedicle (placement, patency) if euthanasia is performed before the end of the study.

Five reasons for consulting the staff veterinarian
1) Before the start of the study to inform her/him of the nature of your study, planned analgesia, and follow-up strategies and expected outcomes
2) Special diet or supplementary nutrients that can help improve the animal condition during the study
3) Unexpected anesthesia-related death
4) Unexplained worsening of the animal condition
5) Surgical complication to assess salvaging possibilities or indications for euthanasia

Table 2: When to call the vet. Communication with the staff is essential for the conduct of an *in vivo* study.

DISCUSSION

Orthotopic limb transplantation models in rodents have been described in the literature[15,16,17]; however, they require a nerve repair, muscle reattachment, and a perfect osteosynthesis of the femur, which can be a very difficult step. These models are also associated with a higher morbidity and mortality rate in rodents[14], especially in the short-term follow-up as the recovery of a normal function of a transplanted hindlimb can take several months[18]. However, they allow for a longer-term assessment of graft function in case of success. The main limitation in heterotopic limb transplantation is the muscle atrophy induced by the lack of reinnervation of the graft. As previously published in the literature, muscle fiber injuries can occur as soon as
studies on tissue preservation[10], tissue bioengineering, and immunosuppression strategies[20].

In the proposed model, both surgical and postoperative complications are limited and can be easily and rapidly addressed by a microsurgical researcher. We estimate that success can be achieved in this model after 3-6 transplantations for a young surgeon, given that they have received basic microsurgery courses. It is also a procedure that can be performed by a single operator in less than 3 h with an ischemia time below 2 h. This model can also be performed by two operators, shortening the ischemia time to the time of microvascular anastomoses only. The critical step in this model is the graft inset and ensuring good placement of the pedicle to avoid any kinking or tension that would cause microvascular thrombosis. For this reason, dissection of the femoral vessels should proceed as proximally and distally as possible in the donor and the recipient, respectively. Cutaneous vascularization should be thoroughly preserved by keeping the skin wrapped around the limb until graft revascularization to avoid any shearing effect on the arterial skin perforators, which can cause low skin perfusion and subsequent skin necrosis.

In the literature, complications in rodent VCA models are not well-reported. The knowledge of every possible outcome is essential to be able to prevent and anticipate complications. This study stresses the need for good communication with the staff veterinarian. Acquiring skills in rat handling and physical examination is crucial for the well-being of the animal and the proper conduct of the study. Once this technique is mastered, this model can be used for VCA research with a success rate close to 100%. Although non-functional models are not suited for long-term evaluations, they are of great interest for early graft assessment in immunological research on full-mismatch VCA transplantation or evaluation of ischemia-reperfusion injuries. The described model

offers a large skin component and muscle volume that can be repeatedly sampled using punch biopsies, generating several histological and immunological assays as well as imaging evaluation techniques[13]. This precise protocol offers a reproducible and reliable VCA model in rats with reduced morbidity once the possible complications are foreseen and actively prevented.

REFERENCES

1. Lantieri, L. et al. Feasibility, reproducibility, risks and benefits of face transplantation: a prospective study of outcomes. American Journal of Transplantation. 11 (2), 367-378 (2011).
2. Park, S. H., Eun, S. C., Kwon, S. T. Hand transplantation: current status and immunologic obstacles. Experimental and Clinical Transplantation. 17 (1), 97-104 (2019).
3. Cetrulo, C. L., Jr. et al. Penis transplantation: first US experience. Annals of Surgery. 267 (5), 983-988 (2018).
4. Grajek, M. et al. First complex allotransplantation of neck organs: larynx, trachea, pharynx, esophagus, thyroid, parathyroid glands, and anterior cervical wall: a case report. Annals of Surgery. 266 (2), e19-e24 (2017).
5. Pribaz, J. J., Caterson, E. J. Evolution and limitations of conventional autologous reconstruction of the head and neck. Journal of Craniofacial Surgery. 24 (1), 99-107 (2013).
6. Lipson, R. A. et al. Vascularized limb transplantation in the rat. I. Results with syngeneic grafts. Transplantation. 35 (4), 293-299 (1983).
7. Lipson, R. A. et al. Vascularized limb transplantation in the rat. II. Results with allogeneic grafts. Transplantation. 35 (4), 300-304 (1983).
8. Adamson, L. A. et al. A modified model of hindlimb osteomyocutaneous flap for the study of tolerance to composite tissue allografts. Microsurgery. 27 (7), 630-636 (2007).
9. Arav, A., Friedman, O., Natan, Y., Gur, E., Shani, N. Rat hindlimb cryopreservation and transplantation: a step toward "organ banking". American Journal of Transplantation. 17 (11), 2820-2828 (2017).
10. Gok, E. et al. Development of an ex-situ limb perfusion system for a rodent model. ASAIO Journal. 65 (2), 167-172 (2019).
11. Gok,E.,Rojas-Pena,A.,Bartlett,R.H.,Ozer,K.Rodent skeletal muscle metabolomic changes associated with static cold storage. Transplantation Proceedings. 51 (3), 979-986 (2019).
12. Brandacher, G., Grahammer, J., Sucher, R., Lee, W. P. Animal models for basic and translational research in reconstructive transplantation. Birth Defects Research. Part C, Embryo Today. 96 (1), 39-50 (2012).
13. Fleissig, Y. et al. Modified heterotopic hindlimb osteomyocutaneous flap model in the rat for translational vascularized composite allotransplantation research. Journal of Visualized Experiments: JoVE. (146), 59458 (2019).
14. Ulusal, A. E., Ulusal, B. G., Hung, L. M., Wei, F. C. Heterotopic hindlimb allotransplantation in rats: an alternative model for immunological research in composite-tissue allotransplantation. Microsurgery. 25 (5), 410-414 (2005).
15. Kern, B. et al. A novel rodent orthotopic forelimb transplantation model that allows for reliable assessment of functional recovery resulting from nerve regeneration. American Journal of Transplantation. 17 (3), 622-634 (2017).
16. Perez-Abadia, G. et al. Low-dose immunosuppression in a rat hind-limb transplantation model. Transplant International. 16 (12), 835-842 (2003).
17. Sucher, R. et al. Orthotopic hind-limb transplantation in rats. Journal of Visualized Experiments. (41), 2022 (2010).
18. Fleissig, Y. Y., Beare, J. E., LeBlanc, A. J., Kaufman, C. L. Evolution of the rat hind limb transplant as an experimental model of vascularized composite allotransplantation: Approaches and advantages. SAGE Open Medicine. 8 2050312120968721 (2020).
19. Lindboe, C. F., Presthus, J. Effects of denervation, immobilization and cachexia on fibre size in the anterior tibial muscle of the rat. Acta Neuropathologica. 66 (1), 42-51 (1985).
20. Nazzal, J. A., Johnson, T. S., Gordon, C. R., Randolph, M. A., Lee, W. P. Heterotopic limb allotransplantation model to study skin rejection in the rat. Microsurgery. 24 (6), 448-453 (2004).

CHAPTER 13

Optimization of Ex Vivo Machine Perfusion and Transplantation of Vascularized Composite Allografts

Laura C. Burlage
Alexandre G. Lellouch
Corentin B. Taveau
Philipp Tratnig-Frankl
Casie A. Pendexter
Mark A. Randolph
Robert J. Porte
Laurent A. Lantieri
Shannon N. Tessier
Curtis L. Cetrulo Jr.
Korkut Uygun

Adapted from in press Journal of Surgical Research

ABSTRACT

Background: Machine perfusion is gaining interest as an efficient method of tissue preservation of Vascularized Composite Allografts (VCA). The aim of this study was to develop a protocol for ex vivo subnormothermic oxygenated machine perfusion (SNMP) on rodent hindlimbs and to validate our protocol in a heterotopic hindlimb transplant model.

Methods: In this optimization study we compared three different solutions during 6 hours of SNMP (n=4 per group). Ten control limbs were stored in a preservation solution on Static Cold Storage [SCS]). During SNMP we monitored arterial flowrate, lactate levels and edema. After SNMP, muscle biopsies were taken for histology examination and energy charge analysis. We validated the best perfusion protocol in a heterotopic limb transplantation model with 30-day follow up (n=13). As controls, we transplanted untreated limbs (n=5) and hindlimbs preserved with either 6 or 24 hours of SCS (n=4 and n=5).

Results: During SNMP, arterial outflow increased, and lactate clearance decreased in all groups. Total edema was significantly lower in the HBOC-201 group compared to the BSA group (p=0.005), 4.9 (4.3-6.1) vs. 48.8 (39.1-53.2) percentage, but not to the BSA + PEG group (p=0.19). Energy charge levels of SCS controls decreased 4-fold compared to limbs perfused with acellular oxygen carrier HBOC-201, 0.10 (0.07-0.17) vs. 0.46 (0.42-0.49) respectively (p=0.002).

Conclusion: Six hours ex vivo SNMP of rodent hindlimbs using an acellular oxygen carrier HBOC-201 results in superior tissue preservation compared to conventional SCS.

INTRODUCTION

Vascularized composite allotransplantation (VCA) remains the most advanced treatment option to restore motor function and aesthetics in patients living with devastating disfigurements. To date, worldwide more than 200 patients have benefited from VCA, the majority receiving hand/upper extremity or face transplants [1, 2]. Since in all fields of transplantation, graft viability prior to transplantation is inextricably linked to post-transplant success, minimization of graft injury prior to transplantation is also key to improve outcomes in VCA [3].

The current gold standard of graft preservation is based on cooling the graft in a cold preservation solution (4° C) on ice, referred to as static cold storage (SCS). The significant drop in temperature lowers the metabolic rate of the tissue, which enable the graft to temporarily cope with the absence of oxygen and nutrients. Muscle cells (the dominant tissue type as per quantity in most VCA grafts) are, however, highly metabolically active which allows only for an extremely limited ischemia time; irreversible cell damage already occurs after as little as 4 hours of ischemia [4]. Moreover, upon reperfusion, the sudden abundance of oxygen will aggravate cell damage even more, by initiating reactive oxygen species (ROS) formation and intracellular calcium influx leading to mitochondrial dysfunction and eventually cell death. Apoptotic and necrotic muscle cells ultimately trigger the immune system, affecting both early and long-term graft function [5–7].

Ex vivo machine perfusion is gaining increasing attention as an alternative method of VCA graft preservation. During ex vivo machine perfusion, the oxygenated perfusate allows maintenance of aerobic metabolism, thereby limiting tissue damage and allowing for quality assessment and possibly viability improvement. Other groups have reported favorable results of both hypothermic and normothermic machine perfusion of VCA grafts compared to SCS [8–10]. Oxygenated subnormothermic machine perfusion (SNMP) is a perfusion modality performed at room temperature (21°C) and aims to enable energy metabolism while keeping tissue metabolic demands easy to fulfill. SNMP of both rat and human livers prior to transplantation has been shown to improve the quality of the liver by reducing ischemia-induced damage [11–13].

In this study, we develop a protocol for 6 hours of SNMP on VCA grafts. First, we compare three media options in terms of perfusion characteristics as well as energy status after perfusion. In all cases we use a muscle culture media as the base, which has not been considered previously in VCA perfusion literature. Differentiating components in the three perfusion solutions are i) polyethylene glycol (PEG) and ii) acellular oxygen carrier HBOC-201 (Hemopure®, HbO2, Therapeutics LLC) with prostaglandin. PEG is a water-soluble nontoxic polymer with multiple beneficial effects. Addition of large PEG molecules in vivo is associated protective effects against I/R injury in both rat hearts and livers [14, 15]. The protective effects were associated with decreased vascular permeability, decreased oxidative stress, and inhibition of cell death [16]. HBOC-201 is a hemoglobin-based oxygen carrier polymer (250 kDa) that has the capacity to unload oxygen in peripheral tissues at sub-physiological temperatures [17]. Finally, for the perfusion protocol with best ex vivo results, we test in a heterotopic hindlimb transplant model.

MATERIALS & METHODS

Animals & Housing. For perfusion optimization, twelve Lewis rats (250-300g) were used as hindlimb donors and muscle biopsies of another 3 rats were used to set reference values (Charles Rivers Laboratories, Wilmington, MA, USA). For transplant studies, thirty-seven male Lewis rats (250-300g) were used as donors and thirty-seven male Lewis rats (300-350g) were used as recipients. Male rats easily facilitate systemic heparinization via the penile vein during the limb procurement, as detailed in the next section. Animals were housed and maintained in accordance with the National Research Council guidelines and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital (Boston, MA, USA).

Limb Procurement

A heterotopic model as previously described by Ulusal and colleagues was used [18]. Our protocol for limb procurement can be found in the supplementary data section.

Perfusion Solutions

In first part of this study, 3 different perfusion solutions were tested ex vivo for 6 hours of subnormothermic machine perfusion (SNMP) on rodent partial hindlimbs. A detailed overview of all perfusion solutions is summarized in Table 1. In all groups, skeletal muscle media with basic epidermal and fibroblast growth factors (PromoCell, C-23160, Heidelberg, Germany) provided the base of the solution. Bovine serum albumin (BSA) was the base colloid component in all groups. Also, additional supplements such as insulin, heparin, dexamethasone, hydrocortisone and antibiotics were same between groups. The main differences between these perfusion solutions were based on the presence or absence of these 2 components:

1. Addition of polyethylene glycol (PEG) with a molecular weight of 35 kDa.
2. Addition of an acellular oxygen carrier, HBOC-201 (Hemopure®, HbO2, Therapeutics LLC) in combination with vasodilator prostaglandin

The total volume of the perfusion solution was 500 mL in all groups.

Machine Perfusion

For 6 hours of SNMP, we used a custom-made machine perfusion system as displayed in Figure 1. Key components for our system were a rotating pump (07522-20 DRIVE MFLEX L/S 600RPM 115/230, Cole-Parmer, Vernon Hills, IL), tubing (Masterflex platinum-cured silicone tubing, L/S 16, Cole-Parmer, Vernon Hills, IL) and a membrane oxygenator, bubble trap chamber and tissue bath (catalog numbers 130144, 130149 and 158400 respectively, Radnoti LTD, Dublin, Ireland). Vascular pressure was measured via a pressure transducer (PT-F, Living Systems Instrumentation, St Albans City, VT) and read by a portable pressure monitor (PM-P-1, Catamount Research and Development, St Albans, VT). Prior to connecting the limb, pressures of the system without the limb were noted at different flow rates (Pressurewithout). During perfusion, pressures with the limb were observed (Pressurewith) and flows were adjusted accordingly to aim for a vascular pressure between 30-40 mmHg. The vascular pressure was calculated as Pressurewith - Pressurewithout. Vascular resistance was calculated as by dividing the vascular pressure by the flow rate.

During 6 hours of perfusion, perfusion samples were collected from both the arterial inflow and venous outflow. An i-STAT analyzer (Albott, Princeton, NJ) was used to measure perfusate levels of potassium and lactate as well as oxygen tension and saturation. At the end of 6 hours of SNMP, biopsies form the rectus femoris muscle were taken. Biopsies were snap-frozen in liquid nitrogen and stored in a -80 ºC freezer for mass spectrometry or stored in formalin for histological analysis.

Transplantation

In total, 13 right partial hindlimbs were transplanted were transplanted after 6 hours of SNMP (HBOC-201 group). Transplant controls included hindlimbs that were preserved for 6 hours of SCS (n=4), 24 hours of SCS (n=5) or hindlimbs that were transplanted directly after harvest referred to as fresh controls (n=5). Heterotopic hindlimb transplantation was performed as described previously by Ulusal and colleagues [18]. Post-operative follow-up was 30 days in all study groups. Viability of the graft was assessed by physical examination: temperature (cold or body temperature), color (pale or blue) and turgor (swelling).

Perfusate Injury Markers

Lactate clearance (μmol/min) was calculated by the difference between the arterial and venous lactate concentration (mmol/L) and corrected for flow (mL/min). Potassium release (μmol/min) was calculated as differences in concentration (mmol/L) between the arterial inflow and venous outflow and corrected for flow (mL/min).

Oxygen Consumption

Total oxygen consumption was calculated by the difference between the arterial and venous oxygen content and corrected for flow. The following formula was used for calculations:

Total oxygen consumption (μL O2/min) = cO2 * (pO2art-ven * flow) + (Hb + cHb + (SO2art-ven * flow)), where cO2 is the oxygen solubility coefficient (3.14 * 10−5 mLO2/mmHg O2/mL), pO2art-ven is the difference in partial oxygen pressure between in artery inflow and venous outflow (mmHg), flow is the arterial inflow (mL/min), Hb is the hemoglobin concentration (g/mL) and cHb is the oxygen binding capacity of Hb (1.26 for HBOC-201).

Energy Charge Analysis

All muscle biopsies were analyzed with liquid chromatography-mass spectrometry for energy cofactors adenosine triphosphate [ATP], adenosine diphosphate [ADP], adenosine monophosphate [AMP], which are previously demonstrated as indicators of viability for liver perfusion and transplant [19].

All frozen tissue biopsies were pulverized, weighted (averaging ~25 mg) and analyzed for energy cofactors using a liquid chromatography-mass spectrometry system (AB Sciex, Foster City, CA), as previously described [19].

Histology Analysis of Muscle Biopsies

Muscle biopsies were fixated in formalin, paraffin embedded, and cross-sectioned. Slides were stained with hematoxylin and eosin (H&E) and apoptosis marker TUNEL by the pathology department.

Statistical Analysis

Continuous data are reported as medians with interquartile range, categorial variables as absolute numbers. Differences between groups were analyzed using a Kruskal-Wallis H test with a Dunn's post-test or Mann-Whitney test when applicable. All statistical analysis was performed using Prism 5.0a for Mac OSX (GraphPad Software, La Jolla, CA). P values less than 0.05 were considered to be significant.

RESULTS

Procurement

Average procurement time was 20 minutes (+/- 5 min) with an average warm ischemia time until machine perfusion of 10-15 minutes.

Optimization of Perfusion Solution

Hemodynamic Parameters

Arterial flow increased in all groups during the first half of perfusion and remained stable thereafter (Figure 2A). After 1 hour of SNMP, median flows were significantly higher in the BSA group compared to the HBOC-201 group (p=0.01), 1.4 (1-2.1) vs. 0.4 (0.2-0.4) mL/min respectively. Median flows continued to be higher in the BSA group compared to the HBOC-201 (p=0.27), but not the BSA + PEG (p=0.43), group until the end of 6 hours perfusion.

Vascular resistance decreased in all groups during the first hour of perfusion and remained stable thereafter (Figure 2B). After 1 hour of SNMP, median vascular resistance was significantly higher in the HBOC-201 compared to the BSA (p=0.01), but not in the BSA + PEG group (p=0.10).

Perfusate Injury Markers

In all groups, lactate clearance increased within the first hour of perfusion and declined thereafter, as presented in Figure 2C. During 6 hours of perfusion, there was no statistical difference in lactate clearance between the groups. It should be noted that the HBOC-201 perfusion fluid had a median lactate concentration of 2.9 mmol/L (2.9-3.0) prior to perfusion while the BSA and BSA + PEG had unmeasurable concentration of lactate prior to perfusion. This is due to the presence of sodium lactate (27 mmol/L) in the HBOC-201 solution as described in the product sheet by the manufacturer (Hemopure, HbO2, Therapeutics LLC). During the 3 hours of SNMP, potassium concentration increased in all groups but levels stabilized thereafter, as presented in Figure 2D. After 1 hour of SNMP, median potassium release was significantly higher in the HBOC-201 group compared to the BSA group (p=0.006), 5.8 (4.3-9.0) vs. 1.8 (1.1-2.1), but not compared to the BSA + PEG group, 5.8 (4.3-9.0) vs. 4.4 (4.2-5.2) (p=0.27). While potassium levels normalized in all groups and after 6 hours of SNMP, median potassium release did not significantly differ between groups (p=0.55).

Weight Gain due to Edema

Limbs were weighed prior to and after 6 hours of SNMP. Median start weight of all limbs prior to perfusion was 19 (17-21) grams and did not differ between groups (p=0.11). Median weight gain (as a percentage of baseline) was significantly lower in the HBOC-201 group compared to the BSA alone group (p=0.005), median increase of 4.9 (4.3-6.1) vs. 48.8 (39.1-53.2), but not compared to the BSA + PEG group (p=0.19), median increase of 27.3 (20.5-41.6) respectively (Figure 2E).

Total Oxygen Consumption

After 2 hours of SNMP, total oxygen consumption was significantly higher in the HBOC-201 group compared to the BSA + PEG group (p=0.03), 55.8 (27.7-63.0) vs. 17.5 (14.3-23.0) μL/min, but not to BSA alone (p=0.87), 55.8 (27.7-63.0) vs. 33.9 (24.0-36.6) μL/min respectively. While oxygen consumption stabilized after the first 2 hours in the BSA and BSA + PEG groups, oxygen consumption in the HBOC-201 continued to increase during the first 4 hours of SNMP before it stabilized for the remainder of SNMP (Figure 2F). After 4 hours of SNMP, total oxygen consumption was significantly higher in the HBOC-201 group compared to both the BSA group (p=0.05), 86.1 (68.1-93.3) vs. 29.4 (26.5-33.8), and the BSA + PEG group (p=0.03), 86.1 (68.1-93.3) vs. 23.7 (16.3-26.6). At the end of 6 hours SNMP, total oxygen consumption continued to be significantly higher in the HBOC-201 group compared to theBSA + PEG group (p=0.03), 74.0 (55.8-87.8) vs. 22.0 (8.6-31.5), but not compared to the BSA alone group (p=0.13), 74.0 (55.8-87.8) vs. 31.9 (21.5-40.0) respectively.

Energy Charge

Energy charge values are displayed in Figure 3. At the end of 6 hours of perfusion, median energy charge levels were comparable between the BSA, BSA + PEG and HBOC-201 groups, 0.25 (0.15-0.47) vs. 0.33 (0.23-0.42) vs. 0.46 (0.42-0.49) (p=0.20) respectively. Interestingly, all energy charge levels of all groups were comparable to the energy charge ratio of in vivo controls (median ratio 0.37 (0.19-0.58)), as indicated by the red dotted line in Figure 3. However, energy charge ratios of SCS control limbs were significantly lower compared to HBOC-201 perfused limbs , 0.10 (0.07-0.17) vs. 0.46 (0.42-0.49) (p=0.002), but not to BSA (p=0.15) and BSA + PEG (p=0.08) limbs.

Perfused Limb Histology Assessment

None of the muscle biopsies showed myocyte injury or degeneration after perfusion. Furthermore, none of the muscle biopsies showed apoptotic cell death. Biopsies of BSA perfused limbs showed, however, more signs of interstitial edema compared to HBOC-201 perfused limbs (Figure 4).

Transplant Survival

Grafts were transplanted and followed for 30 days post-operatively (Figure 5). Mortality rates due to graft failure were 20% in the fresh control group (i.e. untreated control grafts), 15% in the HBOC-201 group and 25% in the SCS group (Figure 6). In the negative control group (24 hours SCS) all animals died as a consequence of graft failure (100%). Automutilation was a significant factor and counted for 50% of the failed experiments in the fresh control group, 20% of the failed experiments in the 24 hours SCS group and 23% of the failed experiments in the HBOC-201 group. Survival was therefore plotted in two separate graphs: first displaying all transplant results (Figure 6A) latter where automutilation is censored (Figure 6B).

Overall survival of the animals that received a graft that was perfused for 6 hours with the HBOC-201 was slightly higher compared to the fresh control group; 80% vs. 75% excluding automutilation (Figure 6B) and 62% versus 60% including automutilation (Figure 6A). Animals that received a graft that was preserved with 6 hours of SCS had a survival of 50% after 30 days while none of the negative controls that received a graft that was preserved using 24 hours of SCS completed the follow up (0% survival) (both Figure 6A and 6B).

DISCUSSION

Rapid decay of graft viability using SCS forms a logistic barrier for expanding the use of VCA transplantation, which has unique matching requirements compared to solid organs. Further, the severe time limit hinders new developments in the field of VCA transplant, in particular because the time is not sufficient for tolerance protocols currently in trials [3]. Here we report the development of a protocol to successfully preserve VCA grafts for up to 6 hours ex vivo using SNMP. Our key findings are 1) that 6 hour ex vivo SNMP is able to maintain energy charge levels comparable to in vivo controls, while energy charge levels dropped significantly in grafts preserved with SCS; 2) addition of HBOC-201 to the preservation solution significantly decreases edema and increases peak oxygen extraction; and 3) transplantation of grafts perfused after 6 hours of SNMP are successful.

In solid organ transplantation, energy charge levels prior to transplantation significantly correlate with post-operative outcome [19]. It is also well known that during cold ischemia, cellular energy levels rapidly decline [20, 21]. In the field of VCA, cold ischemia has been frequently described as a contributor to immune activation, rejection and inhibition of tolerance [22]. Previous studies have reported histopathological changes of both muscle cells and nerves during cold ischemia [23]. To our knowledge this is the first to report to energy charge levels during cold ischemic preservation and machine perfusion on VCA grafts.

Edema is frequently observed upon revascularization of a VCA graft in vivo [24]. During VCA transplantation, important graft edema may reflect an obstructed venous outflow, inadequate lymphatic drainage, or allograft rejection [25, 26]. During ex vivo perfusion, graft edema is, however, more likely to be caused by the diffusion of perfusion solution components into the interstitial space (i.e. rationale of colloids in static cold perfusion solutions) [27]. Such interstitial expansion may result in inadequate tissue perfusion and even cell death, due to compression of delicate, thin-walled capillaries [27]. Other groups have reported reduced edema during ex vivo perfusion of porcine limbs, upon addition of the colloid dextrose to their modified phosphate buffered saline solution [28].

In this study, weight gain due to edema was significantly lower in the PEG and HBOC-201 group, compared to the BSA alone group. In previous studies, PEG has shown to reduce endothelial leakage and HBOC-201 is a large molecule not likely to extravasate [16]. The addition of PEG and HBOC-201 might thus increase viscosity of the solution, thereby compromising vascular flow rates as observed in this study, yet still lead to better tissue perfusion as reflected by increased peak oxygen extraction. Thus, in our opinion high flow vascular flow rates alone do not indicate adequate tissue perfusion.

Potassium and lactate levels are well-known 'real time' parameters of tissue damage during ex vivo organ prefusion. In this study, median potassium release was significantly higher in the BSA group compared to BSA + PEG and HBOC-201 perfused limbs. High potassium release can be an early sign of tissue necrosis and are common signs of hypoxia observed during extra corporal perfusion [29–31]. Blood lactate levels are a balance between lactate production and elimination. Lactate production is a result of anaerobic glucose metabolism, most commonly caused by tissue hypoxia and hypoperfusion. Lactate can be produced by most cells but is mainly produced by tissues with a high metabolic rate such as muscles [32]. In vivo, the majority of the lactate is cleared from the blood by the liver (~80%) and to a lesser degree by the kidneys and muscles [32]. In this study we calculated the lactate clearance of an isolated, inactive limb. Studies have shown that inactive muscles actively participate in lactate clearance although it is highly depended on blood flow, arterial lactate concentration and muscle metabolism [33, 34]. Our

study showed that in all groups, lactate clearance increased during the first hours of perfusion and decreased thereafter.

As part of this study we show that limbs that are perfused for 6 hours with the HBOC-201 protocol can successfully be transplanted. Thirty days post-transplant survival rates in the HBOC-201 group were at least comparable and even slightly higher compared to survival rates of animals that received a limb that was preserved with SCS for an equal amount of time (80% vs 75% survival). None of the negative controls survived past day 18 (survival rate of 0%). Ulusal et al. reported a mortality rate for orthotopic hindlimb transplantation of 26.7% which is comparable to our findings. Regardless, this rat hindlimb transplant model remains in need of further improvements or alternatives in order to minimize the loss of animals especially due to automutilation. Future studies might include hind limb splints or head cones to prevent the animals form biting the graft.

Ozer et al. reported promising results of ex vivo preservation of porcine limbs using near normothermic machine perfusion with heparinized autologous blood [30]. Only recently, the first ex vivo perfusion of a human limb was reported also using near normothermic machine perfusion with a plasma-based perfusate with packed red blood cells (with an average hemoglobin concentration of 4-6 g/dL) [9]. While the use of acellular hemoglobin-based oxygen carriers such as HBOC-201 are gaining increasing attention as an alternative for red blood cells in ex vivo machine perfusion of solid organs [17, 35–37], only limited reports have been published about the use of HBOCs in VCA machine perfusion so far [37, 38].

The study has several limitations. In the HBOC-201 protocol, except from the addition of HBOC-201 we also added prostaglandin to the protocol. Addition of prostaglandin was necessary to overcome high vascular resistance, which was an immediate issue when we used HBOC-201 alone in initial testing. Testing the effect of prostaglandin alone on ex vivo SNMP of VCA grafts could be considered in the protocols we tested, here as well as others in literature. Due to the heterotopic model used and since we did not aim to study graft rejection, and we chose a follow-up duration of 30 days to be sufficient. Our results indicate that shorter periods could be considered sufficient, which may also reduce automutilation artifacts. Moreover, in this experimental set up we used male rats only since systemic heparinization during limb harvest was easily facilitated via the penile vein. While this might be an issue is small animals, we do not expect that our results of one sex only would potentially limitate future VCA research as we expect to upsize the experimental subjects (i.e. swin and eventually human) in which vena puncture is not an issue.

CONCLUSION

This study demonstrates that 6 hours ex vivo SNMP of rodent hindlimbs is feasible using an acellular oxygen carrier, and results in superior tissue preservation compared with conventional cold preservation methods. Moreover, heterotopic transplantation of hindlimbs preserved with the HBOC-201 ex vivo SNMP protocol presented is feasible and shows promising results. Future studies may incorporate machine perfusion as part of a longer protocol to extend the preservation time even more or consider it in combination with mixed chimerism tolerance induction protocols.

HIGHLIGHTS

- Six hours of ex vivo subnormothermic machine perfusion (SNMP) of rodent vascularized composite allografts (VCA) is feasible using acellular oxygen carrier HBOC-201
- SNMP results in superior tissue preservation compared to conventional static cold storage (SCS) preservation
- Successful transplantation of VCA grafts preserved with 6 hours of SNMP is feasible and 30 day survival rates are comparable to SCS preserved graft

REFERENCES

1. Mendenhall SD, Ginnetti MT, Sawyer JD, et al. Prevalence and Distribution of Potential Vascularized Composite Allotransplant Donors, Implications for Optimizing the Donor-recipient Match. Plast Reconstr Surg Glob Open 2018;6:e1833–e1833.
2. Lantieri L, Cholley B, Lemogne C, et al. First human facial retransplantation: 30-month follow-up. Lancet 2020;396:1758–1765.
3. Burlage LC, Tessier SN, Etra JW, Uygun K, Brandacher G. Advances in machine perfusion, organ preservation, and cryobiology: potential impact on vascularized composite allotransplantation. Curr Opin Organ Transplant 2018;23:561–567.
4. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10:620–630.
5. Landin L, Cavadas PC, Garcia-Cosmes P, Thione A, Vera-Sempere F. Perioperative ischemic injury and fibrotic degeneration of muscle in a forearm allograft: functional follow-up at 32 months post transplantation. Ann Plast Surg 2011;66:202–209.
6. Murata S, Miniati DN, Kown MH, et al. Superoxide dismutase mimetic m40401 reduces ischemia-reperfusion injury and graft coronary artery disease in rodent cardiac allografts. Transplantation 2004;78:1166–1171.
7. Panizo A, Pardo FJ, Lozano MD, de Alava E, Sola I, Idoate MA. Ischemic injury in posttransplant endomyocardial biopsies: immunohistochemical study of fibronectin. Transplant Proc 1999;31:2550–2551.
8. Ozer K, Rojas-Pena A, Mendias CL, Bryner BS, Toomasian C, Bartlett RH. The Effect of Ex Situ Perfusion in a Swine Limb Vascularized Composite Tissue Allograft on Survival up to 24 Hours. J Hand Surg Am 2016;41:3–12.
9. Werner NL, Alghanem F, Rakestraw SL, et al. Ex Situ Perfusion of Human Limb Allografts for 24 Hours. Transplantation 2017;101:e68–e74.
10. Kueckelhaus M, Fischer S, Sisk G, et al. A Mobile Extracorporeal Extremity Salvage System for Replantation and Transplantation. Ann Plast Surg 2016;76:355–360.
11. Berendsen TA, Bruinsma BG, Lee J, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res 2012;1:6.
12. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant 2014;14:1400–1409.
13. Bruinsma BG, Avruch JH, Weeder PD, et al. Functional Human Liver Preservation and Recovery by Means of Subnormothermic Machine Perfusion. J Vis Exp 2015.
14. Xu X, Philip JL, Razzaque MA, Lloyd JW, Muller CM, Akhter SA. High-molecular-weight polyethylene glycol inhibits myocardial ischemia-reperfusion injury in vivo. J Thorac Cardiovasc Surg 2015;149:588–593.
15. Bejaoui M, Pantazi E, Folch-Puy E, et al. Protective Effect of Intravenous High Molecular Weight Polyethylene Glycol on Fatty Liver Preservation. Biomed Res Int 2015;2015:794287.
16. Bejaoui M, Pantazi E, Calvo M, et al. Polyethylene Glycol Preconditioning: An Effective Strategy to Prevent Liver Ischemia Reperfusion Injury. Oxid Med Cell Longev 2016;2016.
17. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. Liver Transpl 2018;24:528–538.
18. Ulusal AE, Ulusal BG, Hung L-M, Wei FC. Heterotopic hindlimb allotransplantation in rats: an alternative model for immunological research in composite-tissue allotransplantation. Microsurgery 2005;25:410–414.
19. Bruinsma BG, Avruch JH, Sridharan GV, et al. Peritransplant energy changes and their correlation to outcome after human liver transplantation. Transplantation 2017;101:1637–1644.
20. Vajdová K, Graf R, Clavien P-A. ATP-supplies in the cold-preserved liver: A long-neglected factor of organ viability. Hepatology 2002;36:1543–1552.
21. Berendsen TA, Izamis ML, Xu H, et al. Hepatocyte viability and adenosine triphosphate content decrease linearly over time during conventional cold storage of rat liver grafts. Transplantation proceedings 2011;43:1484–1488.
22. Kueckelhaus M, Fischer S, Seyda M, et al. Vascularized composite allotransplantation: current standards and novel approaches to prevent acute rejection and chronic allograft deterioration. Transpl Int 2016;29:655–662.
23. Hautz T, Hickethier T, Blumer MJF, et al. Histomorphometric evaluation of ischemia-reperfusion injury and the effect of preservation solutions histidine-tryptophan-ketoglutarate and University of Wisconsin in limb transplantation. Transplantation 2014;98:713–720.
24. Azari K, Imbriglia J, Goitz R, et al. Technical Aspects of the Recipient Operation in Hand Transplantation. Journal of Reconstructive Microsurgery 2012;28:27–34.
25. Kaufman CL, Marvin MR, Chilton PM, et al. Immunobiology in VCA. Transplant International 2016;29:644–654.
26. Mundinger GS, Narushima M, Hui-Chou HG, et al. Infrared fluorescence imaging of lymphatic regeneration in nonhuman primate facial vascularized composite allografts. Ann Plast Surg 2012;68:314–319.

27. Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation 1988;45:673–676.
28. Troutman EC. Hypothermic Machine Perfusion of Composite Tissues. :62.
29. Fichter AM, Ritschl LM, Borgmann A, et al. Development of an Extracorporeal Perfusion Device for Small Animal Free Flaps. PLoS One 2016;11:e0147755.
30. Muller S, Constantinescu MA, Kiermeir DM, et al. Ischemia/reperfusion injury of porcine limbs after extracorporeal perfusion. J Surg Res 2013;181:170–182.
31. Constantinescu MA, Knall E, Xu X, et al. Preservation of amputated extremities by extracorporeal blood perfusion; a feasibility study in a porcine model. J Surg Res 2011;171:291–299.
32. Consoli A, Nurjhan N, Reilly JJJ, Bier DM, Gerich JE. Contribution of liver and skeletal muscle to alanine and lactate metabolism in humans. Am J Physiol 1990;259:E677-684.
33. Ahlborg G, Wahren J, Felig P. Splanchnic and peripheral glucose and lactate metabolism during and after prolonged arm exercise. J Clin Invest 1986;77:690–699.
34. Bangsbo J, Aagaard T, Olsen M, Kiens B, Turcotte LP, Richter EA. Lactate and H+ uptake in inactive muscles during intense exercise in man. J Physiol 1995;488 (Pt 1):219–229.
35. Fontes P, Lopez R, van der Plaats A, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant 2015;15:381–394.
36. Laing RW, Bhogal RH, Wallace L, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation 2017;101:2746–2756.
37. de Vries Y, van Leeuwen OB, Matton APM, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. Transplant International 2018;31:1283-1284.
38. Said SA, Ordenana CX, Rezaei M, et al. Ex-Vivo Normothermic Limb Perfusion With a Hemoglobin-Based Oxygen Carrier Perfusate. Mil Med 2020;185:110–120.

TABLE 1

Table 1. Overview machine perfusion solutions

	Group 1 BSA n=4	Group 2 BSA + PEG n=4	Group 3 HBOC-201 n=4
Solution base			
- PromoCell muscle media (mL)	500	500	375
- HBOC-201 (mL)	-	-	125
Differentiating additives			
- Bovine serum albumin (BSA) (g)	10	10	10
- Polyethylene glycol (PEG) (g)	-	15	15
- Prostaglandin[1] (μL/min)	-	-	0.2
Additional supplements			
- Penicillin-Streptomycin (mL)	2	2	2
- L-glutamine (mL)	5	5	5
- Insulin (μL)	100	100	100
- Heparin (mL)	1	1	1
- Hydrocortisone (μL)	100	100	100
- Dexamethasone (μg)	8	8	8

Overview of the different perfusion solutions. Abbreviations used; BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin based oxygen carrier-201. 1Prostaglandin is Alprostadil 500mcg/mL vial is diluted in 50mL of saline according to manufacturing instructions. This mixture was added to the solution via a syringe pump at a flow rate of 0.2 μL/min.

FIGURE 1

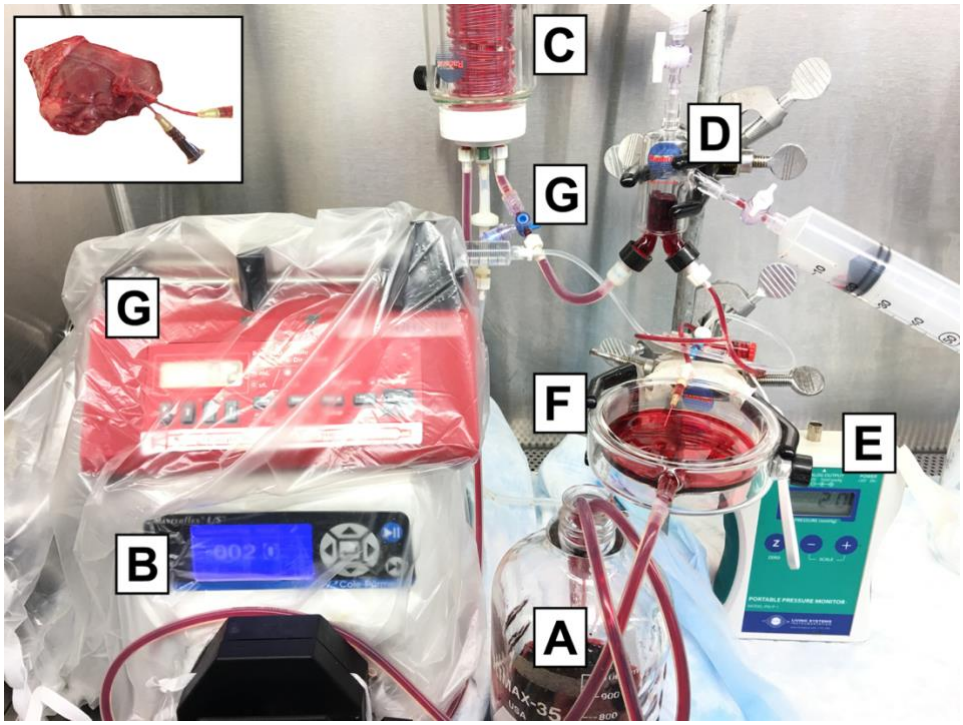


Figure 1. Ex vivo subnormothermic machine perfusion set up with HBOC-201 perfusion solution. The circuit consists of perfusion solution (A) that is pumped via a roller pump (B) to the oxygenator (C), that is oxygenated with a carbogen mixture (5%

CO2 and 95% oxygen). The solution then goes through the bubble trap (D) to prevent air bubbles going into the limb. The pressure is measured (E) at the level of the limb that is laying the basin (F). Inflow samples are measure at the inflow valve (G) with outflow samples are measured directly from the venous outflow canula (as shown in upper left panel).

FIGURE 2

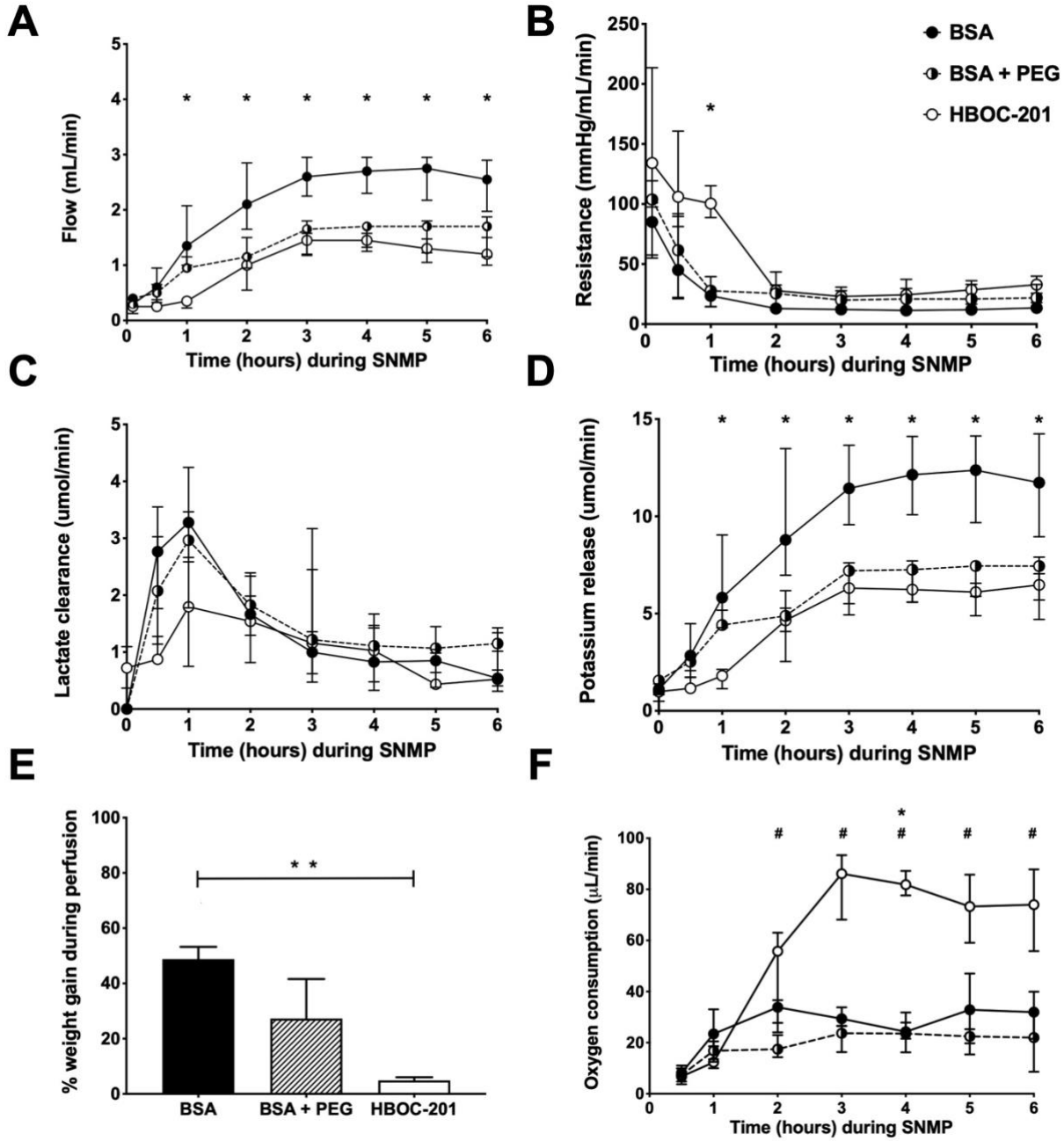


Figure 2. Overview perfusion parameters. In all groups, arterial flow increased while vascular resistance decreased over the course of perfusion (Panel A&B). Lactate levels peaked during the first hour of perfusion and decreased thereafter (Panel C). After 2 hours of SNMP, potassium levels were significantly higher in the BSA group compared to HBOC-201 group, but not compared to the BSA + PEG group (Panel D). Weight gain was calculated as the difference compared to baseline and was significantly higher in the BSA group compared to HBOC-201 group (p=0.005) (Panel E). After 2 hours of SNMP, oxygen

consumption was significantly higher in the HBOC-201 group compared to the BSA (Panel F). Abbreviations used: Abbreviations used; BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin-based oxygen carrier-201. Asterix (*) indicates significance between HBOC-201 and BSA, hashtag (#) indicates significance between HCOC-201 and BSA + PEG.

FIGURE 3

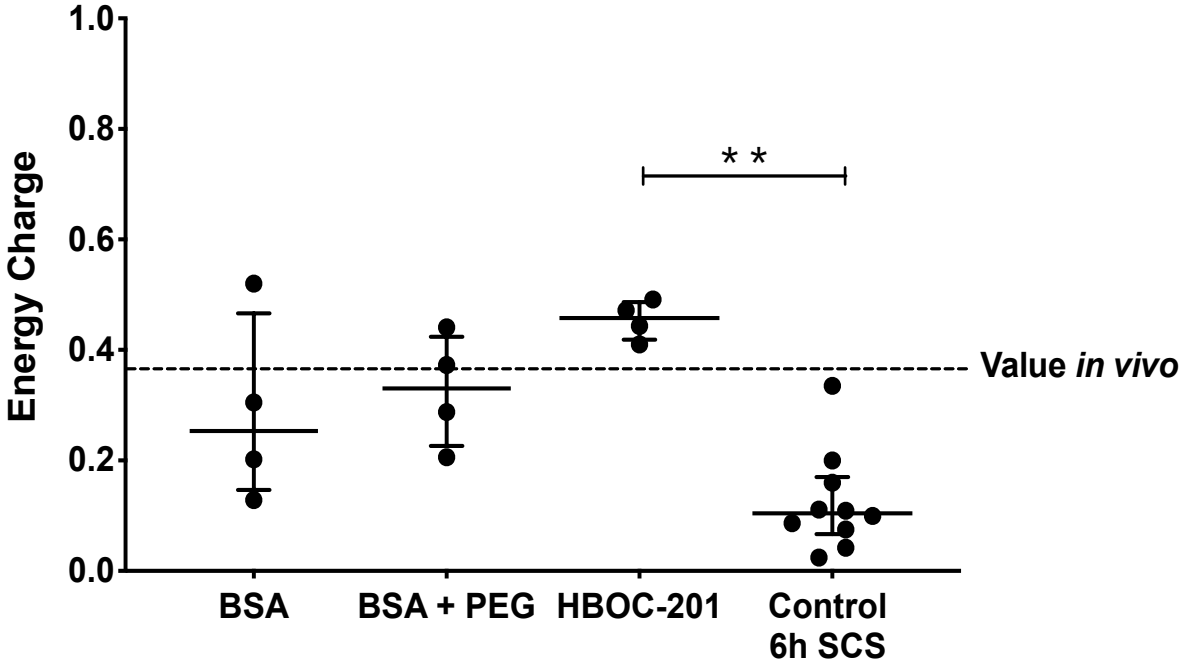


Figure 3. Energy charge values. Energy charge ratios of SCS control limbs were significantly lower compared to HBOC-201 perfused limbs, but not BSA and BSA + PEG limbs (p=0.002) respectively. The red dotted line indicates median energy charge levels in vivo. Abbreviations used: Abbreviations used; BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin-based oxygen carrier-201.

FIGURE 4

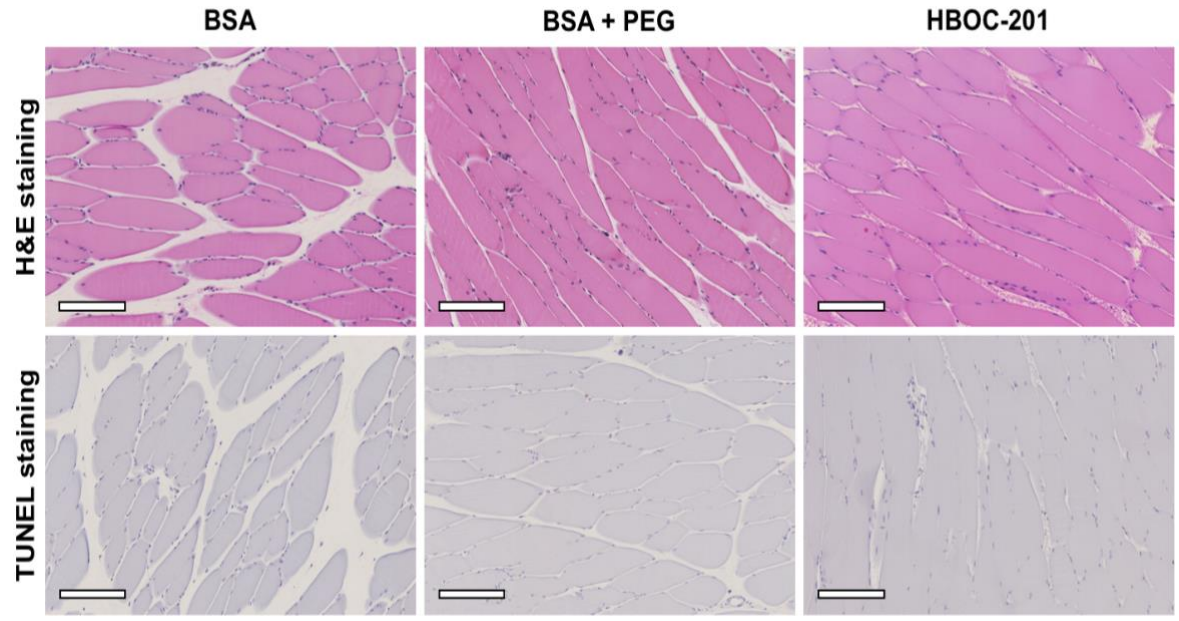


Figure 4. Representative muscle histology of limbs after 6 hours of SNMP with BSA, BSA + PEG and HBOC-201 respectively. Upper panels represent H&E stained biopsies, lower panels show TUNEL stained biopsies. All biopsies show a normal polygonal structure with no signs of apoptosis. All slides are shown at 10x magnification, and the white ox indicates 200 mm. Abbreviations used: H&E = hematoxylin & eosin, BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin-based oxygen carrier – 201.

FIGURE 5

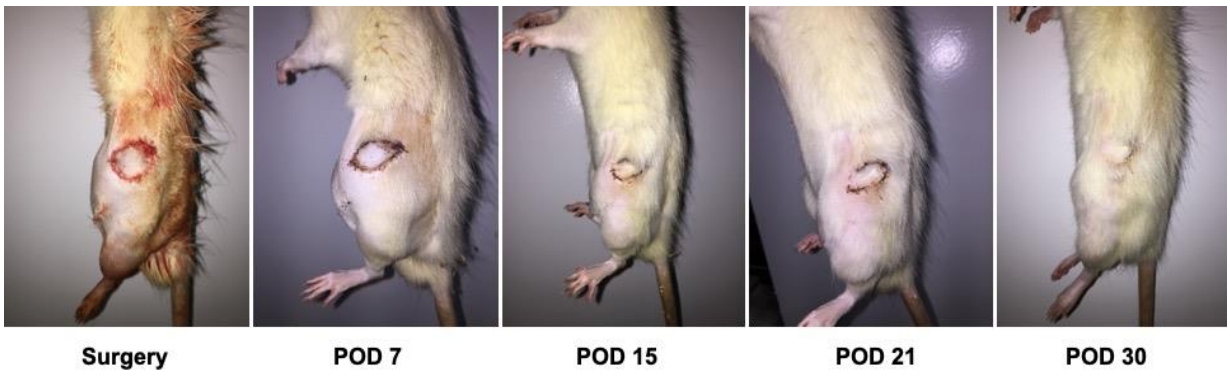


Figure 5. Heterotopic hind limb transplant grafts on post-operative days 0, 7, 15, 21 and 30. Post-operative follow-up of heterotopic hind limb transplant grafts.

FIGURE 6

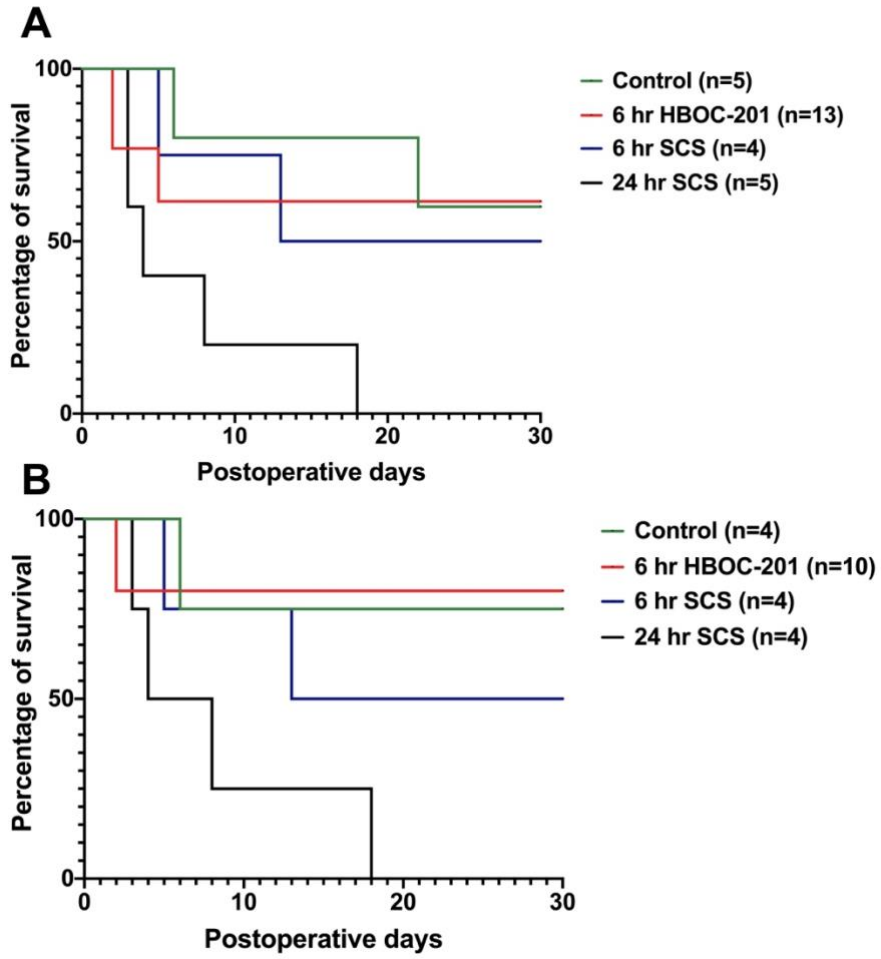


Figure 6. Transplant survival rates after 30 day follow up. Figure 6A shows survival rates including the failed experiments due to automutilation while these are excluded in Figure 6B. Overall survival in the 6 hours HBOC-201 group is 80% excluding the failed experiments due to automutilation (Figure 6B) and 62% including the failed experiments due to automutilation (Figure 6A). The survival of the fresh controls is 75% excluding the failed experiments due to automutilation (Figure 6B) and 60% including the failed experiments due to automutilation (Figure 6A). Overall survival of the 6 hours SCS and 24 hours of SCS are 50% and 0% respectively in both graphs (Figure 6A and B).

APPENDIX

Limb procurement

Animals were anesthetized with isoflurane (Forane, Baxter, Deerfield, IL) using a Tech 4 vaporizer (Surgivet, Waukesha, WI). Animals were placed on a heating pad in a supine position and were shaved from the right ankle with the distal lower ribs as the proximal and midline as the medial landmarks. The animals were prepped in a sterile manner using povidone iodine and surgical drape. The line on medial on medial side of the hindlimb overlies the femoral vessels and the circular lines on mid-thigh and above the ankle, respectively, delineate the skin paddle (4 x 3 cm). We started by a circular skin incision at the location of the medial above the ankle. First, the anterior and posterior tibial pedicles were ligated first using 8/0 ethilon sutures. The Achilles tendon was then sectioned, and the tibial periosteum was exposed by pushing back all tendons using an Obwegeser periosteal elevator. At this point, animals were systemically heparinized (30 IU) via the dorsal penile vein. For the skin paddle, we incised the line on the inner thigh. The fat pad was then dissected out to identify the femoral vessels and all surrounding muscle were cut off. Subsequently, both the femoral artery and vein were skeletonized and cannulated with a 24-gauge intravenous catheter that was secured with 7/0 silk ligation. The graft was mobilized by cutting the bones above the ankle and under the inguinal ligament and flushed with 10mL heparinized saline (10 IU/mL) via the femoral artery till limpid outflow.

CHAPTER 14

In Vivo Activity of Genetically Modified Cells Preseeded in Rat Vascularized Composite Allografts

Corentin B. Taveau
Alexandre G. Lellouch
Ling-Yee Chin
Olivia Mamane
Philipp Tratnig-Frankl
Laurent A. Lantieri
Mark A. Randolph
Korkut Uygun
Curtis L. Cetrulo Jr.
Biju Parekkadan

Adapted from Transplant Proc. 2021 May 10:S0041-1345(21)00230-X. doi: 10.1016/j.transproceed.2021.02.028. PMID: 33985799

ABSTRACT

Transplantation of the hand or face, known as vascularized composite allotransplantation (VCA), has revolutionized reconstructive surgery. Notwithstanding, there are still several areas of improvement to mitigate immune rejection while sparing systemic adverse effects. The goal of this study was to evaluate the engraftment and viability of a genetically modified cell population pre-engrafted into a VCA transplant, to potentially act as a local biosensor to report and modify the graft in vivo. A rat fibroblast cell line genetically modified to secrete Gaussia-Luciferase (gLuc), which served as a constitutive biomarker of cells, was incorporated into a VCA to study the viability of biosensor cells in a syngeneic rat heterotopic partial hindlimb transplantation model. Five perfusions were first performed as engineering runs to have a stable limb perfusion protocol, followed by 3 perfusions to analyze the cell engraftment during machine perfusion, and finally 4 perfusions to study in vivo persistence of the cell biosensors. Blood samples were collected to monitor gLuc secretion during perfusion and postoperatively. A time-dependent increase in gLuc secretion in the limb perfusion outflow during machine perfusion indirectly verified the presence of biosensors within the graft. After the ex vivo perfusion, VCA hindlimbs were analyzed for near infrared fluorescence emission that showed a presence of dyed engineered cells in all areas of the limbs. Postoperatively, gLuc was detectable 4 to 5 days after transplantation ($W = 16$, $P = .02857$). This study demonstrated that engineered cells could be successfully preimplanted into VCAs—an important step toward development of an in vivo biosensor platform to use in modulating acute VCA outcomes.

INTRODUCTION

Vascularized composite allotransplantation (VCA) is an established option for reconstruction of complex injuries that are inadequately addressed by autologous surgery[1,2]. However, VCA is often not performed[3] due to a number of factors: 1) a risk of immunologic rejection (a risk considered higher than in solid organ transplantation due to the immunogenicity of the skin [4]), 2) the need for aggressive immunosuppressive drug regimens with concomitant side effects, and 3) the potential for long term graft loss [5,6] secondary to chronic rejection. Furthermore, the monitoring [7] and management of early VCA rejection lacks specific clinical and biological markers [8,9]. Currently, only gross observations and histological features can confirm a rejection diagnosis[10] and grade using the BANFF classification[11].

This pilot study evaluated a new approach to potentially monitor and modulate VCA rejection by using a genetically-modified cell biosensor to release a protein biomarker in VCA. The primary objective was to verify if biosensors were viable in VCA grafts after transplantation. Machine perfusion, a promising approach for organ preservation[12–14], was used as an ex vivo platform to engraft these genetically modified cells during VCA preparation. As recently described by our group, bioengineered cells can be successfully infused into a liver ex vivo using machine perfusion along with methods to evaluate their engraftment properties[15] but no in vivo attempts were evaluated thus far. Here, we report the use of cell sensors in the preparation of a VCA along with in vivo detection of cell sensors after heterotopic transplantation in a syngeneic rat model.

MATERIAL AND METHODS Animals

All animals were kept in accordance with the National Research Council guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Massachusetts General Hospital. We used 350g to 400g syngeneic male Lewis rats (Charles River Laboratories, Boston, Mass, United States).

Genetically Modified Cell Preparation

Allogeneic rat fibroblasts were sourced from the American Type Culture Collection (Rat2 ATCC CRL-1764; ATCC, Manassas, Va, United States). These cells were engineered via lentiviral transduction to secrete Gaussia-Luciferase (gLuc), as previously described[15], and purified by fluorescence activated cell sorting (FACS) sorting based on the expression of green fluorescent protein. Cells were frozen and used immediately post thaw to simulate an organ transplant setting. Ten million cells were preseeded into the limb through machine perfusion before transplantation.

Procurement

Donor right partial hindlimbs were procured during terminal procedures in a sterile manner as described previously[16]. Five minutes after intravenous infusion of 300 U of heparin, the hindlimb was isolated. The femoral pedicle was cannulated with 24-gauge angiocatheters and flushed with 10 mL of 100 U/mL heparin saline using a pressure monitor to avoid vessel injuries.

Machine Perfusion

The hindlimb was connected to a subnormothermic machine perfusion through the femoral artery and perfused with our preservation media[17]. We realized 12 limb perfusions: 5 perfusions to optimize the protocol, 3 to analyze the cell engraftment during machine perfusion, and eventually 4 limbs perfusions that were transplanted. Ten million bioengineered fibroblasts were added to the media and the perfusion was carried on for a duration of 3 hours. The perfusion flow rate was adjusted for a target pressure of 25 to 35 mm Hg. Venous outflow was collected at 0, 30, 60, 120, and 180 minutes, and analyzed for gLuc.

In Vivo Transplantation

Three animals were then transplanted with VCA in a heterotopic manner. The femoral vessels of the syngeneic recipient rat were dissected out[16] and were ready for transplantation after 3 hours of perfusion. The hindlimb was flushed with heparin saline, and vascular anastomosis was performed under microscope with 10/0 Ethilon (Ethicon, Somerville, NJ). The partial hindlimb was then sutured in heterotopic position in the left flank. Animals were monitored for pain and distress and treated accordingly.

gLuc Assay

Blood samples were collected from the lateral tail vein daily during the first week. Plasma was isolated and stored frozen. A gLuc luminescence assay was performed using its substrate, coelenterazine (NanoLight Technology, Arizona, USA)[15].

Fibroblast Ex Vivo Imaging

Fibroblasts were marked with a near infrared fluorescent membrane dye as previously described[15]. Ex vivo near infrared fluorescence imaging was used to visualize cell engraftment and distribution in the VCA immediately after machine perfusion.

End of Study

Animals were kept alive for more than 1 month to ensure good viability of the transplants, and at the end of study vascular anastomosis patency was confirmed, skin and muscle biopsy specimens were taken, and animals were euthanized.

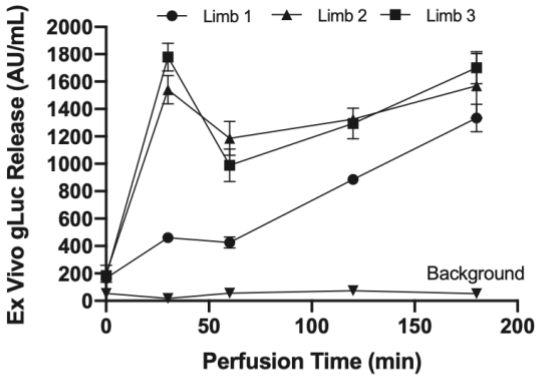


Fig 1. Bioengineered cell engraftment during vascularized composite allotransplantation machine perfusion: Gaussia-Luciferase (gLuc) secretion increase with time of perfusion. Samples were collected in the venous outflow of rat hindlimbs during 3-hour time machine perfusion after addition of 10 million genetically modified fibroblasts at time 0. Three different hindlimb perfusions were performed with cell engraftment and one without cell engraftment that appears as the control on the graph.

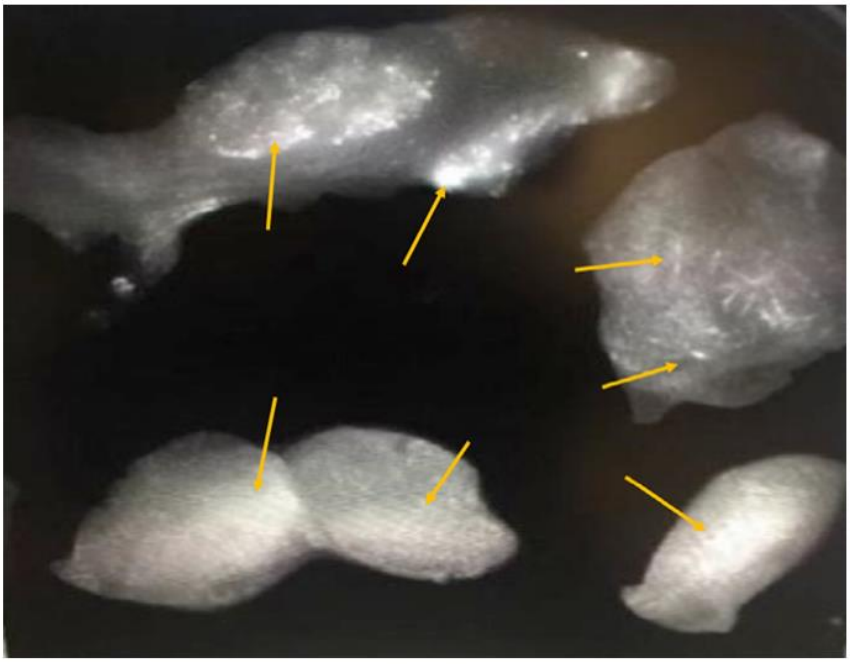


Fig 2. Bioengineered cells successfully engrafted in whole vascularized composite allotransplantation after machine perfusion. Near infrared fluorescence imaging of sliced rat hindlimb immediately after 3-hour machine perfusion showing a global distribution of dyed fibroblasts. Arrows are pointing to different highly fluorescent spots present in every slice representing various clusters of dyed cells.

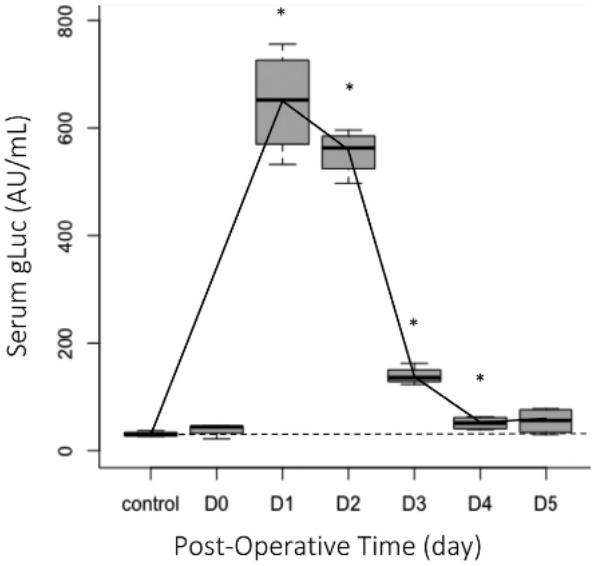


Fig 3. In vivo activity of genetically modified preseeded fibroblasts detectable up to 4 days after transplantation. gLuc luminescence assay performed on postoperative plasma showing the presence of plasmatic expression of gLuc 96 to 120 hours after transplantation. The control is the gLuc expression found in naive rats from the same herd that were not transplanted, nor had genetically modified fibroblasts infusion. The asterisks represent statistically significant differences ($P < .05$) between the gLuc expression on day 1 compared with the control values using a Wilcoxon rank sum test. D0, day of transplantation immediately after surgery; Dx, x day after surgery; gLuc, Gaussia-Luciferase.

Statistical Analysis

Statistical analysis was performed using R software (3.3.1 version; The R Foundation, Vienna, Austria, available online). To calculate if the detection of gLuc in the transplanted rats serum was statistically different compared with naive rats from the herd, we used a Wilcoxon rank sum because of the small number of subjects.

RESULTS

Allogeneic rat fibroblasts were genetically engineered to express a secreted luciferase (gLuc) as a simple biomarker to indirectly assess viability of cell sensors within an organ by measurement of circulating fluids. Allogeneic cells were used to simulate the clinical scenario where an "off-the-shelf" cell therapy could be available in a frozen format for rapid use in transplant settings. Five VCA perfusions were performed to develop a process for engineered fibroblast engraftment that began to show detectable gLuc in the perfusate before conducting a protocol-driven study (data not shown). Upon developing a stable engraftment process for biosensors, a protocol-driven perfusion study was performed with 3 VCA grafts with cell sensors. gLuc was detected in the venous outflow with an initial spike at 30 minutes after cell perfusion and then a linear increase of expression during the duration of the perfusion (Fig 1). The biosensor cells also were labeled with an intracellular, near infrared dye for noninvasive tracking after ex vivo

perfusion. All VCA hindlimbs were analyzed for near infrared fluorescence emission and showed presence of dyed engineered cells distributed across the limbs (Fig 2).

Finally, 4 VCA transplantations performed were successful as evidenced by viable transplanted limbs and absence of wound dehiscence up to euthanasia. All animals were healthy after surgery and underwent a similar postoperative history as previously experienced on this surgical model. Postoperatively, gLuc was detectable during the first several days after transplantation and then became undetectable by approximately day 5 (Fig 3). During the first 4 days after transplantation gLuc expression was significantly different from control rat serum ($W = 16$, $P = .02857$), and at day 5 after transplantation there is no more difference from the control animals ($W = 14$, $P = .1143$). The viability of the VCA was good at the end of the study as noted by gross and microscopic histology (4 weeks postoperative; data not shown).

DISCUSSION

This study evaluated the engraftment of genetically engineered cells in an organ before transplantation while tracking transient cell engraftment and function in vivo. Our results demonstrate that genetically modified cells are viable and detectable after perfusion into organs and transplantation. However, the release of gLuc was transient, which suggests that genetically modified fibroblasts may not have survived or the expression of gLuc was lost in vivo. Potential causes of transient expression include the use of allogeneic cells without immunosuppressive therapy leading to rejection of engineered cells, or apoptosis of cells due to ischemic or reperfusion injury on transplantation, and antibody neutralization of gLuc in rodents. Further studies to explore the mechanism of transient expression and improve the signal for longer-term persistence are warranted. This research establishes that an engineered cell population can remain viable in vivo to potentially help the acute detection and management of VCA rejection.

This pilot study shows early verification data that engineered cells can be preimplanted into VCA, with the aid of machine perfusion, and be tracked after transplantation. The power of the study was limited to minimize animal usage while trying to detect dramatic outcomes in cell viability, though further validation with a greater number of animals and control groups is warranted. The duration of the genetically modified cells engraftment in the limb has to be increased to gain clinical relevance because the objective would be to create a cell line that would ideally survive for at least the first few months, when the rejection episodes are more often and severe, or even better for years.

CONCLUSIONS

This study demonstrates that engineered cells can be preimplanted into VCA, with the aid of machine perfusion, and be tracked after transplantation as a first step toward developing biosensor cells for the early detection and treatment of rejection episodes in VCA.

REFERENCES

1. Lantieri L, Grimbert P, Ortonne N, Suberbielle C, Bories D, Gil-Vernet S, et al. Face transplant: long-term follow-up and results of a prospective open study. Lancet 2016;388:1398–407.
2. Petruzzo P, Sardu C, Lanzetta M, Dubernard JM. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). Curr Transplant Reports 2017;4:294– 303.
3. Krezdorn N, Tasigiorgos S, Wo L, Lopdrup R, Turk M, Kiwa- nuka H, et al. Kidney dysfunction after vascularized composite allo- transplantation. Transplant Direct 2018;4:e362.
4. Lee WP, Yaremchuk MJ, Pan YC, Randolph MA, Tan CM, Weiland AJ. Relative antigenicity of components of a vascularized limb allograft. Plast Reconstr Surg 1991;87:401–11.
5. Morelon E, Petruzzo P, Kanitakis J, Dakpe S, Thaunat O, Dubois V, et al. Face transplantation: partial graft loss of the first case 10 years later. Am J Transplant 2017;17:1935–40.
6. Kanitakis J, Petruzzo P, Badet L, Gazarian A, Thaunat O, Tes- telin S, et al. Chronic rejection in human vascularized composite allo- transplantation (hand and face recipients). Transplantation 2016;100:2053–61.
7. Kaufman CL, Ouseph R, Marvin MR, Manon-Matos Y, Blair B, Kutz JE. Monitoring and long-term outcomes in vascularized com- posite allotransplantation. Curr Opin Organ Transplant 2013;18:652–8.
8. Sarhane KA, Tuffaha SH, Broyles JM, Ibrahim AE, Khalifian S, Baltodano P, et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. Front Immunol 2013;4:406.
9. Hautz T, Zelger BG, Weißenbacher A, Zelger B, Brandacher G, Landin L, et al. Standardizing skin biopsy sampling to assess rejec- tion in vascularized composite allotransplantation. Clin Transplant 2013;27:E81–90.
10. Fischer S, Lian CG, Kueckelhaus M, Strom TB, Edelman ER, Clark RA, et al. Acute rejection in vascularized composite allotrans- plantation. Curr Opin Organ Transplant 2014;19:531–44.
11. Rosales IA, Foreman RK, DeFazio M, Sachs DH, Cetrulo CL, Leonard DA, et al. Systematic pathological component scores for skin- containing vascularized composite allografts. Vasc Compos Allotrans- plantation 2017;3:62–74.
12. de Vries RJ, Tessier SN, Banik PD, Nagpal S, Cronin SEJ, Ozer S, et al. Supercooling extends preservation time of human livers. Nat Biotechnol 2019;37:1131–6.
13. Yeh H, Uygun K. Increasing donor liver utilization through machine perfusion. Hepatology 2019;70:431–3.
14. Sierra Parraga JM, Rozenberg K, Eijken M, Leuvenink HG, Hunter J, Merino A, et al. Effects of normothermic machine perfusion conditions on mesenchymal stromal cells. Front Immunol 2019;10:765.
15. Chin L-Y, Carroll C, Raigani S, Detelich DM, Tessier SN, Wojtkiewicz GR, et al. Ex vivo perfusion-based engraftment of geneti- cally engineered cell sensors into transplantable organs. PLoS One 2019;14:e0225222.
16. Nazzal JA, Johnson TS, Gordon CR, Randolph MA, Lee WPA. Heterotopic limb allotransplantation model to study skin rejection in the rat. Microsurgery 2004;24:448–53.
17. Lellouch AG, Tessier SN, Cronin SE, Schol IM, Pendexter CA, Randolph MA, et al. Optimization of ex-vivo subnormothermic oxy- genated machine perfusion in vascularized composite allograft on rat to prolong preservation duration. Paper presented at: 50th Annual Meeting of the American Burn Association. Chicago, IL: Journal of burn care & research; 2018.

CHAPTER 15

Engineering Vascularized Composite Allografts Using Natural Scaffolds: A Systematic Review

Elise Lupon
Alexandre G. Lellouch
Aylin Acun
Alec R. Andrews
Ruben Oganesyan
Marion Goutard
Corentin B. Taveau
Laurent A. Lantieri
Curtis L. Cetrulo Jr.
Basak E. Uygun

Adapted from Tissue Eng Part B Rev. 2021 Jul 9. doi: 10.1089/ten.TEB.2021.0102.PMID: 34238047

ABSTRACT

Introduction: Vascularized Composite Allotransplantation refers to the transplantation of multiple tissues as a functional unit from a deceased donor to a recipient with a severe injury. These grafts serve as potential replacements for traumatic tissue losses. The main problems are the consequences of the long immunosuppressive drugs medications and the lake of compatible donor. To avoid these limitations, decellularization/recellularization constitute an attractive approach.

The aim of decellularization/recellularization technology is to develop immunogenic free biological substitutes that will restore, maintain, or improve tissue and organ's function.

Methods: A PubMed search was performed for articles on decellularization and recellularization of composite tissue allografts between March and February 2021, with no restrictions in publication year. The selected reports were evaluated in terms of decellularization protocols, assessment of decellularized grafts, and evaluation of their biocompatibility and repopulation with cells both in vitro and in vivo.

Results: The search resulted in a total of 88 articles. Each article was reviewed, 77 were excluded and the remaining 11 articles reported decellularization of 12 different vascular composite allografts in humans (four), large animals (three), and small animals (rodents) (five). The decellularization protocol for vascularized composite allotransplantation varies slightly between studies, but majority of the reports employ 1% sodium dodecyl sulfate as the main reagent for decellularization. The immunological response of the decellularized scaffolds remains poorly evaluated. Few authors have been able to attempt the recellularization and transplantation of these scaffolds. Successful transplantation seems to require prior recellularization.

Conclusion: Decellularization/recellularization is a promising, growing, emerging developing research field in vascular composite allotransplantation.

Impact statement: Tissue engineering for vascular composite allotransplantation using decellularization and recellularization approach is a fast-growing area of interest in the reconstructive surgery field. This review will be a very useful tool to get a clear overview for researchers interested in this field.

1. INTRODUCTION

Vascularized Composite Allotransplantation (VCA) is the transplantation of multiple tissues such as skin, nerve, muscle, tendon, cartilage, and bone, as a functional unit (e.g., a hand, or face) and has emerged as a feasible option to restore anatomically complex defects that cannot be addressed by autologous graft reconstruction[1]. The first VCA surgery took place in 1964 with the first hand allotransplantation, but it was in 1999 when the first successful immunosuppressive therapy protocol enabled long-term graft acceptance of a vascular composite allograft[2]. Over the last two decades, more than 200 VCAs were performed around the world. Despite the immunosuppressive regimen to prevent graft loss, there is still the risk of acute and chronic rejection. Acute rejection of the VCA, with skin as its primary target, occurs in up to 90% of patients, most of whom experience at least one such episode within the first-year post-transplantation and almost 60% develop multiple episodes[3]. Furthermore, the complications associated with the life-long and high dose immunosuppressive therapy make VCA a high-risk procedure posing a significant ethical dilemma regarding this reconstructive procedure[4].

Tissue engineering offers tools to create alternative tissues for VCA that would potentially serve as an autologous tissue and eliminate the need for immunosuppression, minimizing the risks of this life changing procedure for patients. One such tool is tissue decellularization and recellularization, an approach that involves removal of native cells from discarded tissue to yield an extracellular matrix (ECM) scaffold and its subsequent repopulation with healthy and patient derived cells to recreate the tissue microarchitecture[5]. Decellularization is achieved by treating the tissue or organ with detergents, enzymes or chemicals either by simple immersion or perfusion through vasculature[6]. The resulting scaffold is composed of native ECM that has the composition and architecture of the native tissue which has been proven to be difficult to replicate using bottoms up approaches[7]. The advantages of a decellularized ECM scaffold are several. First, the vascular architecture of the tissue is preserved in a perfusion decellularized scaffold which enables perfusion and nutrient delivery to the cells seeded in the scaffolds. The preserved vascular bed also facilitates connection to the blood circulation upon transplantation. Second, the maintenance of ECM components provides tissue-specific biochemical and biomechanical cues which may facilitate cell attachment and tissue remodeling, and subsequent tissue function upon repopulation with cells[5] .

While tissue decellularization has been used in tissue engineering for preparation of biological scaffolds for dermal, vascular, and small intestinal submucosa tissues, its application as perfusion decellularization in solid organs such as the liver, heart, lungs has attracted much attention during the last two decades[8]. More recently, the ability to preserve the native tissue vascular bed and the microarchitecture by perfusion decellularization has fueled its exploration in VCA applications. In this systematic review, we provide an insight on the current status of research in engineered VCA grafts using decellularized scaffolds.

2. METHODS

A PubMed search was performed for articles in all languages on decellularization and recellularization of composite tissue allografts, with no restrictions in publication year using the following keywords: "plastic surgery and decellularization", "plastic surgery and recellularization", "vascularized composite allotransplantation and decellularization", "vascularized composite allotransplantation and recellularization", "vascularized composite allotransplantation and tissue engineering", "composite tissue decellularization", "composite tissue recellularization", "flap decellularization", "flap recellularization".

2.1 Article selection and inclusion/exclusion criteria

Original research articles were selected for review based on the title and abstract. These articles exclusively reported a decellularized specimen that can be used in plastic surgery. Work in the field of orthopedic surgery such as isolated decellularization of bone, tendons and ligaments as well as decellularization of muscle with pedicle and skin biopsies were excluded from the analysis, as they do not meet the exact definition of VCA. Articles explaining generalities of decellularization and recellularization, without describing the specific decellularization of a specimen were excluded. Non-English publications were excluded.

2.2 Data collection

For each publication, we indexed the decellularized specimen, if specified. The decellularization protocol used was detailed (specifying the decellularization method chosen, the agents used, their concentrations and treatment durations). Results in terms of vasculature and ECM integrity and immunogenicity tests were listed. The attempt at recellularization was specified including its protocol. Finally, the transplantation of the specimen, if it took place, was noted.

3. RESULTS

The PubMed search was performed between February and March 2021 using the keywords listed above. The search resulted in a total of 88 articles. Each article was reviewed, 77 were excluded: 50 were out of scope (i.e., review articles), 20 were in the orthopedic field (e.g., tendons, ligaments, muscles, etc.) and 7 involved partial VCA (e.g., skin biopsy, muscle section). The remaining 11 articles reported decellularization of 12 different vascular composite allografts in humans (four), large animals (e.g., pig, NHP) (three), and small animals (rodents) (five) (Figure 1). The details of each study are given in Table 1.

Briefly, the studies included in this review reported decellularization of rat abdominal fasciocutaneous flaps[9, 10], rat upper limbs[11], primate upper limbs[11], rat groin skin/adipose tissue flap[12], porcine fasciocutaneous groin flap[13], human lower face and full face[14], a human ear[15], a full human cadaveric upper extremity[16] , a full-thickness rat hemi-face[17] , a porcine ear[18] and a whole human penis[19]. These reports were evaluated in terms of

decellularization protocols, assessment of decellularized grafts, and evaluation of their biocompatibility and repopulation with cells both in vitro and in vivo.

3.1 Preparation of decellularized VCA scaffolds

3.1.1 Tissue procurement

Tissue procurement technique strongly affects the quality of the decellularized graft as the integrity of the vasculature and overall tissue architecture directly determines the perfusability and the uniformity of the decellularization process. In the publications reviewed, the VCAs from non-human primates, swine models, and rodents were cannulated and decellularized immediately after harvesting except in[12] where the rat skin/adipose tissue flap was subjected to three freeze/thaw cycles before decellularization. Human VCAs present challenges for procurement since they are less frequently donated than internal organs due to the significant visual deterioration of the donor body[20]. In addition, fresh tissue may not be immediately available for harvesting from brain dead donors. Nonetheless, VCAs have been obtained from deceased donors several days post mortem and were successfully decellularized without significant damage to the gross morphology of the graft[14-16]. The preservation of the structural integrity postmortem can be attributed to low cellularity of the tissue and high density of the extracellular matrix proteins in the particular grafts.

3.2.2 Decellularization protocols

The decellularization protocols for VCAs vary slightly between studies, but the majority of the reports employ 1% sodium dodecyl sulfate (SDS) as the main reagent for decellularization[9, 11, 13-19]. SDS is an ionic detergent and has been widely used in decellularization of other organs[21-24]. While it is very effective in removing cellular proteins and nucleic acids, SDS may also lead to removal of extracellular matrix associated components such as growth factors and glycosaminoglycans from the resulting scaffolds[5]. Commonly, the VCA is exposed to the detergent through perfusion via the pedicle of the graft and decellularization is uniformly achieved throughout the graft. While Duisit et al.[17] recommends avoiding decellularization only by immersion in the SDS, no studies have been published showing its ineffectiveness to date. Furthermore, Henderson et al. successfully decellularized fourteen abdominal rat fasciocutaneous flaps through incubation in 0.1% sodium azide, 40 IU/ml DNAse, and 4% sodium deoxycholate [9]. Similarly, rat fasciocutaneous flaps were successfully decellularized using 0.25% trypsin-EDTA through incubation without perfusion[12]. In these studies, the incubation periods in detergent were accompanied with agitation to improve the detergent penetration. However, decellularization by agitation is not recommended as the study by Duisit et al. reports deterioration of decellularized pig ears due to the trauma inherent in this method[17]. The first indicator of the effective removal of cells from the VCA is the progressive change of its color to a white or even transparent color during decellularization as we have also shown with different VCAs including human face (Figure 2A, B), porcine skin (Figure 2C, D), and rat limbs (Figure 2D, E). Fat tissue can remain significantly yellow even in the case of effective decellularization. The color change happens within the first few hours to first few days of detergent treatment[9].

However, complete removal of cells and all cellular materials throughout the VCA requires longer treatment times. The duration of decellularization varies with the surface area of the VCAs, from 7 days for the smallest VCAs (e.g., rodent flap)[12] to 60 days for the most complex (e.g., human arm)[16].

The perfusion pressure is a critical parameter to control in order to avoid damage to the microvascular architecture of the VCAs. The decellularization of human ear, human face, human limb, and porcine ear grafts was achieved by controlling the flow rate of the perfusion to achieve 80 mmHg pressure or less[14-16, 18] throughout the decellularization. The resulting flow rates varied from 0.6 to 8 ml/min, depending on the study. However, some studies reported using higher target pressures, aiming for equal to or less than 200 mmHg throughout the decellularization of rat and primate limbs[11] or porcine skin flaps [11, 13]. It was reported that the pressure balance may be difficult to maintain during SDS perfusion due to sudden changes in internal pressures such that it requires very minimal flowrates[12], showing the importance of constant pressure monitoring throughout the decellularization process.

Duisit et al. reported an additional step post decellularization which is defatting and it involves removal of the fat tissue from the VCA. Contrary to solid organs, fat tissue is an independent anatomical and functional compartment in the face and its removal was justified[25]. The defatting step is achieved by treating the VCA with a polar solvent, 2-propanol, initially described for isolated adipose tissue decellularization[26, 27]. However, perfusion with 2-propanol led to an increase in vascular resistance of the VCA and defatting by an overnight incubation in 2-propanol, rather than perfusion, was shown more suitable[14, 15].At the end of the decellularization protocol, the scaffolds were usually stored at 4°C in PBS containing 1% penicillin/streptomycin until further use in all the reports reviewed here.

3.2.3 Scaffold assessment

Removal of cells

The main objective of decellularization is to remove all cellular material from the tissue and the removal of cells and nucleic acids should be confirmed both qualitatively and quantitatively. The efficiency of the decellularization protocol will play an important role in the modulation of the host response. Since ECM molecules are highly conserved among species, immune reaction will mainly depend on the presence of cellular material[28]. Insufficient removal of cellular material can elicit an inflammatory response and can negatively influence or even terminate the regeneration process. Thus, one method to determine the decellularization efficacy is to detect the remaining DNA presence with quantitative DNA content assay.

It has been widely accepted that the following criteria should be satisfied for successful tissue decellularization: less than 50 ng dsDNA per mg ECM dry weight, less than 200 bp DNA fragment length and clear lack of visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) or H&E in histological sections[29]. While H&E staining indicates removal of cells and gives an overview of tissue architecture, it is not a

precise method to assess the decellularization efficiency. Lack of staining of histological sections using a DNA-binding dye such as DAPI and/or quantification of extracted DNA from decellularized tissue with such dyes yield a more accurate measure of removal of nuclear material of the cells. Based on these criteria, all studies reviewed here demonstrated decellularization of the VCA, except two out of five protocols tested on abdominal fasciocutaneous flap (failure with the short agitation in SDS for 12 h and 72 h). In addition to removal of nuclear material, it is critical to demonstrate the removal of cellular proteins from the decellularized tissue in order to confidently report decellularization efficiency[30]. Accordingly, the absence of cellular proteins after decellularization was also confirmed by negative staining for myosin, alpha-actinin sarcomere, neurofilament H, S100 calcium-binding protein B (in muscle) by immunohistochemical staining of the decellularized VCA. Large specimens are more difficult to evaluate and requires multiple samples from different locations to confirm the decellularization[14].

Preservation of extracellular matrix

Extracellular matrix is a complex non-living biomaterial, secreted by the cells and has numerous roles in determining cellular behavior. It is composed of proteins, proteoglycans, and glycosaminoglycans which constitute the structure of the tissue while providing the biochemical signals that facilitate cell engraftment, viability and function of the tissue. It plays vital roles in maintaining the native-like environment for the cells both in vivo and in vitro[31]. ECM harbors growth factors and has tissue specific mechanical properties to regulate dynamic cellular behaviors, such as proliferation and migration, influencing rapid growth of the cells[32]. Therefore, the goal of successful decellularization is to remove all cellular material while preserving the extracellular matrix composition and architecture to the maximum extent possible.

Depending on the type of detergent used, the impact on the ECM of the tissue can be different[6]. Surfactant agents, such as Triton X-100, SDS and sodium deoxycholate cause disarrangement of the phospholipid cell membrane, thereby lysing cells. SDS is an ionic detergent excellent in removing both cytoplasmic and nuclear material but have the tendency to disturb the native architecture of the tissue by disrupting protein-protein interactions and damaging signaling proteins. As it is cytotoxic, it must be thoroughly washed after the decellularization process. Triton X-100 is a non-ionic detergent described to cause minimal damage to the tissue structure and it acts by mainly disrupting lipid–lipid and lipid–protein interactions rather than protein-protein interactions[33]. It is ineffective on its own, therefore it is often used in combination with other agent such as SDS[10, 11, 13, 15, 16, 18] or trypsin/ethylenediaminetetraacetic acid[12]. Sodium deoxycholate is an ionic surfactant, less damaging and cytotoxic to tissue compared to SDS. It causes DNA agglutination and therefore is commonly used in combination with nucleases, as for rat abdominal fasciocutaneous flap decellularization [10]. Mechanical agitation is also used as a means of facilitation of decellularization. The mechanical agitation lyses cells and destroys cell–matrix adhesive proteins. This approach was tested in two studies[11, 18] but show a risk of disturbing the structural architecture of the ECM. In addition, enzymes are also used to achieve decellularization. The enzymes break down the nucleic acids and proteins. In one study by Duisit and al.[18]

DNAse was used on porcine ear decellularization in combination with SDS (1%) treatment. The acid agents (such as trypsin/ethylenediaminetetraacetic acid) act by solubilization of the cell membrane and nuclear material as their intrinsic charge properties are highly corrosive and strongly oxidizing nature, used in combination with other methods, as Triton X-100[12]. Otherwise, it is known to increase ECM stiffness[34].
The specific ECM components of the decellularized VCA were evaluated qualitatively by histology/immunohistochemistry and quantitatively by biochemical or antibody-based assays such as ELISA. Major ECM components analyzed via histology included total collagen (with Masson Trichrome or Miller's blue and Sirius red stain), glycosaminoglycans (with Alcian blue or safranin O), and elastin (with Miller's elastin/Alcian staining). Immunohistochemical staining confirmed the preservation of basement membrane proteins such as collagen IV, laminin and fibronectin. Growth factors, chemokines and cytokines (e.g., VEGF, TGF-β1 GM-CSF; SDF-1α, TNF-α, IL-8, IL-10, and IL-6) retained on the decellularized VCA were determined via immunohistochemistry and ELISA based microarrays[14, 15, 19].

The majority of the studies, using a protocol including 1% SDS and 1% Triton X-100, reported increased total collagen content, and decreased elastin, GAGs, and growth factors contents after decellularization[11, 13, 14, 15, 16, 19]. However, this result was dependent on the tissue component analyzed; for example, decellularized cartilage showed a significant increase in specific growth factors[15], and the fat tissue showed higher preserved elastin[14] and cytokines[15]. Elastin is a key component of the ECM that provides the elasticity of the tissue and is important in load bearing tissues, such as the skin and the cartilage, where mechanical energy is required to be stored. Although elastin was found to be present in all tissues analyzed, it was found to be decreased after decellularization. The decrease was found to be 30% by Jank et al. [13]. Three of these the studies reported the resilience of the grafts after decellularization[13, 15, 18] and mostly found a decrease in elasticity as a result. Elastin was only lowered in the skin compartment but not in the cartilage in decellularized human ears[15]. Another ECM component is collagen that contributes to the mechanical strength of the tissue and is the main component of cartilage, ligaments, tendons, bone, and teeth. Total amount of collagen was analyzed in all the included studies[9-19] and was found to be preserved or increased except in decellularized human cadaveric upper extremity[16]. Type I collagen is by far the most abundant protein in all vertebrates. It assembles into fibers that form the structural and mechanical scaffold. It has been analyzed in five studies[12, 13, 15, 17, 19]. Collagen type II allows cartilage to entrap the proteoglycan aggregate as well as provide tensile strength to the tissue and it was reported to be present in decellularized human ears[15]. Collagen type IV is a component of the basement membrane plays an essential role in cell adhesion, migration, differentiation, and growth. While it was found to be slightly decreased amount of soluble collagen in the muscles, the impact of this decreased cannot be concluded because the decellularized scaffolds were not recellularized[16].

Glycosaminoglycans are another group of ECM constituents that play a crucial role in the cell signaling process, including regulation of cell growth, proliferation, promotion of cell adhesion, anticoagulation, and wound repair. It was analyzed in many of the included studies[9, 11-16, 18, 19] and was described “preserved”. However, it was retained at only 40% on rat upper limbs[11], 56% on porcine fasciocutaneous groin flap[13] and it was significantly

decreased in the skin and cartilage of the human ear scaffold[15]. This decrease in GAGs does not seem to impact the major functions of the GAG-related ECM since recellularization was successful, with cell attachment and proliferation of cells in the studies that have tried it[11-15, 18, 19]. Laminins are an important and biologically active part of the basal lamina, influencing cell differentiation, migration, and adhesion was found preserved in 4 studies[11, 12, 14, 19]. Fibronectin, which has a role in cell adhesion, growth, migration, and differentiation, was tested in decellularized porcine ears and human penile grafts[18, 19]. It was preserved with a good adhesion of the transferred cells during the recellularization of these scaffolds.

Cytokines are small glycoproteins produced by a number of cell types, predominantly leukocytes, that regulate immunity, inflammation, and hematopoiesis and have been found lowered in decellularized human face and human ear grafts[14, 15]. Treatment of human face grafts[14] with 1%SDS and 1% Triton X-100 caused a decrease in IL-8 and GM-CSF, but SSDF-1α, IL-10, TNF-α, IL-6 contents were stable. In decellularized human ear grafts[15], cytokines were all reduced in skin, stable in fat (except IL-4), and decreased for 22/44 cytokines in cartilage. Thus, it is necessary to consider the possibility that the decrease of specific cytokines could have been at the reason of the good integration of the in vivo study results as described later.

Growth factors played an important role in promoting cellular differentiation and cell division and showed heterogeneous results in the studies that analyzed this component. In the study regarding human ears scaffolds[15], the authors found an increase of TGF-beta1, TGF-beta3, and CXCL5 content. The study with the decellularized human penile[19], reported retention of growth factors VEGF (45%), EGF (57%), and TGF-beta1 (42%) on ELISA. The cavernosa and urethra had the significantly highest level for all three growth factors compared to the tunica. Interestingly, cells formed small clusters in the cavernosa and urethra parts when they proceed with recellularization, with tube-like structure formation observed in the cavernosa region. That can be explained by the increased presence of these growth factors at these sites. On rat groin Skin/adipose tissue flap[12], bFGF and VEGF, which play important roles in angiogenesis and neovascularization, were analyzed. These growth factors were retained after decellularization, along vessels, nerves, and nanofibrous structures, indicating a pre-angiogenic property.

Further analysis of the components using mass spectrometry is necessary to ensure the exact effect of detergents on the preservation of the ECM components[35]. However, the initial composition after decellularization might not be so crucial since once the cells are seeded, they secrete their own ECM and remodel the scaffold[36].

The structural integrity and general morphology of decellularized VCA scaffolds were assessed through scanning electron microscopy (SEM) of whole-organ human penile tissue[19], human ear grafts[15], rat groin skin/adipose tissue flap[12], porcine fasciocutaneous groin flap[18] and porcine ear sample[18]. SEM images of these decellularized scaffolds revealed the characteristic porous structure of the scaffold generated left behind after removal of the cells. The stromal layers were easily identifiable. The ECM structure appeared intact in the SEM images on these four tissue types. In particular, Duisit et al. pointed out the dense fibrous matrix and reticular fibers and the preservation of

functional structure (dermal papilla) in decellularized porcine ear samples[18]. None of the studies performed transmission electron microscopy to assess deeper the ultrastructural properties of the scaffolds.

Vascular integrity

Integrity of the vascular bed in decellularized VCA is critical for the success of downstream repopulation and the transplantation of the engineered grafts. The vascular architecture of the decellularized VCAs was evaluated via contrast enhanced computed tomography (CT) and micro CT (with microfil)[12, 16, 19], microtomography[11] , fluoroscopy[14, 15], perfusion of PBS or PBS and 0.5% Evans blue dye[9] . Microscopic architecture of decellularized VCA was a visualized via Scanning Electron Microscopy (SEM)[12, 15]. Each study reports the preservation of macrovasculature in the decellularized VCAs but the degree of preservation of the microvasculature was found to be variable[13]. Qu et al. recognized major damage to the vasculature of the fasciocutaneous flaps after decellularization with agitation[10]. Similarly, Duisit et al. described a vessel leakage during perfusion recellularization of decellularized lips[14]. Jank et al. found that three-dimensional composite tissue architecture was generally preserved with decreased volume and signal intensity in soft tissues in microtomographic imaging of the decellularized rat and primate limbs[11]. The microtomographic angiography showed perfusable vascular channels throughout the entire graft flowing from larger to smaller vessels.

Mechanical properties of the decellularized VCAs

The mechanical strength of the decellularized VCAs was evaluated in a number of the studies reviewed[11, 13, 15, 18]. All of them used a decellularization protocol based on 1% SDS and 1% Triton X-100 and one study used an enzymatic decellularization agent additionally[18]. The most extensive evaluation was done by Jank et al. for the decellularized limbs[11] which were exposed to detergent for more than 2 days (1% SDS for 50 h and 1% Triton X-100 for 1 h). They carried out peripheral dual-energy X-ray absorptiometry (pDXA) assessment for bone mineral density and bone mineral content and tested the bone with a radii assessment for bending stiffness, modulus of elasticity, bending strength, and fracture energy. They also tested passive tension in tendon, a morphometric measurement of myofibers, and an isometric force measurement with an in vitro culture. They found no significant effect of decellularization on mechanical strength, mineral content, and geometric bone characteristics in pDXA. The passive mechanical testing showed functional preservation of the whole skeletomuscular apparatus with intact osteotendinous junctions. Decellularized limbs maintained stability and a full range of motion in joints of wrist, and digits. Another study by the same group[13] described biaxial mechanical testing of the decellularized porcine skin flaps treated with surfactants for 11 days (1% SDS for 10 days and 1% Triton X-100 for 1 day) showing the same stiffness as the native tissue.

Duisit et al. used a ball burst test and a tensile test on native and decellularized human ears to evaluate the mechanical strength of the scaffold which was treated with 1% SDS for 88 hours and 1% Triton X-100 for 25 hours[15]. They found a significant decrease in

stiffness, a substantial increase in maximum engineering stress, and a considerable increase in dispersion after decellularization.
The same group reported that the Young's modulus was not different between decellularized and native porcine ear in the cartilage but was significantly lower in the skin component of the decellularized VCA than in the native counterpart[18]. It is interesting to note that scaffolds were treated with 1 liter of Type I bovine DNase in addition to the 1% SDS (44.5 L) and the 1%Triton X-100 (3.5 L).

Immunological compatibility

The decellularized VCAs were evaluated in only four out of 11 studies to determine the immunological characteristics[13-15, 18]. Upon decellularization, the decellularized VCAs were tested for the lack of major histocompatibility complex class I (MHC-I)[15, 17] or swine leukocyte antigen (SLA Ic) [18] to ensure the removal of immunogenic antigens. Jank et al. tested the immunological response in vivo by implanting decellularized porcine flap scaffolds in rats and by analyzing them after two weeks[13]. When compared to native

skin flap, Permacol, and Alloderm samples, the decellularized scaffolds revealed a highly integrated graft with almost 100% cellular infiltration (including 89% of immune cell) and neovascularization within the sample. Decellularized and native human ear samples were also tested immunologically through subcutaneous implantation in 16 adult Wistar rats[15]. The implants were harvested on days 15, 30, and 60 for histological evaluation. The analysis showed vascular and cellular infiltration within the decellularized implants, a limited remodeling, a CD68+ macrophage infiltration, and a poor anti-CD3- response. Duisit et al. also tested the allocompatibility of decellularized porcine ear grafts by implantation in a swine pouch for 30 days[18]. As a result, they found no SLA markers at the analysis post-implantation and no production of anti-donor SLA specific antibodies at one month. The implantation was well tolerated with no inflammatory processes. Interestingly, in the two swine studies[13, 18], α-gal epitope, the main antigen causing hyperacute rejection of porcine xenografts in primates[37] was not evaluated.

3.2.4 Recellularization of decellularized VCAs

Recellularization protocols

Eight out of 11 studies have attempted recellularization of decellularized VCAs (Table 1) [11-15, 17-19]. The scaffolds were first sterilized in 0.1% peracetic acid supplemented with or without 1% antibiotic-antimycotic solution. Then the cell type of choice was introduced to the scaffolds following two different protocols. The cells were seeded through perfusion via the vasculature of the decellularized VCA in a bioreactor[11, 13-15, 18] or through manual injection of cells at different sites of the scaffold followed by static culture[12, 17, 19]. In one study, the cells were seeded in multiple batches and electrical stimulation was used post seeding to improve functional muscle formation[11].

The cell types used in the recellularization of the VCA scaffolds included endothelial cells (e.g., human umbilical vein endothelial cells (HUVECs)), fibroblasts, myoblasts, adipose derived stem cells (ASC), and mesenchymal stem cells. In one study, rat upper limb

scaffolds were perfused with 5x106 HUVECs on day 0 followed by the perfusion with 10x106 C2C12 cells (an immortalized mouse myoblast cell line) on day two. These grafts were later seeded with 0.5x106 mouse embryonic fibroblasts[11]. Duisit and al. perfused rat hemifacial grafts with 5x105 human adipose-derived stem cells[17] , seeded human ear grafts with 3.6x104 cells/cm2 rat adipose-derived stem cells and 30x106 human arterial endothelial cells[15] and porcine ear scaffolds with 2.6x106 Cos-7 cells (a fibroblast-like cell line derived from monkey kidney tissue) and 1.5x105 green fluorescent protein positive porcine bone marrow mesenchymal stem cells (pBM-MSCs) [18]. The decellularized human penis graft received 1x106 cells/cm2 human adipose-derived stromal vascular cells[19]. The decellularized porcine groin flaps were perfused with 40x106 millions HUVECs before orthotopic transplantation[13].
Cell seeding on human face grafts was performed statically by injecting 50x104 NIH-3T3 dermal fibroblast and 4x106 C2C12 myoblast progenitor cells[14]. Jank et al. first injected 10x106 HUVECs on day 0 in 20 injections, followed by injection of HUVECs, mouse embryonic fibroblasts and C2C12 on day 2[11]. To re-endothelialize the decellularized rat groin flaps, Zhang et al. injected 2x105 HUVECs in 200 µl medium into the artery and vein followed by 1x106 hASCs and 2x105 HUVECs into 10 different sites throughout the flaps [12].

In a slightly different approach, in vivo recellularization through implantation was reported for porcine ear scaffolds[18]. The authors noted that the scaffolds were infiltrated with a significant number of cells with signs of vascularization. Similarly, decellularized human ear scaffolds displayed vascular infiltration upon subcutaneous implantation[15]. The repopulation of implanted VCA scaffolds was also observed by Jank et al. when a 6-mm decellularized skin flap scaffold was sutured in a full thickness skin defect in a rat model[13]. When the implants were tested for cellular infiltration via histology, and immunohistochemistry 14 days after implantation, the scaffolds were found to have extensive cellular infiltration, local inflammation (CD68+ and CD3+ by IHC) and neovascularization. The tissue morphology was retained. Altogether, the results indicated a fully integrated graft, with neovascularization and a fully regenerated epidermis with an efficient barrier function.

Evaluation of recellularized VCAs

The recellularized VCAs were assessed for cell engraftment via histology (H&E or DAPI staining) and quantification of cells via image analysis of stained sections[12, 15, 17]. The cell proliferation was tested via reduction of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by viable cells[15], live/dead assay based on calcein AM/ethidium homodimer-1 staining[12, 15, 17, 19] and/or via Ki67 staining for proliferating cells[18]. The recellularized VCAs were also stained via IHC and assayed via quantitative reverse transcriptase polymerase chain reaction for collagen (ECM), von Willebrand factor, CD31 (endothelial cells), SM22 and desmin (smooth muscle cells) for evaluation of cellular distribution and confirm the presence of the cells[12, 15, 17, 18]. Jank et al. attempted to reconstruct an epidermis in vitro by cultivating human keratinocytes on decellularized skin flaps during a 7-day culture. The epidermal stratification was induced by raising the scaffold seeded with cells to an air/liquid

interface. Successful formation of an epidermal layer was reported as confirmed by biotin permeability assay[13]. Some limitations of various recellularization protocols have been noted. Although the attachment and proliferation of cells within the scaffolds was shown, the cell density was found to be low[15]. Migration of cells within scaffolds was only observed with fibroblasts seeded statically but not in perfusion seeded cells[14]. Jank et al. reported that recellularization through vascular infusion was not suitable for muscle repopulation because of the marginal distribution of myoblasts into muscle ECM with the majority of cells being retained in the vascular conduit[11]. When the same group injected cells into the muscle ECM (with a surgical microscope), they found good engraftment of cells and muscle like tissue formation but also observed local mechanical disruption of the matrix due to the injection pressure. They also noted that the microcirculation was disturbed with areas of cell apoptosis near the injection site[13]. Endothelization of the vascular bed was achieved in vessels down to 300 μm but microvasculature and capillary beds with much smaller vascular diameters (6 μm) were not endothelialized[13].

3.2.5 Transplantation

Fives studies attempted transplantation of decellularized and/or recellularized VCAs in animals (Table 1)[11-15]. Systemic transplantation involved the anastomotic connection of the scaffold's vessels with those of the recipient (manual or with cuff). The operations were performed under general anesthesia. The transplantations were often orthotopic [11-13, 18] but could be performed heterotopically[14]. In two of the reports, a recellularized VCA was transplanted; a porcine skip flap reendothelized with HUVECs[13] and a rat flap scaffold recellularized with HUVECs and hASCs[12]. Duisit et al.[14, 18] and Jank et al. [11] transplanted decellularized VCAs without the addition of cells.

The transplantation of VCAs were mostly terminated by vascular thrombosis with no immediate venous return[13], at 3 h[15], at 4 h[14] post transplantation. Only one study reported long-term survival of the engineered VCA after transplantation[12]. The study was ended at 3 months for analysis of the recellularized grafts via histology and immunohistochemistry which showed neovascularization and a constructive remodeling with predominant infiltration of M2 macrophages along with significant adipose tissue formation within the scaffold.

4. DISCUSSION

VCAs are complex tissues used to restore anatomically complex defects that cannot be addressed by autologous graft reconstruction and are an emerging feasible tool in reconstructive surgery[38, 39]. Biological scaffolds, which are composed of decellularized extracellular matrices, have long been in use in plastic surgery to repair various tissues, including the skin[40]. In addition, several such scaffolds have received FDA approval for clinical use in humans including decellularized dermal tissue (Alloderm®; LifeCell) and decellularized porcine bladder (bladder matrix; ACell) for wound healing[41, 42]. Although quite a few skin substitutes and dermal matrices have been produced and commercialized, none has truly functioned as full-thickness skin. Indeed, VCAs differ from these commercially available scaffolds by their structural complexity, which poses a real

challenge in the need to functionally replace multiple tissue types in a single unit and the need for transplantation with microsurgical anastomoses. To date, no decellularized and/or recellularized VCA has been successfully transplanted in humans[43] and only one study reports successful transplantation of an engineered VCA in a rat[12]. In this systematic literature review, we analyzed 11 reports of VCA decellularization and recellularization that differ in the type of VCA and its origin. We compared the reports in terms of scaffold preparation methods, recellularization methodologies and their transplantation.

The decellularization protocols differ depending on the tissue origin and size but in general decellularization is effectively achieved using a strong ionic detergent, SDS. The duration of exposure to the detergents is determined by the size of the VCA such that larger tissues require longer exposure times. Exposing the tissue to mechanical forces such as perfusion or gentle agitation during the detergent treatment aids in the effectiveness of cell removal. However, the mechanical forces applied during decellularization should not be too aggressive to preserve the components of the ECM[44]. Indeed, the preservation of ECM proteins plays an essential role in maintaining the mechanical properties of the decellularized scaffold and facilitates appropriate tissue integration[45].

An ideal decellularization protocol should not disrupt the vascular architecture of the VCA which is necessary for the successful perfusion of the engineered grafts and would enable better recellularization and transplantation outcomes. The maintenance of an intact vasculature is one of the biggest challenges in whole organ engineering. A functional vasculature facilitates the delivery of oxygen and nutrients to the different cells throughout the bioengineered tissue and is essential to maintain the viability of the developing tissue in vitro and in vivo. A lack of vascularity leads to metabolically inactive cells and necrotic tissue. Therefore, obtaining a scaffold with an intact and functional vasculature is critical for successful organ engineering[46]. Compared with the vessels of solid organs, the vasculature present in the dermis of a VCAs is thinner[47] and exceptional care must be taken to avoid damage to the microcapillaries. High concentrations of detergents and high perfusion pressures can lead to the destruction of the microvasculature[14]. Maintaining a maximum pressure of 80mmHg during detergent perfusion was found to be ideal for VCA decellularization.

The characterization of decellularized ECM scaffolds has been focused on confirming the removal cells and DNA and preservation of ECM components by histological and biochemical assays. Most studies report successful decellularization with minimal removal of ECM components. A very limited number of studies examine the removal of the detergents from the final decellularized graft[24]. A final extensive washing step with deionized water and phosphate buffered saline is required to ensure the removal of the chemical agents. In addition to removal of remaining detergents and nuclear material, the removal of cellular proteins abundantly found in the native tissue should also be confirmed via immunohistochemistry or western blots[48]. One limitation of the studies reviewed here is that the immunological response to the decellularized VCA scaffolds has been poorly studied. This is concerning because the skin is the most immunogenic organ

of the human body[49] and the non-immunogenicity of the decellularized skin should be established prior to further evaluations.

Recellularization is challenging with composite tissues like VCA because it requires repopulation with multiple cell types in different compartments to form a complex cellular architecture[46]. The large number of cells needed to regenerate the clinical size complex tissues also represents a major hurdle on the path towards successful recellularization in vitro[50]. Adult tissue stem cells, such as the adipose derived stem cells and mesenchymal stem cells, are considered to hold great potential in repopulating the engineered VCA due to their potential to generate the different cell types. In addition, these stem cells not only directly participate in mesenchymal tissue regeneration but also modulate the host immune response to the engineered constructs[12, 51-56]. Indeed, Zhang et al. advocates that hASCs and decellularized skin flaps worked synergistically to activate M2 macrophage polarization and provide a pro-regenerative environment for adipose tissue formation[12]. Other cell types that have not been tested so far in VCA repopulation are lymphatic endothelial cells which hold great promise to engineer functional lymphatic vessels in hydrogel-based skin implants[57] and induced pluripotent stem cells which allows for generating patient specific engineered VCA with minimal to no immunological rejection[57]. Regarding the methodology of scaffold repopulation, it will be necessary to make further assessments to understand whether static seeding or dynamic perfusion is more effective. Perfusion pressure is critical to distribute the cells into the tissue in dynamic seeding such that the perfusion pressure should be high enough to force the cells to go into the extravascular space but moderate enough not to create ECM damage. Likewise, static seeding through multiple injections of cells into the tissue may achieve uniform distribution of the cells in anatomically correct positions, but it has been reported that injection causes localized ECM damage. It is expected that partial recellularization ex vivo (e.g., endothelialization and skin repopulation) followed by maturation in vivo would achieve a functional graft upon transplantation since a VCA does not need to be fully functional before implantation unlike solid organs such as kidney, liver, heart, lung.

5. CONCLUSION

The decellularization of VCAs is a growing, emerging developing field. The immunological response of the decellularized scaffolds remains poorly evaluated. Few authors have attempted the recellularization and transplantation of these scaffolds, highlighting the difficulty of obtaining a scaffold that has been decellularized and properly recellularized. Establishing of standardized assays with well-defined endpoints is necessary for accurate estimation of the quality and success of the engineered grafts. Overall, although results so far are promising, successful use of VCAs in clinic will require more extensive evaluation of immunological response to decellularized scaffolds following decellularization, effective re-endothelialization, and complete recellularization with multiple cell types.

REFERENCES

1. Diaz-Siso, J.R., Borab, Z.M., Plana, N.M., et al. Vascularized Composite Allotransplantation: Alternatives and Catch-22s. Plast Reconstr Surg 142, 1320, 2018.
2. Dubernard, J.M., Owen, E., Herzberg, G., et al. Human hand allograft: report on first 6 months. Lancet 353, 1315, 1999.
3. Petruzzo, P., and Dubernard, J.M. The International Registry on Hand and Composite Tissue allotransplantation. Clin Transpl, 247, 2011.
4. Howsare, M., Jones, C.M., and Ramirez, A.M. Immunosuppression maintenance in vascularized composite allotransplantation: what is just right? Curr Opin Organ Transplant 22, 463, 2017.
5. Badylak, S.F., Taylor, D., and Uygun, K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. Annu Rev Biomed Eng 13, 27, 2011.
6. Gilpin, A., and Yang, Y. Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications. Biomed Res Int 2017, 9831534, 2017.
7. Uygun, B.E., Yarmush, M.L., and Uygun, K. Application of whole-organ tissue engineering in hepatology. Nat Rev Gastroenterol Hepatol 9, 738, 2012.
8. Acun, A., Oganesyan, R., and Uygun, B.E. Liver Bioengineering: Promise, Pitfalls, and Hurdles to Overcome. Curr Transplant Rep 6, 119, 2019.
9. Henderson, P.W., Nagineni, V.V., Harper, A., et al. Development of an acellular bioengineered matrix with a dominant vascular pedicle. J Surg Res 164, 1, 2010.
10. Qu, J., Van Hogezand, R.M., Zhao, C., Kuo, B.J., and Carlsen, B.T. Decellularization of a Fasciocutaneous Flap for Use as a Perfusable Scaffold. Ann Plast Surg 75, 112, 2015.
11. Jank, B.J., Xiong, L., Moser, P.T., et al. Engineered composite tissue as a bioartificial limb graft. Biomaterials 61, 246, 2015.
12. Zhang, Q., Johnson, J.A., Dunne, L.W., et al. Decellularized skin/adipose tissue flap matrix for engineering vascularized composite soft tissue flaps. Acta Biomater 35, 166, 2016.
13. Jank, B.J., Goverman, J., Guyette, J.P., et al. Creation of a Bioengineered Skin Flap Scaffold with a Perfusable Vascular Pedicle. Tissue Eng Part A 23, 696, 2017.
14. Duisit, J., Maistriaux, L., Taddeo, A., et al. Bioengineering a Human Face Graft: The Matrix of Identity. Ann Surg 266, 754, 2017.
15. Duisit, J., Amiel, H., Wuthrich, T., et al. Perfusion-decellularization of human ear grafts enables ECM-based scaffolds for auricular vascularized composite tissue engineering. Acta Biomater 73, 339, 2018.
16. Gerli, M.F.M., Guyette, J.P., Evangelista-Leite, D., Ghoshhajra, B.B., and Ott, H.C. Perfusion decellularization of a human limb: A novel platform for composite tissue engineering and reconstructive surgery. PLoS One 13, e0191497, 2018.
17. Duisit, J., Amiel, H., Orlando, G., et al. Face Graft Scaffold Production in a Rat Model. Plast Reconstr Surg 141, 95, 2018.
18. Duisit, J., Orlando, G., Debluts, D., et al. Decellularization of the Porcine Ear Generates a Biocompatible, Nonimmunogenic Extracellular Matrix Platform for Face Subunit Bioengineering. Ann Surg 267, 1191, 2018.
19. Tan, Y., Landford, W.N., Garza, M., et al. Complete Human Penile Scaffold for Composite Tissue Engineering: Organ Decellularization and Characterization. Sci Rep 9, 16368, 2019.
20. McDiarmid, S.V., and Azari, K.K. Donor-related issues in hand transplantation. Hand Clin 27, 545, 2011.
21. Ott, H.C., Clippinger, B., Conrad, C., et al. Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med 16, 927, 2010.
22. Peloso, A., Petrosyan, A., Da Sacco, S., et al. Renal Extracellular Matrix Scaffolds From Discarded Kidneys Maintain Glomerular Morphometry and Vascular Resilience and Retains Critical Growth Factors. Transplantation 99, 1807, 2015.
23. Peloso, A., Urbani, L., Cravedi, P., et al. The Human Pancreas as a Source of Protolerogenic Extracellular Matrix Scaffold for a New-generation Bioartificial Endocrine Pancreas. Ann Surg 264, 169, 2016.
24. Uygun, B.E., Soto-Gutierrez, A., Yagi, H., et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16, 814, 2010.
25. Rohrich, R.J., and Pessa, J.E. The fat compartments of the face: anatomy and clinical implications for cosmetic surgery. Plast Reconstr Surg 119, 2219, 2007.
26. Flynn, L.E. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31, 4715, 2010.
27. Porzionato, A., Sfriso, M.M., Macchi, V., et al. Decellularized omentum as novel biologic scaffold for reconstructive surgery and regenerative medicine. Eur J Histochem 57, e4, 2013.
28. Badylak, S.F. Decellularized allogeneic and xenogeneic tissue as a bioscaffold for regenerative medicine: factors that influence the host response. Ann Biomed Eng 42, 1517, 2014.
29. Crapo, P.M., Gilbert, T.W., and Badylak, S.F. An overview of tissue and whole organ decellularization processes. Biomaterials 32, 3233, 2011.

30. Philips, C., Cornelissen, M., and Carriel, V. Evaluation methods as quality control in the generation of decellularized peripheral nerve allografts. J Neural Eng 15, 021003, 2018.
31. Rijal, G. The decellularized extracellular matrix in regenerative medicine. Regen Med 12, 475, 2017.
32. Chaudhuri, O., Koshy, S.T., Branco da Cunha, C., et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. Nat Mater 13, 970, 2014.
33. Seddon, A.M., Curnow, P., and Booth, P.J. Membrane proteins, lipids and detergents: not just a soap opera. Biochim Biophys Acta 1666, 105, 2004.
34. Garcia-Gareta, E., Abduldaiem, Y., Sawadkar, P., et al. Decellularised scaffolds: just a framework? Current knowledge and future directions. J Tissue Eng 11, 2041731420942903, 2020.
35. Acun, A., Oganesyan, R., Uygun, K., et al. Liver donor age affects hepatocyte function through age-dependent changes in decellularized liver matrix. Biomaterials 270, 120689, 2021.
36. Li, Q., Chang, Z., Oliveira, G., et al. Protein turnover during in vitro tissue engineering. Biomaterials 81, 104, 2016.
37. McPherson, T.B., Liang, H., Record, R.D., and Badylak, S.F. Galalpha(1,3)Gal epitope in porcine small intestinal submucosa. Tissue Eng 6, 233, 2000.
38. Cetrulo, C.L., Jr., Ng, Z.Y., Winograd, J.M., and Eberlin, K.R. The Advent of Vascularized Composite Allotransplantation. Clin Plast Surg 44, 425, 2017.
39. Chim, H., Amer, H., Mardini, S., and Moran, S.L. Vascularized composite allotransplant in the realm of regenerative plastic surgery. Mayo Clin Proc 89, 1009, 2014.
40. Livesey, S.A., Herndon, D.N., Hollyoak, M.A., Atkinson, Y.H., and Nag, A. Transplanted acellular allograft dermal matrix. Potential as a template for the reconstruction of viable dermis. Transplantation 60, 1, 1995.
41. Gilbert, T.W., Sellaro, T.L., and Badylak, S.F. Decellularization of tissues and organs. Biomaterials 27, 3675, 2006.
42. Yang, Q., Peng, J., Guo, Q., et al. A cartilage ECM-derived 3-D porous acellular matrix scaffold for in vivo cartilage tissue engineering with PKH26-labeled chondrogenic bone marrow-derived mesenchymal stem cells. Biomaterials 29, 2378, 2008.
43. Rajab, T.K., O'Malley, T.J., and Tchantchaleishvili, V. Decellularized scaffolds for tissue engineering: Current status and future perspective. Artif Organs 2020.
44. Freedman, B.R., Bade, N.D., Riggin, C.N., et al. The (dys)functional extracellular matrix. Biochim Biophys Acta 1853, 3153, 2015.
45. Chan, B.P., and Leong, K.W. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. Eur Spine J 17 Suppl 4, 467, 2008.
46. Lovett, M., Lee, K., Edwards, A., and Kaplan, D.L. Vascularization strategies for tissue engineering. Tissue Eng Part B Rev 15, 353, 2009.
47. Kanitakis, J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol 12, 390, 2002.
48. Geerts, S., Ozer, S., Jaramillo, M., Yarmush, M.L., and Uygun, B.E. Nondestructive Methods for Monitoring Cell Removal During Rat Liver Decellularization. Tissue Eng Part C Methods 22, 671, 2016.
49. Shimada, S., and Katz, S.I. The skin as an immunologic organ. Arch Pathol Lab Med 112, 231, 1988.
50. Pellegata, A.F., Tedeschi, A.M., and De Coppi, P. Whole Organ Tissue Vascularization: Engineering the Tree to Develop the Fruits. Front Bioeng Biotechnol 6, 56, 2018.
51. Brown, B.N., Valentin, J.E., Stewart-Akers, A.M., McCabe, G.P., and Badylak, S.F. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. Biomaterials 30, 1482, 2009.
52. Faulk, D.M., Londono, R., Wolf, M.T., et al. ECM hydrogel coating mitigates the chronic inflammatory response to polypropylene mesh. Biomaterials 35, 8585, 2014.
53. Fishman, J.M., Lowdell, M.W., Urbani, L., et al. Immunomodulatory effect of a decellularized skeletal muscle scaffold in a discordant xenotransplantation model. Proc Natl Acad Sci U S A 110, 14360, 2013.
54. Hanson, S.E., King, S.N., Kim, J., et al. The effect of mesenchymal stromal cell- hyaluronic acid hydrogel constructs on immunophenotype of macrophages. Tissue Eng Part A 17, 2463, 2011.
55. King, S.N., Hanson, S.E., Chen, X., et al. In vitro characterization of macrophage interaction with mesenchymal stromal cell-hyaluronan hydrogel constructs. J Biomed Mater Res A 102, 890, 2014.
56. Valles, G., Bensiamar, F., Crespo, L., et al. Topographical cues regulate the crosstalk between MSCs and macrophages. Biomaterials 37, 124, 2015.
57. Marino, D., Luginbuhl, J., Scola, S., Meuli, M., and Reichmann, E. Bioengineering dermo-epidermal skin grafts with blood and lymphatic capillaries. Sci Transl Med 6, 221ra14, 2014.

Figure Legends

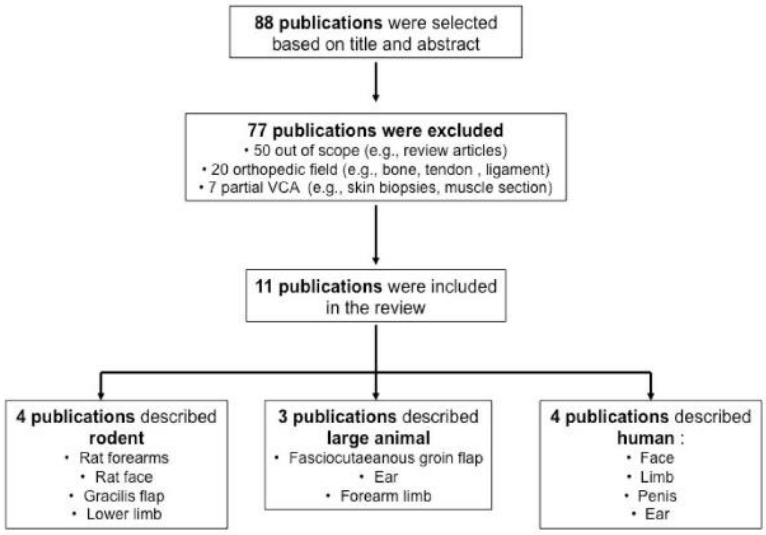


Figure 1. Flow Chart

CHAPTER 16

Acellular nipple scaffold development, characterization, and preliminary biocompatibility assessment in a swine model

Ruben V. Oganesyan
Alexandre G. Lellouch
Aylin Acun
Elise Lupon
Corentin B. Taveau
Laura C. Burlage
Laurent A. Lantieri
Curtis L. Cetrulo Jr.
Mark A. Randolph
Basak E. Uygun

In Press in PRS

ABSTRACT

Background: The gold standard in nipple reconstruction remains the autologous skin flap. Unfortunately the results are not satisfying with up to 75% loss of nipple projection over time.[1–8] Existing studies investigated the use of primates as a source of implants.[9,10] We hypothesized that porcine nipple can serve as a perfect shape-supporting implant due to functional similarities to human nipple.[11,12] Our group developed a decellularization protocol to obtain an acellular nipple scaffold (ANS) for nipple reconstruction.

Methods: Tissue samples were collected from 8 disease-free female Yorkshire pigs (60–70 kg) and then decellularized. The decellularization efficiency and extracellular matrix (ECM) characterization was performed histologically and quantitatively (DNA, total collagen, elastin, and glycosaminoglycan content). In vitro and in vivo biocompatibility was determined by human dermal fibroblast culture and subcutaneous implantation of six ANS in a single Yorkshire pig (60–70 kg) respectively. Inflammation and adverse events were monitored daily based on local clinical signs.

Results: We showed that all cellular structures and 96% of DNA (321.7±57.6 vs. 11.7±10.9 ng DNA/mg wet tissue, in native and ANS, respectively, $p<0.001$) can be successfully removed. However, this was associated with a decrease in collagen (89.0±11.4 and 58.8±9.6 μg collagen/mg ($p<0.001$)) and elastin (14.2±1.6 and 7.9±2.4 μg elastin/mg ($p<0.05$)) and increase in GAG content (5.0±0.7 and 6.0 ± 0.8 ng/mg ($p<0.05$)). ANS can support continuous cell growth in vitro and during preliminary biocompatibility tests in vivo.

Conclusions: This is a preliminary report a novel promising ANS for nipple reconstruction, but more research is needed to validate results.

INTRODUCTION

In 2017, according to the American Society of Plastic Surgery, over 106,000 breast reconstructions were performed.[13,14] A significant number of patients undergo further nipple reconstructions since this procedure gives patient a sense of completeness and patients report higher general (72.2% vs 52.8%, P<0.0001) and aesthetic (70.5% vs. 46.5%, P<0.0001) satisfaction scores compared to patients without nipple reconstruction.[15–19] The most common methods for nipple-areolar complex reconstruction are limited to autologous local skin flaps, secondary site grafting, and 3D tattooing.[20–23] These strategies provide a highly variable long-term results.[1–7] In existing reports the loss of projection ranges from 32 to 71.3% with the use of C-V flaps over the course of 1-3.2 years.[1,24–26] Almost 36% of patients who underwent nipple reconstruction were dissatisfied because of inadequate nipple projection.[17,27] Alternative approaches based on implantation of solid materials have also been explored.[3,28–32] However, it was shown that all these methods still lead to significant nipple mount reduction.[33] Winocour et al. showed that nipple projection was decreased by 22.8-48% with hard implants like auricular cartilage, acellular dermal matrix, costal cartilage, collagen cylinders, and silicone rods.[33] Moreover, these implants had relatively high complication rates of 4% (18/454) due to ischemic flap necrosis.[3] Synthetic hard materials such as silicone rods had even higher complication rates of 33%.[34,33]

Alternative approaches based on implantation of bioengineered structures become more attractive. The bioengineering technique known as decellularization utilized to generate acellular scaffolds has proven to be safe, as commercially available products based on this technology for skin reconstruction (AlloDerm®) are widely available.[35–37] However, it lead to 50-70% loss of nipple projection at the 1-year follow up.[38,39]

In a recent study by Pashos et al. rhesus macaque nipple areolar complexes were used to create decellularized nipple scaffolds with promising results.[9,10] The apical portion of decellularized nipples were removed and onlay engrafted onto each surgically prepared dermal bed on a dorsum of the animal and were explanted at 1-, 3-, and 6-weeks post engraftment. However, no evaluation of mechanical properties and shape retention was done and use of a rhesus macaque as a source of nipple implants can become a limiting factor in the future because of its relatively high price.[40]

The potential biological healing mechanism of acellular scaffold integration was described by Johnson et al.[41]

We hypothesize that ANS is an ideal graft for nipple reconstruction due to its microarchitecture and material composition. We describe a novel decellularization protocol using optimized de-epithelization method of swine nipples to obtain a tissue-engineered ANS. Our approach addresses limitations of the previous studies by providing a more solid support to the nipple mount than commonly used C-V flap, more site specific and dense tissue than Alloderm®.

METHODS

Overall study design

In the first part of the study nipple samples were harvested, de-epithelized using an optimized protocol, and then decellularized to get ANS. The primary and secondary endpoints were achieving complete decellularization of the tissue (less than 50ng of double-stranded DNA per mg of dry weight, and no visible nuclear material in histological analysis with DAPI or H&E.[42] In the second part of the study, the ANS was characterized qualitatively and quantitatively for ensuring preservation of ECM components and architecture. The biocompatibility of the ANS was assessed in vitro and in vivo in a swine model. The endpoint of ECM characterization was qualitative and quantitative ECM analysis, in vitro and in vivo ANS biocompatibility assessment. The study overview is depicted in Figure 1.

Animals and nipple harvest

All animal care and surgical procedures were in accordance with the local Institutional Animal Care and Use Committee (IACUC). Tissue samples were collected from 8 disease-free female Yorkshire pigs (Cummings School of Veterinary Medicine, Tufts University, Grafton MA) weighing 60-70kg. A circle shaped skin incision was made, and the nipples were harvested with subcutaneous fat tissue stored in sterile PBS.

Decellularization of pig nipples

Pig nipples 25-30 mm in size were washed in sterile phosphate buffered saline (PBS) solution for 30 min and then decellularized according to a newly developed protocol, involving initial decellularization, de-epithelialization and final gradual decellularization. We employed a protocol by Chen et al. as a starting point for our new nipple decellularization protocol.[43] Since the epithelial layer is relatively impermeable to detergents, we hypothesized that de-epithelization would allow better penetration of decellularization solutions. We optimized the de-epithelization of nipples by testing different methods described in a study by Purohit et al.[44] (Supplementary Table 1).

First, nipples were washed with deionized water (dH2O) for 12h, then incubated in 0.2% (w/v) sodium dodecyl sulfate (SDS) for 48h, then in de-epithelialization solution containing 0.5M sodium chloride (NaCl), 0.5% (w/v) SDS, 0.25% ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA), and 50mM Tris buffer (T60050, Research Products International, Mt Prospect, IL, USA), followed by 2M NaCl solution for 24h at room temperature (RT) and then manually de-epithelized. Nipples were further incubated in a series of detergent and enzyme solutions as follows: 0.2% SDS for 72h, 0.5% SDS for 24h, 1% Triton X-100 for 48h, PBS for 6h followed by 1h incubation in 1mg/ml DNAse I (Sigma-Aldrich, St. Louis, MO, USA) with addition of 5mM Mg2+ as an activator for 6h. Finally, ANS were extensively washed in PBS overnight and stored in a PBS solution containing 2% penicillin and streptomycin at 4°C until use. All incubations during decellularization process were done at RT with constant agitation at 240rpm unless otherwise noted.

DNA quantification and DAPI staining

Using a 3-mm surgical biopsy punch, 3 wet acellular and 3 wet native (controls) nipples were sampled at three random locations (a total of 18 samples). The samples were digested with proteinase K for 6h at 56°C and processed using Qiagen Dneasy Blood & Tissue Kit (Thermofisher Scientific, Waltham, MA, US) to extract the DNA followed by purification using QIAprep 2.0 Spin Miniprep Columns (Thermofisher Scientific, Waltham, MA, USA). The purified DNA in each sample was then quantified using Nanodrop (Thermofisher Scientific, Waltham, MA, US). Separate tissue samples (3 native and 3 decellularized) were snap frozen, embedded in OCT and sectioned. Ten micrometer tissue sections were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for the visualization of intact nuclei. DAPI stained sections were imaged using an Invitrogen™ EVOS™ FL Imaging System (Leica Microsystems, Buffalo Grove, IL, USA).

Biochemical assays for collagen, glycosaminoglycan, and elastin quantification
Using a 3-mm surgical biopsy punch, 3 wet acellular and 3 wet native nipples were sampled at three random locations (18 samples) for each of the biochemical assays. Total collagen content was measured using the total collagen kit (QuickZyme Biosciences, Leiden, The Netherlands), glycosaminoglycan (GAG) content was measured via a colorimetric assay following a modified protocol by Farndale et al.[45], and elastin content was quantified using the Fastin Elastin Assay Kit (Biocolor Life Science Assays, Carrickfergus, UK) following manufacturer's recommendations.

Histology and immunohistochemistry

The native (n=3) and acellular (n=3) nipple tissue samples were fixed with 10% neutral buffered formalin for 24h, dehydrated, embedded in paraffin, micro sectioned (5µm) and processed for staining with hematoxylin and eosin (H&E), Masson's Trichrome, Verhoeff's Van Gieson. Alcian blue staining for GAGs was completed with Alcian blue 8GX (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with Nuclear-fast red (Sigma-Aldrich, St. Louis, MO, USA). Immunohistochemical (IHC) staining was done using snap frozen samples (n=3) sectioned at 5µm. All histological sections were imaged using Nikon Eclipse E800 microscope (10X and 20X magnification) and then analyzed qualitatively. Moreover, H&E (3 native and 3 decellularized) and DAPI (3 native and 3 decellularized) images were analyzed quantitatively by counting cell nuclei using ImageJ ITCN plug-in.[46,47] IHC images (Collagen IV (3 images), Laminin (3 images)) were analyzed quantitatively by measuring % of stained area using ImageJ software. [48]

Scanning electron microscopy

Electron microscopy was done at the Schepens Eye Institute core facility supported by NIH National Eye Institute Core Grant #P30EY003790. Briefly, samples were dehydrated in graded ethanol solutions and critical point dried using a Samdri 795 Critical Point Dryer (Tousimis, Rockville, Md USA), then mounted onto aluminum stubs and chromium coated with a Gatan high Resolution lon Beam Coater (Gatan Inc, Pleasanton, CA USA). Samples were imaged using a JEOL JSM-7401F Field Emission Scanning Electron Microscope (JEOL Inc, Peabody, MA USA) to provide a qualitative assessment of the scaffold architecture after decellularization.

In vitro testing of ANS

Six ANS samples were cut using surgical 6-mm biopsy punch and were sterilized according to a previously published method.39 Samples were placed into the wells of an ultralow attachment 96-well plate, 3 on the dermal side, and 3 on the epithelial side in preparation for cell seeding. The scaffolds were preconditioned with fibroblast basal medium supplemented with serum (ATCC, Manassas, VA, US) in a cell culture incubator (37oC, 5% CO2) for 30 min prior to seeding. Primary human dermal fibroblasts (ATCC, Manassas, VA, US) were cultured in fibroblast basal medium (ATCC, Manassas, VA, US) supplemented with low serum fibroblast growth kit (ATCC, Manassas, VA, US). The cells were collected using 0.05 % trypsin-EDTA (Thermofisher Scientific, Waltham, MA, US) and resuspended in culture media for seeding on the ANS. Cells were seeded at 6x105 cells/scaffold for achieving full coverage of the scaffolds, cultured for 3 days, and histologically analyzed for overall cell coverage of the scaffolds. In addition, to monitor cell proliferation on the scaffolds, cells were seeded at a density of 4.5x104 cells/scaffold. The cells were cultured for 72h at 37oC and 5% CO2. The growth of the cells was measured via Presto Blue assay (Thermofisher Scientific, Waltham, MA, US) performed daily following manufacturer's instructions. At the end of the 72h culture, the scaffolds were fixed in 10% neutral buffered formalin and further processed for histology to qualitatively assess the morphology of the cells.

In vivo implantation

For this experiment 1 pig was positioned in prone position, so the ANS could be implanted on its back. Six subcutaneous pockets were prepared for 6 ANS and were spaced at least 4 cm apart from each other. The samples were sutured with the dorsal side on to the subcutaneous flap and pockets were closed using absorbable sutures. Scaffold implantation sites were monitored daily and photographed in a standardized manner (magnification / angle) every day for the 3 weeks (end of study). The implantation sites were assessed qualitatively for clinical signs of inflammation (edema, redness) and adverse events (necrosis) by Board-certified Plastic surgeons (AGL, CLC). In a study by Klar et al. was shown that 3 weeks was sufficient for a complete vascular network formation so we used 3 weeks as a final time point, at which the samples were explanted and analyzed histologically.[49]

Statistical Analysis

All experiments were done in three or more replicates. Numerical values are presented as the mean ± standard error of the mean (SEM). After confirming that the datasets were normally distributed using Shapiro-Wilk normality test, statistical significance was

Acellular nipple scaffold development, characterization, and preliminary biocompatibility assessment in a swine model

determined by 2-tailed student's t-test. Difference with a p-value less than or equal to 0.05 was considered statistically significant. All statistical analysis was completed using GraphPad Prism version 8.3.1. For all our experiments a sample size of minimum 3 native and 3 ANS was used to reach statistical significance. The semiquantitative measurements on histological sections were done blinded.

RESULTS

ANS was obtained using a new decellularization protocol.
The efficiency of NaCl solution increased with increasing concentration, prolonged time of exposure, and agitation. 2M NaCl demonstrated the highest de-epithelization efficiency at RT after 24h of exposure, thus we proceeded with this de-epithelization solution. In order to reduce the time of exposure of basement membrane to decellularization solutions, we kept the de-epithelization procedure as the second step of the decellularization protocol and first decellularized the dermal side of the tissue followed by de-epithelization which subsequently exposes the BM to the decellularization solutions. We first washed the nipples with dH2O for 12h, then incubated in 0.2% (w/v) SDS for 48h. As the second step we incubated the samples in the de-epithelization solution containing 2M NaCl, 0.25% EDTA, and 50mM Tris buffer. However, we observed a drastic decrease in the efficiency of our de-epithelization solution following the first step. To overcome this issue, we further optimized our protocol and added a pretreatment solution (0.5M NaCl, 0.5% (w/v) SDS, 0.25% EDTA acid, and 50mM Tris) before applying our de-epithelization solution. The final protocol is given in Table 1.

As a result, nipples were successfully decellularized evidenced by their uniform white color (Figure 2, 3). Complete cell removal was confirmed by H&E (Figure 4, 5) and DAPI staining (Figure 6, 7). Quantitative assessment of histological images showed significant decrease in the number of nuclei in H&E (8798±656 vs. 1 in native samples and ANS respectively, $p<0.001$), and DAPI (2240±312 vs. 1 in native samples and ANS samples respectively, $p=0.027$). Quantitative assessment of DNA content confirmed successful decellularization with removal of 96% of DNA (321.7±33.3 vs. 11.7±6.3 ng DNA/mg wet tissue, in native and acellular tissues, respectively, $p<0.001$) (Figure 8).

ECM components and microarchitecture are preserved after decellularization.
Preservation of ECM components is crucial for cell attachment, proliferation, and function. We observed only a slight decrease in the staining intensity in the ANS compared to native samples in Masson's trichrome staining (Supplementary Figure 1, 2), similar level of color intensity for GAGs in Alcian staining (data not shown), and a decrease in color intensity in Verhoeff's Van Gieson staining (Supplementary Figure 3, 4).

We confirmed our histological observations by quantitative assays determining the total collagen content, which demonstrated a decreased amount of collagen (Figure 9). Native and ANS contained 89.0±6.6 and 58.8±5.5 μg collagen/mg wet tissue, respectively ($p<0.001$). There was a significant increase in GAG content after decellularization, 5.2±0.4 μg/mg total amount for the native tissue and 6.1±0.5 μg/mg total amount for the ANS ($p=0.022$) (Figure 10). Lastly, elastin content assay showed a significant reduction in elastin content from 14.2±0.9 μg/mg of wet tissue in native samples to 7.9±1.4 μg/mg in ANS ($p<0.001$) (Figure 11). Scanning electron microscopy images demonstrated preservation of dermal rete ridges following de-epithelization procedure. (Supplementary Figure 5, 6). IHC staining displayed pronounced expression of collagen IV in both native and acellular samples with no difference in collagen IV distribution (Figure 12, 13). Laminin was found to be present throughout the matrix of both the native and acellular samples with concentrated staining in the basement membrane lining and around the large blood vessels (Figure 14, 15). Quantitative assessment of IHC images showed no significant difference in percent of collagen IV (3.96±0.43 vs. 3.46±0.13 in

native and ANS respectively, p=0.458) and laminin-stained area (4.53±0.13 vs. 3.44±0.39 in native and ANS respectively, p=0.164).

ANS support cell growth in vitro.

We seeded the nipples with dermal fibroblasts on the epithelial (3 samples at each time point) or dermal side (3 samples at each time point) to test biocompatibility. Histological analysis of the scaffolds seeded with 6x105 cells/scaffold on day 3 of culture demonstrated a full coverage of the epithelial surface (Figure 16). In addition, to monitor cell proliferation, cells were seeded at a density of 4.5x104 cells/scaffold. We observed a 5.66±0.55 and 5.72±0.18 -fold increase in the number of cells after 2 days of culture and 8.21±1.07 and 7.64±0.74-fold after 3 days on the epithelial and dermal surfaces, respectively (Figure 17).

ANS show in vivo biocompatibility upon implantation in swine.

Bioactive properties of the ANS were assessed for cell infiltration after 3 weeks. Macroscopically, there were no clinical signs of inflammation during the course postoperative observation. The implanted samples were found to be surrounded by the host connective tissue and none of the six implanted samples showed any sign of inflammatory tissue reaction (Figure 18). ANS implants retained their shape and size and have not been subjected to fibrosis and deformation (Figure 19). Histological examination of the obtained samples revealed extensive cellular infiltration from both dermal (data not shown) and epithelial sides of the scaffold. (Figure 20).

DISCUSSION

Decellularization of nipple tissues posed distinct challenges, which included the lack of an accessible vasculature for the distribution of decellularization solutions, relatively impermeable epidermal layer, and thick subdermal fat layer. Livesey et al. and Chakrabarty et al. used 1M NaCl for human skin de-epithelization, whereas Purohit at el. applied 2% trypsin. In our study, we achieved complete de-epithelization using NaCl solution at a higher concentration (2M) compared to previous studies. This difference can be explained by high tissue density and thickness compared to the skin samples. Pashos et al. did not apply de-epithelization as part of their decellularization protocol, which might have required the use of aggressive decellularization agents leading to the damage of the ECM.[9] We demonstrated that the nipple samples were successfully decellularized with over 96% decrease in DNA content (321.7±33.3 vs. 11.7±6.3 ng DNA/mg wet tissue, in native and ANS, respectively, p<0.001), which was similar to results shown by Pashos et al. (1,905±422 ng DNA/mg lyophilized tissue vs. 56.51±8.45 ng/mg in decellularized NAC). The amount of residual DNA in ANS was even smaller than in commercially available acellular products, such as AlloDerm®, which had 273±169 ng DNA/mg dry tissue weight.[50–52] This shows that ANS will have a minimal risk for immune-mediated implant rejection and inflammatory response.[42]

ECM components promote cellular proliferation, contribute to cell function, and play an important role in mechanical properties of the scaffold.[53–57] In this study, the scaffolds' chemical components were mainly preserved, however there was a decrease in the collagen (from 89.0±6.6 to 58.8±5.5 μg collagen/mg wet tissue, in native and ANS

respectively, p<0.001) which is a common consequence of detergent based decellularization process.[58,59] Pashos et al. also reported changes in ECM contents, with a non-significant increase in average collagen content of 46.12±13.88 to 128.57±55.05 μg collagen/mg dry sample for native and decellularized NAC, respectively. In our study there was a significant increase in GAG content after decellularization, 5.2±0.4 μg/mg total protein and 6.1±0.5 μg/mg total protein in native and ANS respectively (p=0.022), whereas in a study by Pashos et al. there was no difference between native and decellularized NAC (0.068±0.027 and 0.025±0.004, respectively). De-epithelization allowed us to use detergents at lower concentrations and resulted in less elastin loss compared to other studies (from 14.2±0.9 μg/mg of wet tissue in native samples to 7.9±1.4 μg/mg in ANS, p<0.001). For example, Pashos et al. reported more significant loss of elastin 25.03±0.93 to 7.77±2.84 μg/mg of dry tissue compared to 14.16±1.59 to 7.88±2.43 μg/mg of wet tissue in our study.[9] These differences in elastin preservation may be attributed to differences in decellularization detergents. Although all detergents used in decellularization dissolve the ECM components to some extent, SDS has been shown to be a relatively safer option leading to the greatest preservation of collagen, laminin, and several other extracellular matrix components in lungs compared to other detergents.[60] Moreover, Jank et al. showed that even significant decrease in elastin content (70% loss) did not lead to a change in biomechanical properties of the scaffold because it was restored by the recipient.[59,61] Collagen IV is the main component of basal membranes stimulating cell adhesion, migration, and differentiation.[62] In our study, collagen IV content and distribution are almost fully preserved in ANS along with laminin, which was found to be present throughout the matrix of both native and ANS with concentrated staining in the basement membrane and vessel lining. Quantitative assessment of IHC images showed no significant difference in percent of collagen IV (3.96±0.43 vs. 3.46±0.13 respectively, p=0.458) and laminin-stained area between native and ANS (4.53±0.13 vs. 3.44±0.39 respectively, p=0.164) (data not shown). These results imply that the basal membrane remained intact. Compared to a similar study by Pashos et al., we observed more intense immunohistochemical staining of laminin in the basement membrane region closely resembling the pattern seen in the native sample.
The biocompatibility and bioactivity of the ANS play a crucial role in proper recellularization and vascularization in vivo. We showed that ANS were not cytotoxic and supported cell proliferation in vitro and in vivo. Similar biocompatibility results were shown by Pashos et al. where the ability of these scaffolds to initiate host-mediated neovascularization and re-epithelialization in a murine model was tested.[10]

For future clinical application we suggest covering ANS with a split thickness skin graft and perform 2-step procedure with in vivo prevascularization for 3 weeks as performed with Integra® dermal regeneration template. Developed ANS could be used in both autologous and implant-based reconstruction.

We acknowledge some limitations of our study; first in our study we utilize the allogenic (swine to swine) model but in clinical setting our scaffold will be considered a xenograft (swine to human), so further tests on removal of pig-specific antigens and biocompatibility in a humanized model will be needed. Also, since our study was aimed on scaffold ECM assessment and preliminary biocompatibility tests, we did not analyze shape retention. Longer in vivo experiments would be necessary to analyze implant integration and safety/toxicity (local/systemic) in a long-term perspective.

CONCLUSIONS

We have demonstrated that with our novel protocol swine nipples can be decellularized effectively while preserving important ECM components. ANS could serve as a scaffold for tissue ingrowth and anatomical restoration of the nipple mount. However, our study shows only a preliminary evidence of ANS application, and more research is needed to validate results.

REFERENCES

1. Few JW, Marcus JR, Casas LA, Aitken ME, Redding J. Long-term predictable nipple projection following reconstruction. Plast Reconstr Surg. 1999;104(5):1321-1324. doi:10.1097/00006534-199910000-00012
2. Zhong T, Antony A, Cordeiro P. Surgical outcomes and nipple projection using the modified skate flap for nipple-areolar reconstruction in a series of 422 implant reconstructions. Ann Plast Surg. 2009;62(5):591-595. doi:10.1097/SAP.0b013e31819fb1c9
3. Guerra AB, Khoobehi K, Metzinger SE, Allen RJ. New technique for nipple areola reconstruction: arrow flap and rib cartilage graft for long-lasting nipple projection. Ann Plast Surg. 2003;50(1):31-37. doi:10.1097/00000637-200301000-00006
4. Sisti A, Grimaldi L, Tassinari J, et al. Nipple-areola complex reconstruction techniques: A literature review. Eur J Surg Oncol EJSO. 2016;42(4):441-465. doi:10.1016/j.ejso.2016.01.003
5. Butz DR, Kim EK, Song DH. C-Y Trilobed Flap for Improved Nipple-Areola Complex Reconstruction. Plast Reconstr Surg. 2015;136(2):234-237. doi:10.1097/PRS.0000000000001408
6. Yang JD, Ryu JY, Ryu DW, et al. Our Experiences in Nipple Reconstruction Using the Hammond flap. Arch Plast Surg. 2014;41(5):550-555. doi:10.5999/aps.2014.41.5.550
7. Elizabeth Clark S, Turton E. The CC-V Flap: A Novel Technique for Augmenting a C-V Nipple Reconstruction Using a Free Dermal Graft. World J Plast Surg. 2014;3(1):8-12.
8. Farhadi J, Maksvytyte GK, Schaefer DJ, Pierer G, Scheufler O. Reconstruction of the nipple-areola complex: an update. J Plast Reconstr Aesthet Surg. 2006;59(1):40-53. doi:10.1016/j.bjps.2005.08.006
9. Pashos NC, Scarritt ME, Eagle ZR, Gimble JM, Chaffin AE, Bunnell BA. Characterization of an Acellular Scaffold for a Tissue Engineering Approach to the Nipple-Areolar Complex Reconstruction. Cells Tissues Organs. 2017;203(3):183-193. doi:10.1159/000455070
10. Pashos NC, Graham DM, Burkett BJ, et al. Acellular Biologic Nipple–Areolar Complex Graft: In Vivo Murine and Nonhuman Primate Host Response Evaluation. Tissue Eng Part A. Published online February 28, 2020:ten.tea.2019.0222. doi:10.1089/ten.tea.2019.0222
11. Meyer W, Schwarz R, Neurand K. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. Curr Probl Dermatol. 1978;7:39-52. doi:10.1159/000401274
12. Barbero AM, Frasch HF. Pig and guinea pig skin as surrogates for human in vitro penetration studies: a quantitative review. Toxicol Vitro Int J Publ Assoc BIBRA. 2009;23(1):1-13. doi:10.1016/j.tiv.2008.10.008
13. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29. doi:10.3322/caac.20138
14. 2017 Plastic Surgery Statistics Report. Plast Surg. Published online 2017:25.
15. Clarkson DJ, Smith PM, Thorpe RJ, Daly JC. The use of custom-made external nipple-areolar prostheses following breast cancer reconstruction. J Plast Reconstr Aesthetic Surg JPRAS. 2011;64(4):e103-105. doi:10.1016/j.bjps.2010.12.016
16. Wellisch DK, Schain WS, Noone RB, Little JW. The psychological contribution of nipple addition in breast reconstruction. Plast Reconstr Surg. 1987;80(5):699-704. doi:10.1097/00006534-198711000-00007
17. Momoh A, Colakoglu S, Blacam C de, et al. The Impact of Nipple Reconstruction on Patient Satisfaction in Breast Reconstruction. Ann Plast Surg. 2012;69(4):389-393. doi:10.1097/SAP.0b013e318246e572
18. Buck DW, Shenaq D, Heyer K, Kato C, Kim JYS. Patient-subjective cosmetic outcomes following the varying stages of tissue expander breast reconstruction: the importance of completion. Breast Edinb Scotl. 2010;19(6):521-526. doi:10.1016/j.breast.2010.05.017
19. Shaikh-Naidu N, Preminger BA, Rogers K, Messina P, Gayle LB. Determinants of aesthetic satisfaction following TRAM and implant breast reconstruction. Ann Plast Surg. 2004;52(5):465-470; discussion 470. doi:10.1097/01.sap.0000123901.06133.b7
20. Halvorson EG, Cormican M, West ME, Myers V. Three-dimensional nipple-areola tattooing: a new technique with superior results. Plast Reconstr Surg. 2014;133(5):1073-1075. doi:10.1097/PRS.0000000000000144
21. Shay P, Jacobs J. Autologous reconstruction following nipple sparing mastectomy: a comprehensive review of the current literature. Gland Surg. 2018;7(3):316-324. doi:10.21037/gs.2018.05.03
22. Craft RO, May JW. Staged nipple reconstruction with vascularized SurgiMend acellular dermal matrix. Plast Reconstr Surg. 2011;127(6):148e-149e. doi:10.1097/PRS.0b013e3182131e74
23. Seaman BJ, Akbari SR, Davison SP. A novel technique for nipple-areola complex reconstruction: the acellular dermal matrix onlay graft. Plast Reconstr Surg. 2012;129(3):580e-581e. doi:10.1097/PRS.0b013e3182419c68
24. Valdatta L, Montemurro P, Tamborini F, Fidanza C, Gottardi A, Scamoni S. Our experience of nipple reconstruction using the C-V flap technique: 1 year evaluation. J Plast Reconstr Aesthet Surg. 2009;62(10):1293-1298. doi:10.1016/j.bjps.2008.03.064
25. Banducci DR, Le TK, Hughes KC. Long-Term Follow-up of a Modified Anton–Hartrampf Nipple Reconstruction. Ann Plast Surg. 1999;43(5):467-470.
26. Rubino C, Dessy LA, Posadinu A. A modified technique for nipple reconstruction: the 'arrow flap.' Br J Plast Surg. 2003;56(3):247-251. doi:10.1016/S0007-1226(03)00094-8
27. Jabor MA, Shayani P, Collins DR, Karas T, Cohen BE. Nipple-areola reconstruction: satisfaction and clinical determinants. Plast Reconstr Surg. 2002;110(2):457-463; discussion 464-465. doi:10.1097/00006534-200208000-00013

28. Collis N, Garrido A. Maintenance of nipple projection using auricular cartilage. Plast Reconstr Surg. 2000;105(6):2276-2277. doi:10.1097/00006534-200005000-00079
29. Jones AP, Erdmann M. Projection and patient satisfaction using the "Hamburger" nipple reconstruction technique. J Plast Reconstr Aesthetic Surg JPRAS. 2012;65(2):207-212. doi:10.1016/j.bjps.2011.09.014
30. Mori H, Uemura N, Okazaki M. Nipple reconstruction with banked costal cartilage after vertical-type skin-sparing mastectomy and deep inferior epigastric artery perforator flap. Breast Cancer Tokyo Jpn. 2015;22(1):95-97. doi:10.1007/s12282-011-0329-7
31. Tierney BP, Hodde JP, Changkuon DI. Biologic collagen cylinder with skate flap technique for nipple reconstruction. Plast Surg Int. 2014;2014:194087. doi:10.1155/2014/194087
32. Vidya R, Salem F, Iqbal FM, Sircar T. Acellular dermal matrix based nipple reconstruction: A modified technique. JPRAS Open. 2017;13:62-67. doi:10.1016/j.jpra.2017.05.004
33. Winocour S, Saksena A, Oh C, et al. A Systematic Review of Comparison of Autologous, Allogeneic, and Synthetic Augmentation Grafts in Nipple Reconstruction. Plast Reconstr Surg. 2016;137(1):14e-23e. doi:10.1097/PRS.0000000000001861
34. Jankau J, Jaśkiewicz J, Ankiewicz A. A new method for using a silicone rod for permanent nipple projection after breast reconstruction procedures. The Breast. 2011;20(2):124-128. doi:10.1016/j.breast.2010.10.001
35. Pittman TA, Fan KL, Knapp A, Frantz S, Spear SL. Comparison of Different Acellular Dermal Matrices in Breast Reconstruction: The 50/50 Study. Plast Reconstr Surg. 2017;139(3):521-528. doi:10.1097/PRS.0000000000003048
36. Lee K-T, Mun G-H. A Meta-analysis of Studies Comparing Outcomes of Diverse Acellular Dermal Matrices for Implant-Based Breast Reconstruction. Ann Plast Surg. 2017;79(1):115-123. doi:10.1097/SAP.0000000000001085
37. Sorkin M, Qi J, Kim HM, et al. Acellular Dermal Matrix in Immediate Expander/Implant Breast Reconstruction: A Multicenter Assessment of Risks and Benefits. Plast Reconstr Surg. 2017;140(6):1091-1100. doi:10.1097/PRS.0000000000003842
38. Garramone CE, Lam B. Use of AlloDerm in primary nipple reconstruction to improve long-term nipple projection. Plast Reconstr Surg. 2007;119(6):1663-1668. doi:10.1097/01.prs.0000258831.38615.80
39. Nahabedian MY. Secondary nipple reconstruction using local flaps and AlloDerm. Plast Reconstr Surg. 2005;115(7):2056-2061. doi:10.1097/01.prs.0000164490.99581.f9
40. Chen WF, Barounis D, Kalimuthu R. A novel cost-saving approach to the use of acellular dermal matrix (AlloDerm) in postmastectomy breast and nipple reconstructions. Plast Reconstr Surg. 2010;125(2):479-481. doi:10.1097/PRS.0b013e3181c82da6
41. Johnson PC, Klein GL. Orthotopic grafting of decellularized human nipple: Setting the stage and putative mechanism of healing. Breast Edinb Scotl. 2020;54:96-98. doi:10.1016/j.breast.2020.09.007
42. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials. 2006;27(19):3675-3683. doi:10.1016/j.biomaterials.2006.02.014
43. Chen Y, Geerts S, Jaramillo M, Uygun BE. Preparation of Decellularized Liver Scaffolds and Recellularized Liver Grafts. In: Turksen K, ed. Decellularized Scaffolds and Organogenesis. Vol 1577. Methods in Molecular Biology. Springer New York; 2017:255-270. doi:10.1007/7651_2017_56
44. Purohit S, Kumar N, Sharma AK, Sharma K. Development of Acellular Dermal Matrix from Skin of Different Species of Animals Using Biological Detergents and Enzymes Combinations. Published online 2016:10.
45. Farndale R, Buttle D, Barrett A. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta BBA - Gen Subj. 1986;883(2):173-177. doi:10.1016/0304-4165(86)90306-5
46. Warden A, Truitt J, Merriman M, et al. Localization of PPAR isotypes in the adult mouse and human brain. Sci Rep. 2016;6(1):27618. doi:10.1038/srep27618
47. Zhang H-T, Gui T, Liu R-X, et al. Sequential targeting of YAP1 and p21 enhances the elimination of senescent cells induced by the BET inhibitor JQ1. Cell Death Dis. 2021;12(1):1-18. doi:10.1038/s41419-021-03416-1
48. Sean Lauber:ImageJ - Threshold Analysis - OpenWetWare. Accessed May 16, 2021. https://openwetware.org/wiki/Sean_Lauber:ImageJ_-_Threshold_Analysis
49. Klar AS, Güven S, Biedermann T, et al. Tissue-engineered dermo-epidermal skin grafts prevascularized with adipose-derived cells. Biomaterials. 2014;35(19):5065-5078. doi:10.1016/j.biomaterials.2014.02.049
50. Choe JM, Bell T. Genetic material is present in cadaveric dermis and cadaveric fascia lata. J Urol. 2001;166(1):122-124.
51. Derwin KA, Baker AR, Spragg RK, Leigh DR, Iannotti JP. Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties. J Bone Joint Surg Am. 2006;88(12):2665-2672. doi:10.2106/JBJS.E.01307
52. Moore MA, Samsell B, Wallis G, et al. Decellularization of human dermis using non-denaturing anionic detergent and endonuclease: a review. Cell Tissue Bank. 2015;16(2):249-259. doi:10.1007/s10561-014-9467-4
53. Ruszczak Z. Effect of collagen matrices on dermal wound healing. Adv Drug Deliv Rev. 2003;55(12):1595-1611. doi:10.1016/j.addr.2003.08.003
54. Hynes RO. The Extracellular Matrix: Not Just Pretty Fibrils. Science. 2009;326(5957):1216-1219. doi:10.1126/science.1176009
55. Watt FM, Huck WTS. Role of the extracellular matrix in regulating stem cell fate. Nat Rev Mol Cell Biol. 2013;14(8):467-473. doi:10.1038/nrm3620

56. Wang L, Johnson JA, Zhang Q, Beahm EK. Combining decellularized human adipose tissue extracellular matrix and adipose-derived stem cells for adipose tissue engineering. Acta Biomater. 2013;9(11):8921-8931. doi:10.1016/j.actbio.2013.06.035
57. Youngstrom DW, Barrett JG, Jose RR, Kaplan DL. Functional characterization of detergent-decellularized equine tendon extracellular matrix for tissue engineering applications. PloS One. 2013;8(5):e64151. doi:10.1371/journal.pone.0064151
58. Petersen TH, Calle EA, Zhao L, et al. Tissue-Engineered Lungs for in Vivo Implantation. Science. 2010;329(5991):538-541. doi:10.1126/science.1189345
59. Reimer J, Syedain Z, Haynie B, Lahti M, Berry J, Tranquillo R. Implantation of a Tissue-Engineered Tubular Heart Valve in Growing Lambs. Ann Biomed Eng. 2017;45(2):439-451. doi:10.1007/s10439-016-1605-7
60. Gilpin SE, Guyette JP, Gonzalez G, et al. Perfusion decellularization of human and porcine lungs: Bringing the matrix to clinical scale. J Heart Lung Transplant. 2014;33(3):298-308. doi:10.1016/j.healun.2013.10.030
61. Ghazanfari S, Driessen-Mol A, Sanders B, et al. In Vivo Collagen Remodeling in the Vascular Wall of Decellularized Stented Tissue-Engineered Heart Valves. Tissue Eng Part A. 2015;21(15-16):2206-2215. doi:10.1089/ten.TEA.2014.0417
62. Reing JE, Brown BN, Daly KA, et al. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. Biomaterials. 2010;31(33):8626-8633. doi:10.1016/j.biomaterials.2010.07.083

Acellular nipple scaffold development, characterization, and preliminary biocompatibility assessment in a swine model

Figure 1

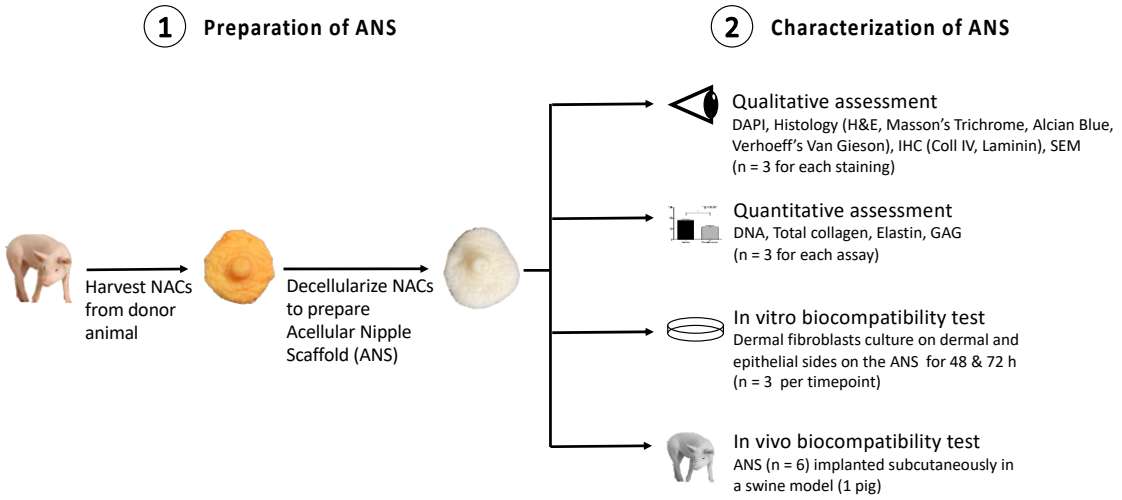


Figure 2

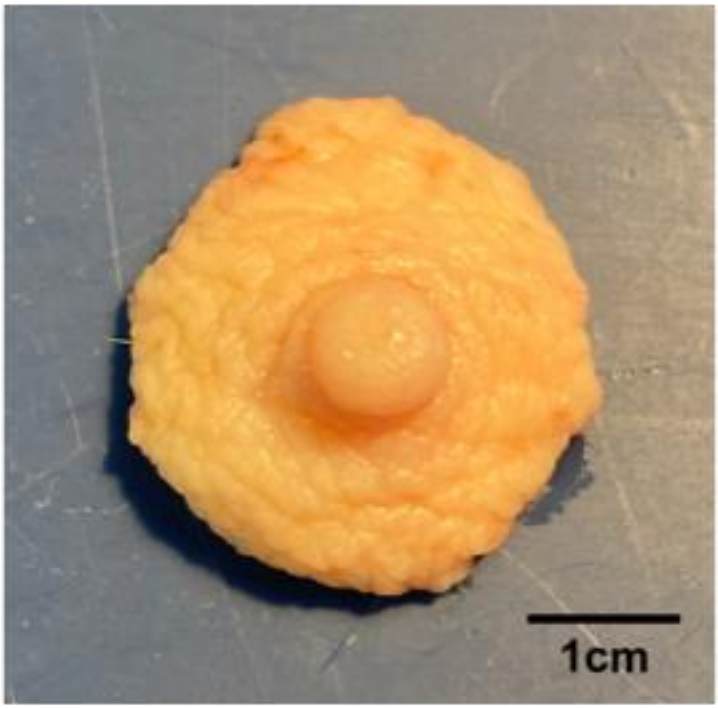


Figure 3

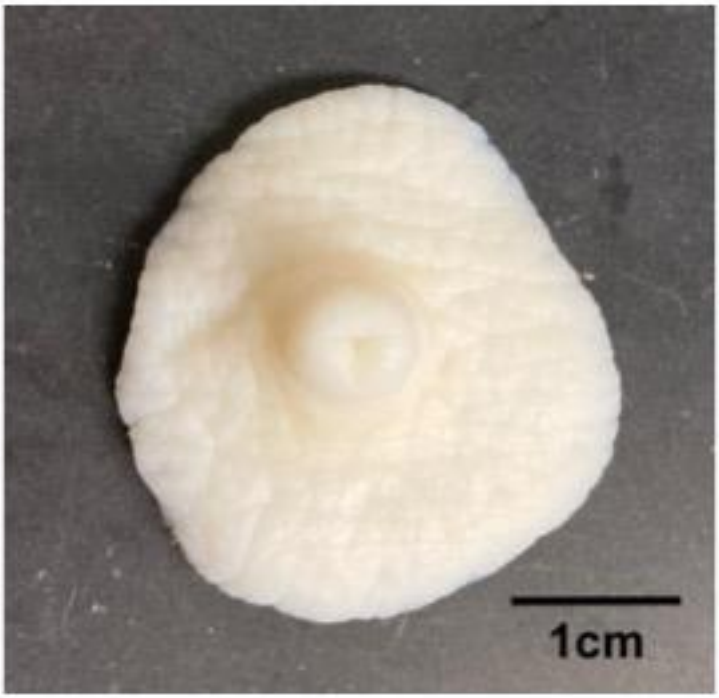


Figure 4

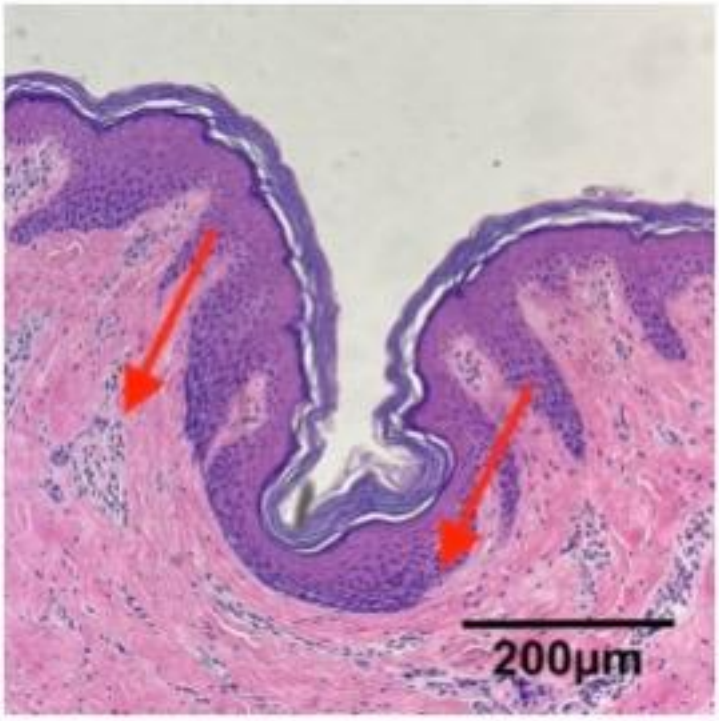


Figure 5

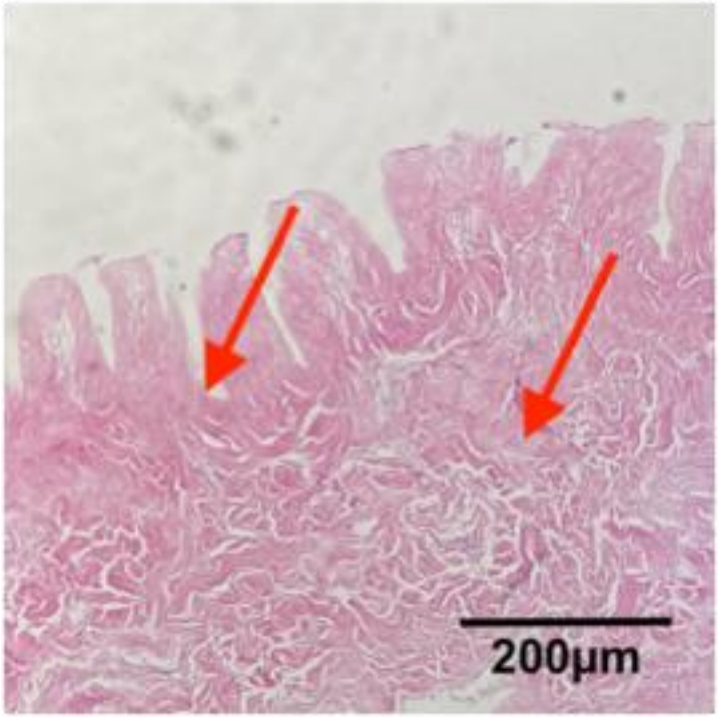


Figure 6

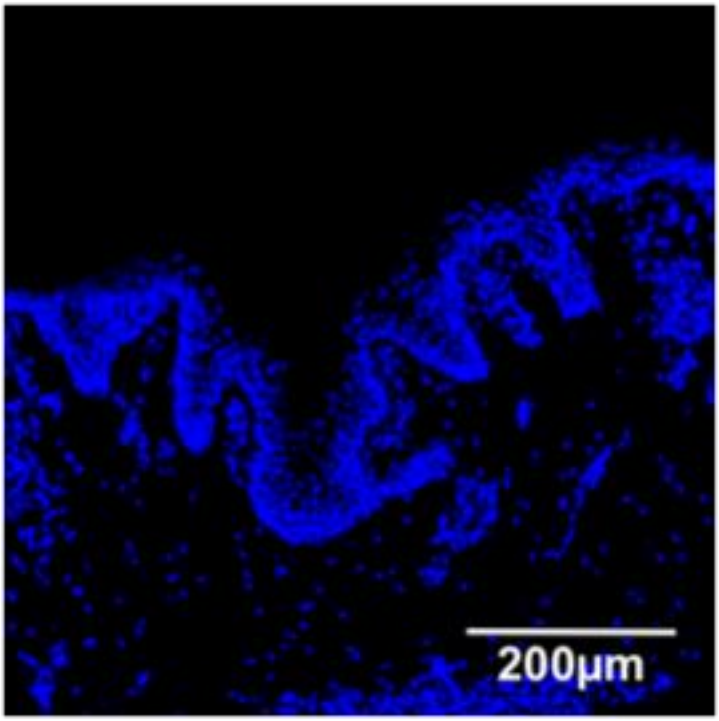


Figure 7

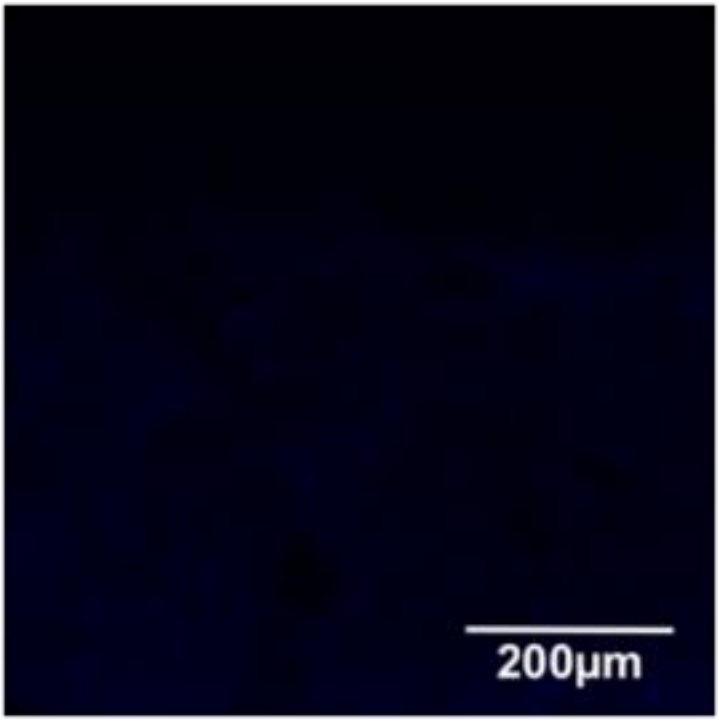


Figure 8

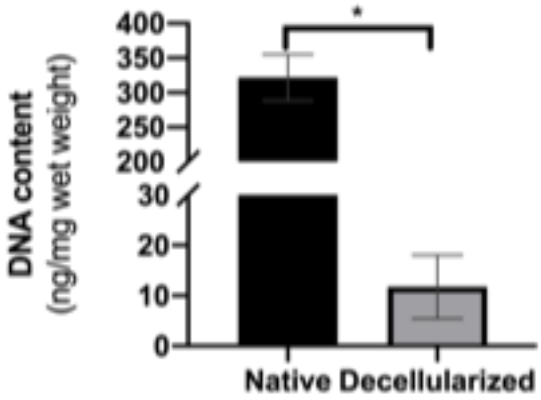


Figure 9

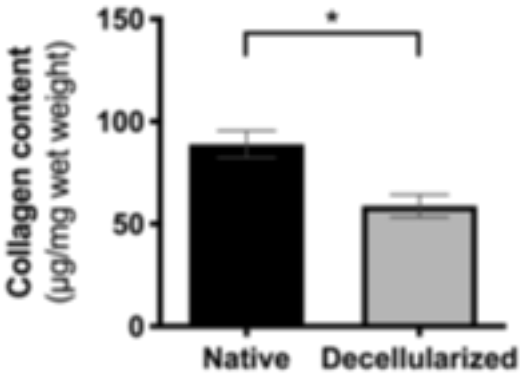


Figure 10

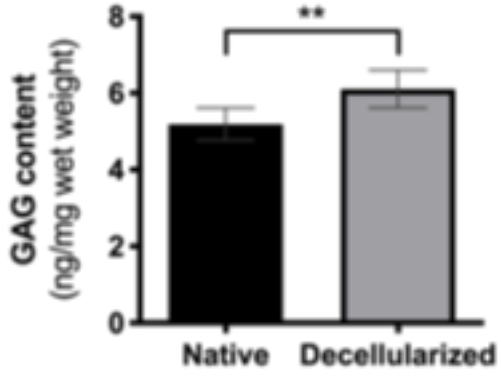


Figure 11

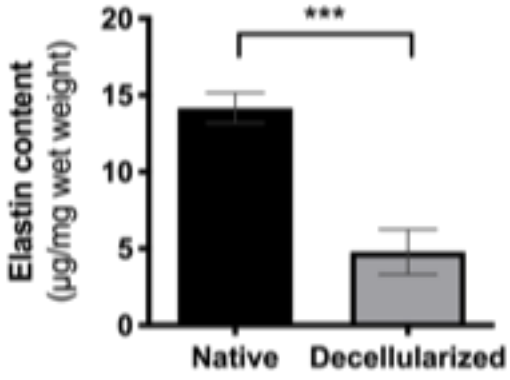


Figure 12

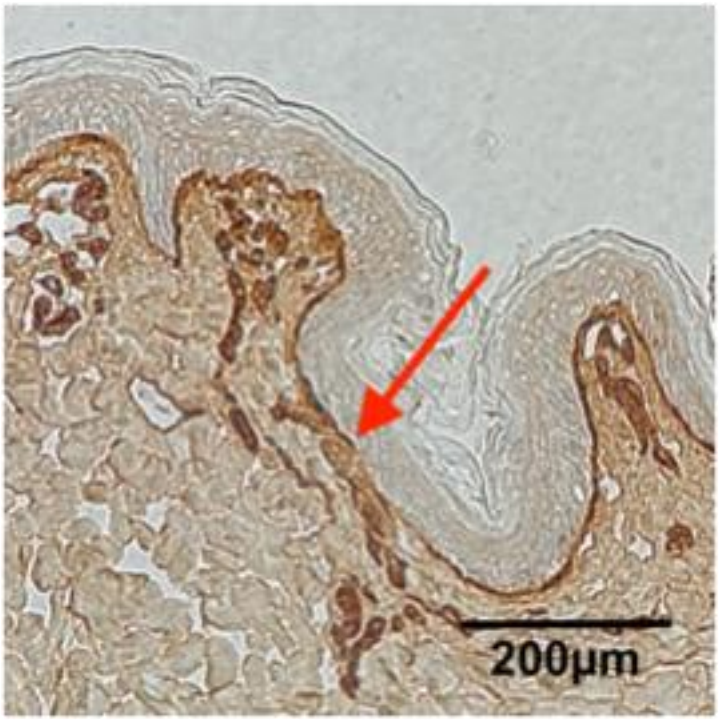


Figure 13

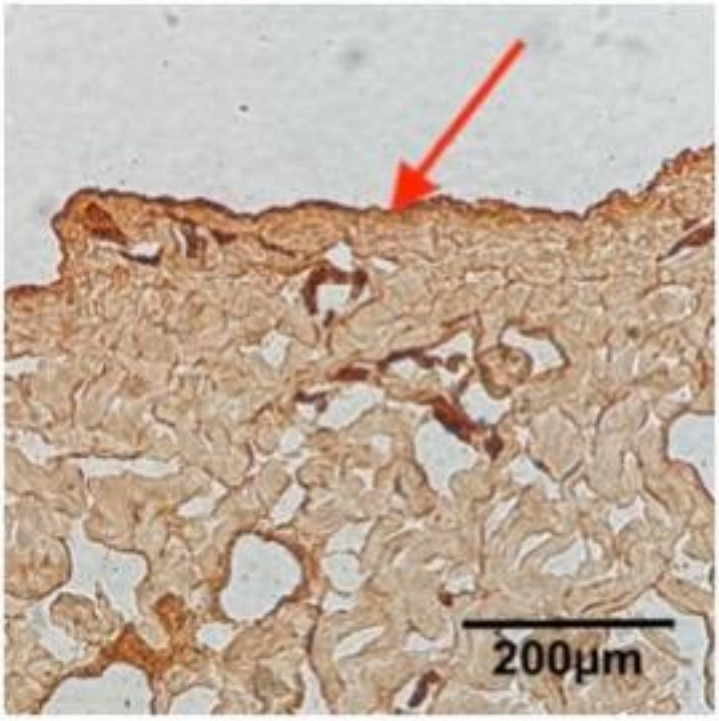


Figure 14

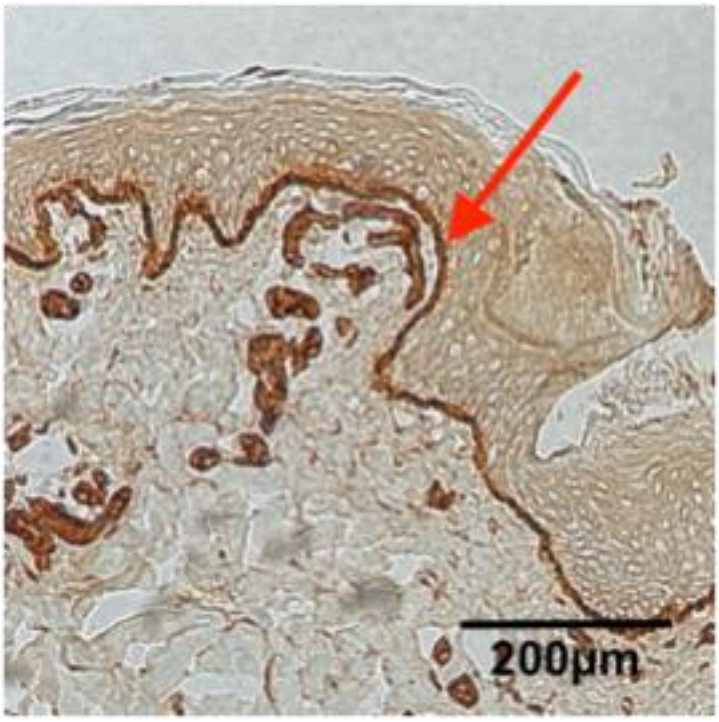


Figure 15

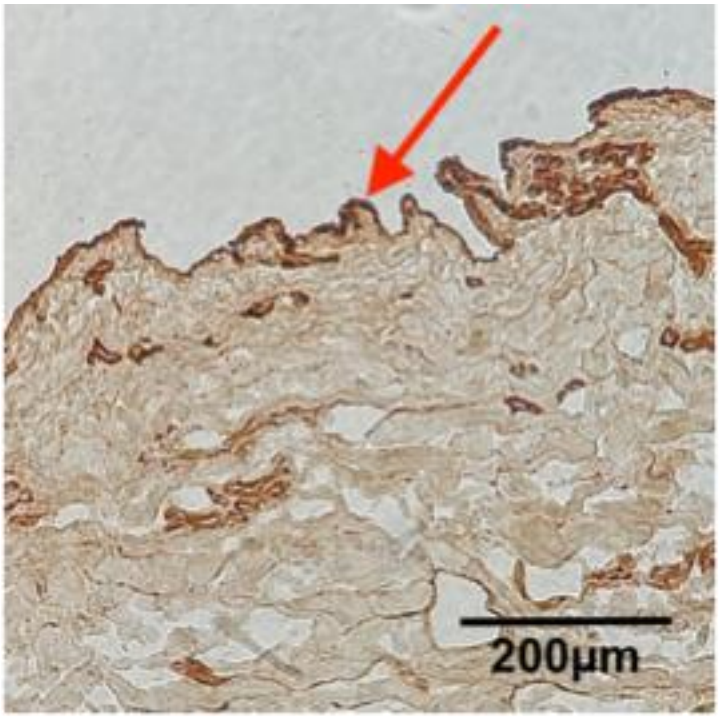


Figure 16

Figure 17

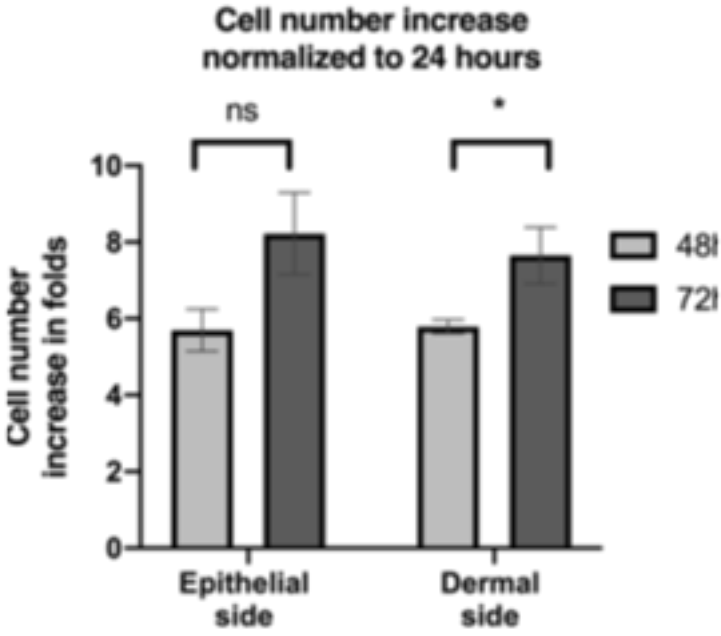


Figure 18

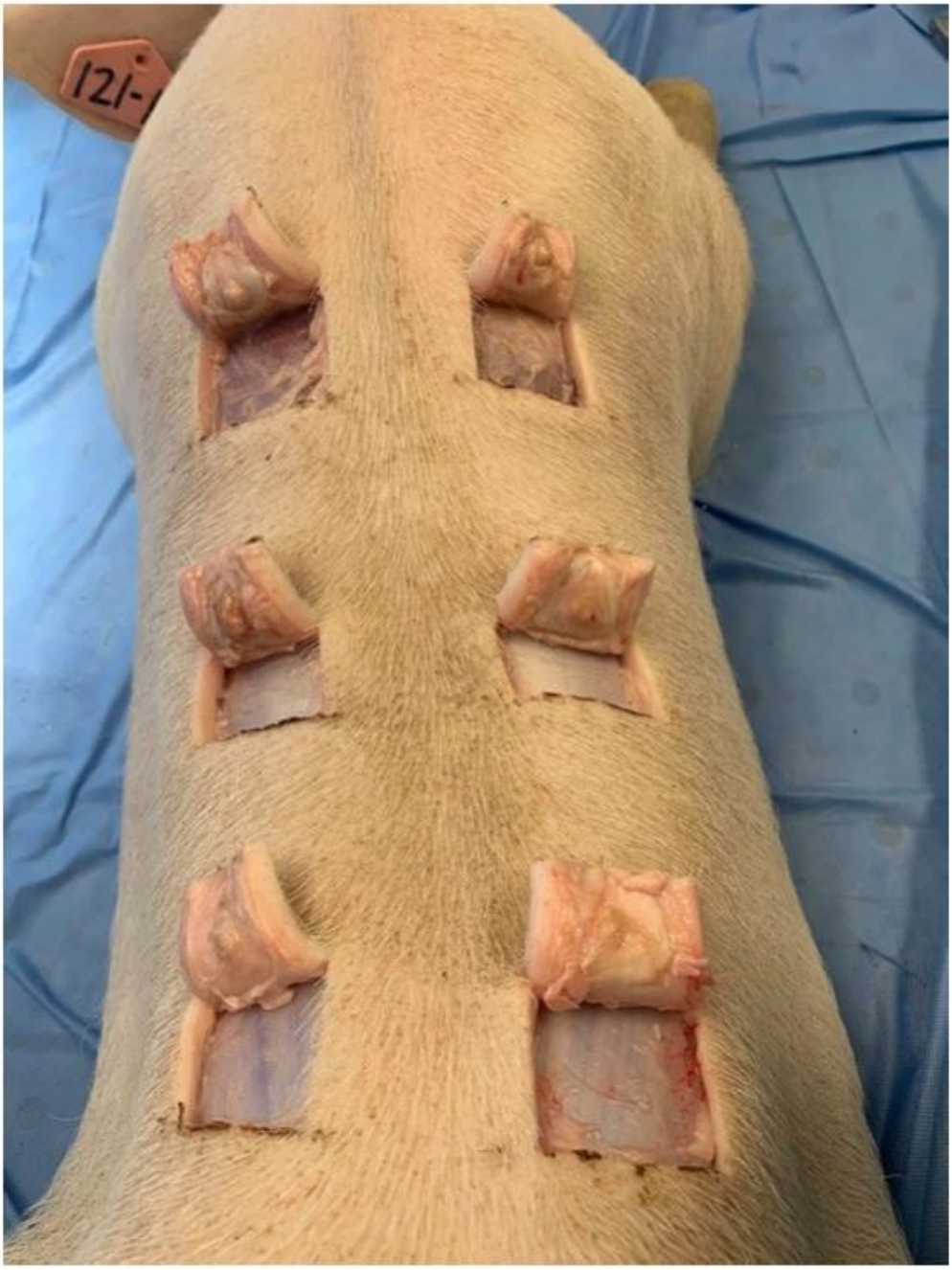


Figure 19

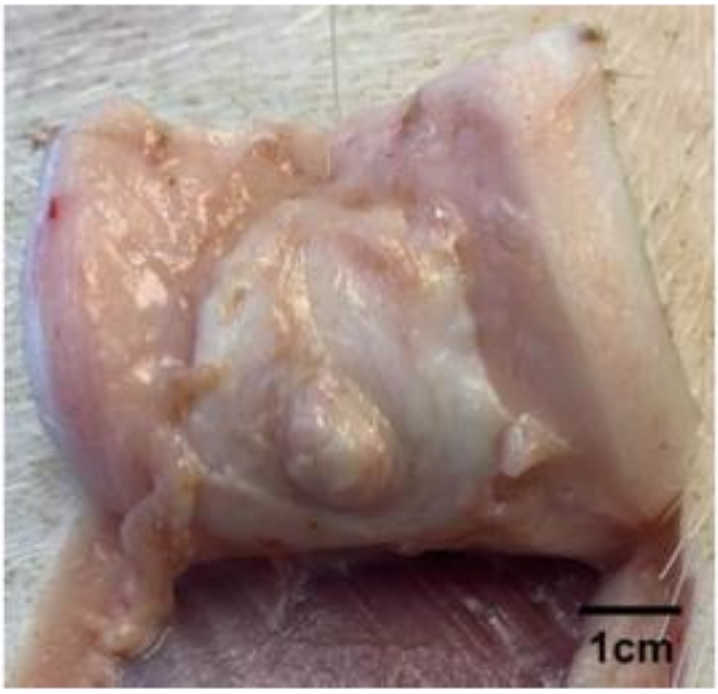
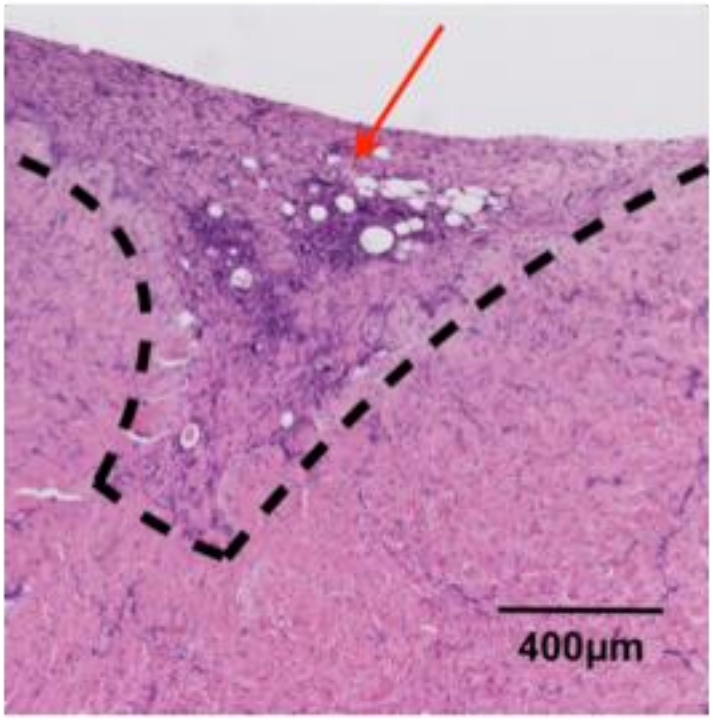


Figure 20



CHAPTER 17

Patent – Artificial Skin

Alexandre G. Lellouch
Lara Jobeili
Laurent A. Lantieri
Walid Rachidi

(12) DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITÉ DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)

(19) Organisation Mondiale de la Propriété Intellectuelle
Bureau international

(43) Date de la publication internationale
07 octobre 2021 (07.10.2021)

WIPO | PCT

(10) Numéro de publication internationale
WO 2021/198177 A1

(51) Classification internationale des brevets :
A61K 35/36 (2015.01) *A61P 17/02* (2006.01)
A61K 35/33 (2015.01) *A61K 8/98* (2006.01)
A61L 27/24 (2006.01) *A61L 24/00* (2006.01)

(21) Numéro de la demande internationale :
PCT/EP2021/058151

(22) Date de dépôt international :
29 mars 2021 (29.03.2021)

(25) Langue de dépôt : français

(26) Langue de publication : français

(30) Données relatives à la priorité :
2003125 30 mars 2020 (30.03.2020) FR

(71) Déposants : **UNIVERSITE GRENOBLE ALPES** [FR/FR] ; 621 Avenue Centrale, 38400 SAINT MARTIN D'HERES (FR). **COMMISSARIAT A L'ENERGIE ATOMIQUE ET AUX ENERGIES ALTERNATIVES** [FR/FR] ; 25 rue Leblanc, Bâtiment "Le Ponant D", 75015 PARIS (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE** [FR/FR] ; 3 Rue Michel Ange, 75016 PARIS (FR).

(72) Inventeurs : **JOBEILI, Lara Marwa** ; 14 rue Irvoy, 38000 GRENOBLE (FR). **LELLOUCH, Alexandre Gaston Mickaël** ; 17 rue Clément Bayard, 92300 LEVALLOIS-PERRET (FR). **RACHIDI, Walid** ; 15 Chemin de Bérivière, 38240 MEYLAN (FR). **LANTIERI, Laurent Alexandre** ; 40 Avenue Charles Floquet, 75007 PARIS (FR).

(74) Mandataire : **DECOBERT, Jean-Pascal** et al. ; CABINET HAUTIER, 20 RUE DE LA LIBERTE, 06000 NICE (FR).

(81) États désignés *(sauf indication contraire, pour tout titre de protection nationale disponible)* : AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,

(54) **Title:** BIOMATERIAL COMPRISING A POROUS RESORBABLE MATRIX AND ASSOCIATED MANUFACTURING METHOD

(54) **Titre :** BIOMATÉRIAU COMPRENANT UNE MATRICE POREUSE RÉSORBABLE ET PROCÉDÉ DE FABRICATION ASSOCIÉ

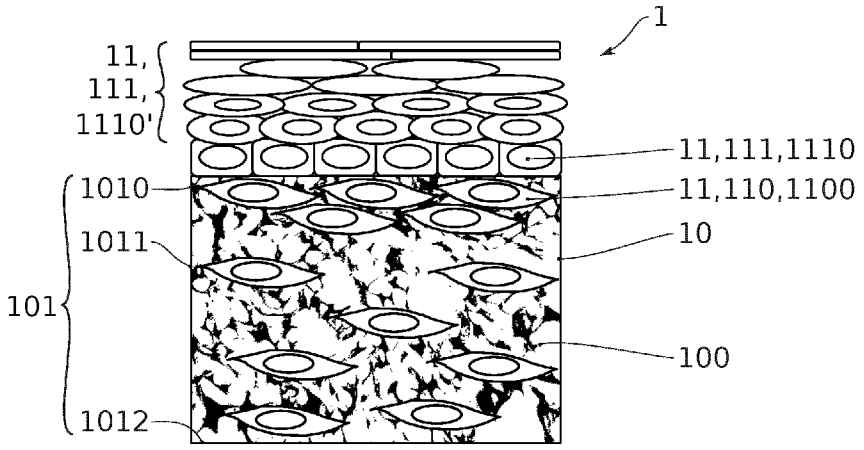


FIG. 5B

(57) **Abstract:** The invention relates to a biomaterial (1) comprising a porous resorbable matrix (10) formed from a material comprising collagen, and having an inner volume (100) and an outer surface (101). Advantageously, the biomaterial (1) comprises at least one type (110) of living biological cells (11) of a tissue, arranged in the inner volume (100) and, alternatively or additionally, on the surface (101) of the porous matrix (10). The biomaterial (1) forms a tissue substitute similar to a native tissue, particularly in terms of the biological structure present in the tissue, and the physiological functions.

(57) **Abrégé :** L'invention concerne un biomatériau (1) comprenant une matrice poreuse (10) résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur (100) et une surface extérieure (101). Avantageusement, le biomatériau (1) comprend au moins un type (110) de cellules biologiques (11) vivantes d'un tissu, disposées dans le volume intérieur (100) et en alternative ou en complément sur la surface (101) de la matrice poreuse (10). Le biomatériau (1) forme un substitut tissulaire se rapprochant d'un tissu natif, notamment en termes de structure biologique présente dans le tissu, et de fonctions physiologiques.

WO 2021/198177 A1

[Suite sur la page suivante]

OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **États désignés** *(sauf indication contraire, pour tout titre de protection régionale disponible)* : ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), eurasien (AM, AZ, BY, KG, KZ, RU, TJ, TM), européen (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Publiée:

— *avec rapport de recherche internationale (Art. 21(3))*

Biomatériau comprenant une matrice poreuse résorbable et procédé de fabrication associé

DOMAINE TECHNIQUE

La présente invention concerne le domaine des biomatériaux comprenant du collagène et leur fabrication. Elle trouve pour application particulièrement avantageuse le domaine des substituts dermo-épidermiques.

ETAT DE LA TECHNIQUE

Les plaies chroniques représentent un problème majeur de santé publique affectant plus de 2 millions de personnes uniquement en France métropolitaine. Ceci inclut les brûlures physiques, par exemple induites par un rayonnement ultra-violet, et chimiques, par exemple induites par des détergents ou des acides, les ulcères, et notamment les ulcères de pression, les ulcères veineux et des ulcères diabétiques. Ces plaies chroniques nécessitent généralement le remplacement des tissus lésés, par exemple par une greffe cutanée.

Pour le cas particulier des greffes de peau, il existe différents types de greffes classés en fonction de l'épaisseur de peau prélevée sur la personne :

- la greffe de peau mince est prélevée par un dermatome électrique avec une épaisseur de 0,2 à 0,3 mm sur une zone donneuse du corps du patient, généralement une face interne de cuisse ou du scalp. Ce prélèvement est

ensuite transféré sur une zone receveuse (à l'aide de suture ou d'agrafe) du patient pour une greffe autologue. Cette technique peut être complétée avec l'ajout d'un substitut dermique artificiel tel que l'integra®. Le principal inconvénient réside dans les séquelles induites par le prélèvement et la surface limitée des zones donneuses;

- la greffe de peau totale, ne nécessite pas de prélèvement au dermatome comme pour une greffe de peau mince. Une incision fusiforme est réalisée dans un pli de peau, par exemple le pli sus claviculaire. La peau est ensuite dégraissée (pour permettre une meilleure prise de greffe), puis suturée sur la zone receveuse. Cette technique est considérée comme particulièrement adaptée pour le visage.

Les prélèvements des tissus cutanés dans le cadre des greffes restent toutefois limités, notamment en termes de surface disponible de zones donneuses et des douleurs induites chez la personne prélevée.

Par ailleurs, depuis 2013, les tests de l'efficacité et de la sécurité des produits dermo-cométiques sont interdits sur les animaux. Pour tester ces substances, l'industrie pharmaceutique ou cosmétique doit donc avoir recours à des substituts tissulaires mimant au mieux les propriétés et les réactions d'un tissu natif.

Il existe donc un besoin pour développer un substitut tissulaire au plus proche d'un tissu natif, par exemple pour le traitement de plaies chroniques ou pour des applications pharmacologiques ou cosmétiques.

Il est connu, du document WO 88/10123 A1, un biomatériau comprenant un composé constitué de collagène, de chitosan acétylé et de glycosaminoglycanes. Ce biomatériau peut constituer la couche dermique d'une peau artificielle, et être associé à un pseudo épiderme biodégradable, par exemple un film de chitosan. Toutefois, ce biomatériau reste perfectible pour mimer une peau native.

Un objet de la présente invention est donc de proposer un biomatériau se rapprochant davantage d'un tissu natif.

Les autres objets, caractéristiques et avantages de la présente invention apparaîtront à l'examen de la description suivante et des dessins d'accompagnement. Il est entendu que d'autres avantages peuvent être incorporés.

RESUME

Pour atteindre cet objectif, selon un premier aspect on prévoit un biomatériau comprenant une matrice poreuse résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur et une surface extérieure.

Avantageusement, le biomatériau comprend au moins un type de cellules biologiques vivantes d'un tissu, disposées dans le volume intérieur et, en alternative ou en complément, sur la surface de la matrice poreuse.

Ainsi, le biomatériau comprend une matrice lui conférant une structure permettant sa manipulation, et des cellules biologiques d'un tissu ensemencées dans la matrice poreuse. Le biomatériau forme donc un substitut tissulaire se rapprochant d'un tissu natif, notamment en termes de structure biologique présente dans le tissu, et de fonctions physiologiques.

En outre, la matrice étant résorbable, la matrice poreuse peut être dégradée pour faciliter l'intégration d'une partie du biomatériau dans l'organisme, et plus particulièrement d'un tissu formé par les cellules biologiques, par exemple suite à une greffe du biomatériau. Le biomatériau forme donc un substitut tissulaire pouvant être greffé sur un organisme, puis agir directement pour réaliser sa fonction de tissu, sans nécessiter en outre le retrait du biomatériau, et plus particulièrement de la matrice poreuse.

Selon un exemple, l'au moins un type de cellules biologiques vivantes d'un tissu sont disposées dans le volume intérieur de la matrice poreuse.

Un deuxième aspect de l'invention concerne un procédé de fabrication d'un biomatériau comprenant :

- une fourniture d'une matrice poreuse résorbable formée d'un matériau comprenant du collagène, puis
- un ensemencement de la matrice poreuse par au moins un type de cellules biologiques vivantes d'un tissu, puis
- une croissance cellulaire de l'au moins un type de cellules biologiques.

Le procédé permet ainsi d'obtenir le biomatériau selon le premier aspect de l'invention. Par ensemencement et croissance, on peut en outre former un biomatériau de taille et de forme variable en fonction des dimensions de la matrice poreuse. On obtient ainsi un biomatériau formant un substitut tissulaire se rapprochant d'un tissu natif.

Un troisième aspect concerne l'utilisation d'un biomatériau, le biomatériau comprenant :

- une matrice poreuse résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur et une surface, et
- au moins un type de cellules biologiques vivantes d'un tissu, disposées dans le volume intérieur et, en alternative ou en complément, sur la surface de la

matrice poreuse, pour une application in vitro d'au moins une substance choisie parmi une substance pharmaceutique et une substance cosmétique.

Un quatrième aspect concerne l'utilisation d'un biomatériau pour le traitement de lésions, et plus particulièrement le traitement de lésions cutanées, le biomatériau comprenant :

- une matrice poreuse résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur et une surface, et
- au moins un type de cellules biologiques vivantes d'un tissu, disposées dans le volume intérieur et, en alternative ou en complément, sur la surface de la matrice poreuse.

Un cinquième aspect concerne un biomatériau destiné à être greffé sur un organisme, par exemple pour le traitement d'une lésion, et plus particulièrement le traitement de lésions cutanées, le biomatériau comprenant :

- une matrice poreuse résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur et une surface, et
- au moins un type de cellules biologiques vivantes d'un tissu, disposées dans le volume intérieur et, en alternative ou en complément, sur la surface de la matrice poreuse.

Un aspect combinable ou séparable concerne un substitut dermo-épidermique comprenant une matrice poreuse résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur et une surface extérieure, le substitut dermo-épidermique étant caractérisé en ce qu'il comprend des cellules dermiques vivantes, disposé dans le volume intérieur de la matrice poreuse, une matrice extracellulaire disposées dans le volume intérieur, la matrice extracellulaire étant sécrétée par les cellules dermiques, et des cellules épidermiques disposées à la surface extérieure de la matrice poreuse.

BREVE DESCRIPTION DES FIGURES

Les buts, objets, ainsi que les caractéristiques et avantages de l'invention ressortiront mieux de la description détaillée d'un mode de réalisation de cette dernière qui est illustré par les dessins d'accompagnement suivants dans lesquels :

La figure 1 représente une vue en coupe transversale de la matrice poreuse selon un mode de réalisation de l'invention.

La figure 2 représente un premier ensemencement de la matrice poreuse illustrée en figure 1 par un premier type de cellules biologiques, selon un mode de réalisation de l'invention.

La figure 3 représente la croissance cellulaire des cellules biologiques suite à l'ensemencement illustré en figure 2, selon un mode de réalisation de l'invention.

La figure 4 représente un deuxième ensemencement de la matrice poreuse par un deuxième type de cellules biologiques et une première sous-croissance cellulaire subséquente du deuxième type de cellules biologiques, suite à la croissance cellulaire illustrée en figure 3, selon un mode de réalisation de l'invention.

La figure 5A représente une maturation du deuxième type de cellules biologiques suite à la première sous-croissance cellulaire illustrée en figure 4, selon un mode de réalisation de l'invention.

La figure 5B représente le biomatériau, selon un mode de réalisation de l'invention.

La figure 6 représente les étapes du procédé de fabrication du biomatériau, selon un mode de réalisation de l'invention.

La figure 7 représente une chronologie des étapes du procédé de fabrication du biomatériau, selon un mode de réalisation de l'invention.

La figure 8 représente une image immunohistologique de microscopie à fluorescence en coupe transversale du biomatériau, dans laquelle les noyaux des cellules biologiques et la cytokératine 14 sont marqués.

La figure 9 représente une image immunohistologique de microscopie à fluorescence en coupe transversale du biomatériau, dans laquelle les noyaux des cellules biologiques, la filaggrine et le collagène de type 1 sont marqués.

La figure 10 représente une image immunohistologique de microscopie à fluorescence en coupe transversale du biomatériau, dans laquelle les noyaux des cellules biologiques, la fibrilline 1 et l'élastine sont marqués.

Les dessins sont donnés à titre d'exemples et ne sont pas limitatifs de l'invention. Ils constituent des représentations schématiques de principe destinées à faciliter la compréhension de l'invention et ne sont pas nécessairement à l'échelle des applications pratiques. En particulier, les dimensions relatives des cellules biologiques entre elles et par rapport à la matrice poreuse ne sont pas représentatives de la réalité.

DESCRIPTION DÉTAILLÉE

Avant d'entamer une revue détaillée de modes de réalisation de l'invention, sont énoncées ci-après des caractéristiques optionnelles qui peuvent éventuellement être utilisées en association ou alternativement pour chacun des aspects de l'invention :

- l'au moins un premier type de cellules biologiques peut être disposé au moins dans le volume intérieur de la matrice poreuse, voire uniquement dans le volume intérieur de la matrice,

- l'au moins un premier type de cellules biologiques peut être disposé au moins sur une surface extérieure de la matrice poreuse, voire uniquement sur une surface de la matrice, et de préférence sur une face supérieure de la matrice poreuse,
- la matrice poreuse peut être biocompatible,
- le matériau de la matrice poreuse peut comprendre au moins 90%, de préférence au moins 95 %, et plus préférentiellement au moins 99 % en masse de collagène, par rapport à la masse sèche de la matrice poreuse,
- le matériau de la matrice poreuse peut être exempt de chitosan,
- la matrice poreuse peut être choisie parmi un gel, une poudre, et une éponge. De préférence, la matrice poreuse est choisie parmi une poudre et une éponge,
- la matrice poreuse peut être une éponge synthétique, et plus particulièrement une éponge hémostatique. Ainsi, la matrice poreuse peut être disponible commercialement et à un coût réduit,
- la matrice poreuse peut être un produit suivant les Bonnes Pratiques de Fabrication (BPF). La matrice poreuse peut être utilisable cliniquement chez l'humain ou l'animal. Le biomatériau peut ainsi être utilisable cliniquement chez l'humain ou l'animal,
- les cellules biologiques peuvent comprendre des cellules choisies parmi des cellules de tissu conjonctif, des cellules épithéliales et endothéliales. Selon un exemple, les cellules de tissu conjonctif sont des cellules dermiques appelées fibroblastes et/ou des cellules du tissu adipeux sous cutané appelées cellules souches du tissu adipeux. Selon un exemple, les cellules épithéliales sont des cellules d'épiderme comprenant les kératinocytes et/ou les mélanocytes. Selon un exemple, les cellules endothéliales sont des cellules des vaisseaux. De préférence, les cellules biologiques sont des cellules humaines ou animales,
- l'au moins un type de cellules biologiques peut être disposé au moins dans le volume intérieur de la matrice poreuse, voire uniquement dans le volume intérieur de la matrice, le biomatériau peut comprendre en outre une matrice extracellulaire au moins dans ledit volume intérieur, la matrice extracellulaire étant sécrétée par l'au moins un premier type de cellules biologiques. Les cellules biologiques et leur matrice extracellulaire permettent de former un tissu dans la matrice poreuse, et se rapprocher encore des propriétés d'un tissu natif,

- un premier type de cellules biologiques peut être disposé dans le volume intérieur de la matrice poreuse, et le biomatériau peut comprendre au moins un deuxième type de cellules biologiques disposé sur la surface, par exemple la face supérieure, de la matrice poreuse, le deuxième type de cellules biologiques pouvant en outre être distinct du premier type de cellules biologiques. Ainsi, le biomatériau peut en outre comprendre une couche cellulaire en surface de la matrice poreuse formant une limite externe d'un tissu, et se rapprocher encore d'un tissu natif. Selon un exemple, le deuxième type de cellules biologiques est un type de cellules épithéliales. Ainsi, le biomatériau présente des propriétés barrières. Le biomatériau permet donc le remplacement de la fonction de barrière, par exemple sur une plaie ne cicatrisant pas spontanément, telle qu'une plaie chronique,
- le premier type de cellules biologiques peut comprendre des cellules dermiques, par exemple des fibroblastes, et le deuxième type de cellules biologiques peut comprendre des cellules épithéliales, par exemple des kératinocytes, voire en outre des mélanocytes. Selon un exemple, le premier type de cellules biologiques peut comprendre en outre au moins un type de cellules biologiques parmi des cellules endothéliales, des cellules immunitaires et des cellules souches du tissu adipeux. Selon un exemple, l'au moins un deuxième type de cellules biologiques peut comprendre en outre des mélanocytes,
- les cellules biologiques sont choisies parmi des cellules dérivées de cultures de lignée cellulaire standard et préférentiellement des cellules primaires isolées d'un échantillon biologique d'un organisme. Selon un exemple, le biomatériau peut comprendre des cellules autologues ou allogéniques. Selon un exemple, les cellules biologiques sont isolées depuis un échantillon de peau d'une personne.

Des caractéristiques optionnelles du deuxième aspect de l'invention sont énoncées ci-après, qui peuvent éventuellement être utilisées en association ou alternativement :

- le procédé peut comprendre une hydratation de la matrice poreuse de façon à équilibrer le pH et l'osmolarité de la matrice poreuse, préalablement à l'ensemencement. Selon un exemple, l'hydratation est réalisée avec au moins une solution tamponnée, puis avec un milieu de culture,

- l'hydratation de la matrice poreuse peut être réalisée par une solution d'hydratation présentant un pH compris entre 6,8 et 7,5, et/ou une osmolarité comprise entre 270 et 360 mmol/kg,
- le procédé peut comprendre en outre un séchage de la matrice poreuse entre l'hydratation de la matrice poreuse et l'ensemencement de la matrice poreuse. Selon un exemple, le séchage de la matrice poreuse est effectué par aspiration, par exemple de la solution d'hydratation,
- l'ensemencement de la matrice poreuse peut être réalisé par dépôt d'une suspension de l'au moins un type de cellules biologiques sur la matrice poreuse. Selon un exemple, de l'au moins un type de cellules biologiques peuvent être ensemencés à une densité cellulaire comprise entre $0,5.10^6$ cellules/cm² et 1.10^6 cellules/cm², les cm² étant donnés en référence à la surface de la matrice poreuse,
- le procédé peut comprendre en outre, après un premier ensemencement par un premier type de cellules biologiques, voire après le premier ensemencement et une partie au moins d'une croissance cellulaire du premier type de cellules biologiques, un deuxième ensemencement de la matrice poreuse par au moins un deuxième type de cellules biologiques, distinct d'un premier type de cellules biologiques ensemencées. Le procédé peut comprendre en outre ensuite une croissance cellulaire de l'au moins un deuxième type de cellule biologiques,
- le procédé peut comprendre en outre un séchage de la matrice poreuse préalablement au deuxième ensemencement de la matrice poreuse,
- le séchage, de préférence chaque séchage, de la matrice poreuse peut comprendre une aspiration de la solution ou du milieu présent dans la matrice poreuse, et le placement de la matrice poreuse sur un support absorbant, de préférence la matrice poreuse est placée sur le support absorbant après l'aspiration,
- le support absorbant peut comprendre une compresse stérile,
- le support absorbant peut comprendre un papier buvard stérile,
- la compresse stérile est de préférence déposée sur un papier buvard stérile,
- le deuxième ensemencement peut être réalisé par dépôt d'une suspension d'au moins un deuxième type de cellules biologiques sur la matrice poreuse,
- les cellules biologiques peuvent comprendre des cellules choisies parmi des cellules de tissu conjonctif et des cellules épithéliales,

- lorsque les cellules biologiques sont des cellules de tissu conjonctif, la croissance cellulaire peut comprendre une immersion de la matrice poreuse dans un milieu de culture. De préférence, la matrice poreuse est entièrement immergée. En outre, la matrice poreuse peut être immergée pendant la majorité de la croissance cellulaire,
- lorsque les cellules biologiques sont des cellules épithéliales, la croissance cellulaire peut comprendre une première sous-croissance pendant laquelle la matrice poreuse est immergée dans un milieu de culture, jusqu'à une confluence des cellules biologiques en surface de la matrice poreuse,
- la croissance cellulaire peut comprendre, suite à la première sous-croissance, une deuxième sous-croissance, pouvant de façon équivalente être désignée comme maturation, durant laquelle la matrice poreuse est placée à une interface entre de l'air et un milieu de culture. La maturation permet la différenciation des cellules épithéliales pour former un épiderme plus complexe, voire complet, et se rapprocher d'un tissu natif

Il est précisé que dans le cadre de la présente invention, le terme « matrice résorbable » désigne le fait que la matrice est biodégradable des cellules, par exemple d'un organisme.

Par une matrice « biocompatible », on entend que la matrice est configurée pour ne pas interférer, voire ne pas dégrader, un milieu biologique au contact duquel la matrice est disposée. Plus particulièrement, la matrice est tolérée par un organisme dans ou sur lequel la matrice est en contact, par exemple par greffe. Plus particulièrement l'organisme présente un faible niveau de réaction inflammatoire, voire une absence de réaction inflammatoire induit par le contact de la matrice, par exemple par greffe.

On entend par un paramètre « sensiblement égal/supérieur/inférieur à » une valeur donnée, que ce paramètre est égal/supérieur/inférieur à la valeur donnée, à plus ou moins 10 % près, voire à plus ou moins 5 % près, de cette valeur.

Par porosité de la matrice, on entend la proportion de vide ou de gaz dans la matrice.

Dans la suite de la description, il sera fait usage de termes tels que, « transversal », « supérieur » et « inférieur ». Ces termes doivent être interprétés de façon relative en relation avec la position normale d'utilisation du biomatériau. Par exemple, dans le cas la matrice poreuse est destinée à être déposée sur un support, la face inférieure correspond à la face destinée à être tournée vers le support. Selon cet

exemple, la face supérieure correspond à la face destinée à être tournée à l'opposé du support.

Le procédé 2 de fabrication du biomatériau 1 est maintenant décrit en référence aux figures 1 à 6. À titre d'exemple, le procédé est illustré par la figure 6, où des étapes optionnelles sont indiquées en pointillé.

Le procédé 2 comprend la fourniture 20 d'une matrice poreuse 20. La matrice poreuse 20 présente des pores délimitant un volume intérieur 100, comme par exemple illustré en figure 1. De façon équivalente, le volume intérieur 100 représente le volume de vide ou de gaz à l'intérieur de la matrice 20, et donc la porosité de la matrice. La matrice poreuse 20 présente une surface extérieure 101, comprenant une face supérieure 1010, et une face inférieure 1012. La surface extérieure 100 de la matrice poreuse 10 peut en outre comprendre au moins une face latérale 1011, voire une pluralité de faces latérales 1011, comme par exemple illustré en figure 1.

Selon un exemple, la porosité de la matrice poreuse 10 est homogène, c'est à dire que la porosité par unité de volume est sensiblement identique en en toute portion d'une même taille déterminée de la matrice poreuse 10. Ainsi, on exclut que le volume intérieur soit ménagé dans la matrice poreuse sous la forme d'une cavité. La dimension des pores de la matrice poreuse 10 est de préférence supérieure à la dimension de cellules biologiques 11, et notamment de cellules biologiques eucaryotes. Ainsi, les pores de la matrice poreuse 10 autorisent la migration des cellules biologiques 11 dans le volume intérieur 100. Des caractéristiques additionnelles de la matrice poreuse 10 sont décrites ultérieurement.

La matrice poreuse 10 peut être préparée en vue d'un ensemencement 23 de cellules biologiques 11. Pour cela, le procédé 2 peut comprendre une hydratation 21 configurée pour équilibrer le pH et l'osmolarité de la matrice poreuse autour de valeurs correspondant à l'environnement natif des cellules. Cela permet d'obtenir une osmolarité homogène dans la matrice poreuse 10, et ainsi une meilleure migration et colonisation des cellules 11 ensemencées. Ceci est particulièrement avantageux lorsque la matrice poreuse 10 est épaisse, par exemple lorsque la matrice poreuse 10 est une éponge. À titre d'exemple, pour la fabrication d'un substitut dermo-épidermique, ce pH peut être compris entre 6,8 à 7,5, et l'osmolarité peut être comprise entre de 270 à 360 mmol/kg. Pour comparaison, *in vivo* l'osmolarité cellulaire est sensiblement égale à 290 mmol/kg et le pH cellulaire est sensiblement égal à 7,4. Durant l'hydratation 21, la matrice poreuse 10 peut être immergée dans une solution tamponnée pendant plusieurs heures, voire plusieurs jours. La matrice poreuse 10 peut en outre être immergée dans un milieu de culture 3 configuré pour correspondre à

l'environnement natif des cellules. À titre d'exemple, pour la fabrication d'un substitut dermo-épidermique, le milieu de culture 3 peut être un milieu équivalent au milieu du derme.

Suite à son hydratation 21, le procédé 2 peut comprendre un séchage 22 de la matrice poreuse 2 pour favoriser l'adhésion des cellules biologiques 11 sur la matrice poreuse lors de leur ensemencement 23, et donc leur croissance cellulaire 24 subséquente. Par exemple, le séchage de la matrice poreuse 10 peut être effectué par aspiration de la solution d'hydratation.

Le procédé 2 comprend ensuite un premier ensemencement 23 de la matrice poreuse par des cellules biologiques 11 vivantes d'un tissu, et plus particulièrement par au moins premier type 110 de cellules biologiques 11. Ainsi, des cellules biologiques 11 sont ajoutées à la matrice poreuse 10 pour former un biomatériau 11 se rapprochant d'un tissu natif, par rapport à un biomatériau formé uniquement d'un matériau inerte.

Les cellules biologiques 11 peuvent être des cellules eucaryotes, humaines ou animales. Dans la suite, on se réfère à l'exemple non limitatif où les cellules biologiques 11 sont des cellules humaines.

Les cellules biologiques 11 peuvent être choisies parmi des cellules dérivées de cultures de lignée cellulaire standard et des cellules isolées d'un échantillon biologique d'un organisme. Un biomatériau 1 comprenant des cellules 11 dérivées de cultures de lignées cellulaires standard permet de former un substitut tissulaire performant, tout en restant de coût réduit, ce qui est particulièrement avantageux pour des applications pharmaceutiques ou cosmétiques. Les cellules de lignée cellulaire standard peuvent être disponibles sur le marché chez des vendeurs connus de l'homme du métier.

Selon un exemple, les cellules biologiques peuvent être autologues ou allogéniques. Les cellules biologiques allogéniques peuvent par exemple être isolées de résidus opératoire, notamment indépendamment d'une greffe de peau. Un biomatériau 1 comprenant des cellules 11 autologues permet d'être personnalisé pour une personne donnée, ce qui est particulièrement avantageux pour des applications médicales comme par exemple une greffe du biomatériau 1. Dans le cas de cellules autologues, les cellules biologiques peuvent être isolées depuis un échantillon biologique d'une personne, par exemple suite à une ou plusieurs biopsie et notamment une biopsie de peau.

Les cellules biologiques 11 peuvent comprendre des cellules choisies parmi des cellules de tissu conjonctif et des cellules épithéliales. À titre d'exemple, on peut citer comme tissu conjonctif le derme, le tissu adipeux sous cutané, le stroma cornéen, les

os, et comme tissu épithélial, l'épiderme et l'épithélium de la cornée. Selon un exemple, les cellules de tissu conjonctif sont des cellules de derme nommées fibroblastes. Selon un exemple, les cellules épithéliales sont des cellules de l'épiderme nommées kératinocytes. Ainsi, et comme décrit plus en détail dans la suite, le biomatériau 1 peut former un substitut dermique, voire dermo-épidermique pour des applications *in vitro* ou *in vivo*.

Dans la suite, on se réfère à l'exemple non-limitatif dans lequel un premier type de cellule comprend, voire est constitué, des cellules de derme, et plus particulièrement des fibroblastes, et un deuxième type de cellule comprend, voire est constitué, des cellules d'épiderme, et plus particulièrement des kératinocytes. Notons que pour la fabrication d'un substitut dermique, voire dermo-épidermique, on peut prévoir que le premier type de cellules biologiques comprenne en addition aux cellules du derme, au moins un type de cellules biologiques parmi :

- des cellules endothéliales, pour obtenir un derme vascularisé,
- des cellules immunitaires, pour obtenir un derme immunocompétent,
- des cellules souches du tissu adipeux, pour obtenir une structure adipeuse sous cutanée.

Selon un exemple, le deuxième type de cellules biologiques peut comprendre en outre des mélanocytes, pour obtenir un épiderme pigmenté. Le biomatériau est ainsi complexifié et se rapproche encore de la peau native, notamment en termes de diversité des cellules biologiques présentes dans le tissu, et de fonctions physiologiques.

Les fibroblastes 1100 peuvent être ensemencés 23 par le dépôt 230 d'une suspension cellulaire, par exemple en goutte à goutte sur la surface 101 de la matrice poreuse, et notamment sur sa face supérieure 1010 selon l'exemple illustré en figure 2. Selon un exemple, les fibroblastes 1100 peuvent être ensemencés à une densité cellulaire comprise entre 500 000 et 1 000 000 cellules/cm^2, voire sensiblement 800 000 cellules/cm^2. Notons que selon le type de cellules biologiques ensemencées, la densité cellulaire peut être adaptée. Ainsi, les fibroblastes 1100 peuvent pénétrer dans le volume intérieur 100 de la matrice poreuse 10. Pour faciliter encore l'adhésion des fibroblastes 1100 ensemencées 23, le procédé peut en outre comprendre une étape d'adhésion des cellules 11, durant laquelle la matrice poreuse 10 est laissée à l'air libre pendant un temps déterminé, par exemple plusieurs minutes, voire une heure, voire de préférence dans une atmosphère contenant 5 % en CO_2.

Le procédé 2 comprend ensuite une croissance cellulaire 24 des fibroblastes 1100 ensemencés 23. Les fibroblastes 1100 peuvent se multiplier par division cellulaire

au moins dans le volume intérieur 100 de la matrice poreuse 10 pour coloniser au moins en partie le volume intérieur 100, voire sa totalité, comme illustré par la figure 3. Lors de la croissance cellulaire 24, les fibroblastes peuvent en outre synthétiser 241, ou de façon équivalente sécréter, une matrice extracellulaire 112 au moins dans le volume intérieur 100. Les cellules biologiques 11, et notamment les fibroblastes 1100, et leur matrice extracellulaire 112 permettent de former un tissu dans la matrice poreuse 10, et se rapprocher encore des propriétés d'un tissu natif, et notamment d'une peau native. Les fibroblastes 1100 dans le volume intérieur 100 de la matrice poreuse 10, voire les fibroblastes 1100 et leur matrice extracellulaire 112, forment ainsi un substitut dermique équivalent au derme natif.

Pour permettre la croissance cellulaire 24 des fibroblastes 1100, la matrice poreuse 10 avec les fibroblastes 1100 ensemencées 23 peut être immergée au moins partiellement, et de préférence totalement, dans un milieu de culture 3 configuré pour mimer l'environnement natif des fibroblastes 1100, comme illustré en figure 3. La matrice 10 étant poreuse, le milieu de culture 3 est ainsi réparti de façon homogène dans la matrice poreuse 10 pour faciliter la croissance cellulaire 24. Par exemple, le milieu de culture est un milieu équivalent au milieu du derme, c'est à dire dont les propriétés, voire la composition, est sensiblement équivalente au milieu du derme. Durant la croissance cellulaire 24, le milieu de culture 3 peut en outre être renouvelé 242, typiquement à une fréquence de plusieurs fois par semaine. Suite à une première immersion dans un milieu de culture 3 exempt acide ascorbique et de facteur de croissance épidermique (abrégé EGF, de l'anglais *Epidermal Growth Factor*), les renouvellements 242 de milieu ultérieurs, peuvent être effectués avec un milieu de culture 3 comprenant de l'acide ascorbique et de l'EGF. Selon un exemple, le premier renouvellement 242 de milieux à lieu 24 à 48h après l'ensemencement 23 des fibroblastes 1100.

Notons que selon le type des autres types cellulaires susceptibles d'être ensemencés avec les fibroblastes 1100, le milieu peut être configuré pour s'adapter et mimer l'environnement plus favorable aux types cellulaires ensemencés.

Le procédé 2 peut comprendre en outre un deuxième ensemencement 26, par exemple effectué après une ou plusieurs semaines de croissance cellulaire 24 des fibroblastes 1100. Préalablement au deuxième ensemencement 26, la matrice poreuse 10 peut être séchée 25 de façon similaire au séchage 22 décrit précédemment. Ensuite, un deuxième type 111 de cellules biologiques 11 peut être ensemencé 26, comprenant notamment des kératinocytes 1110.

Les séchages 22, 25 préalables aux ensemencements permettent de favoriser l'adhésion cellulaire des cellules 11 ensemencées sur la matrice poreuse, et donc leur croissance cellulaire subséquente, comme indiqué précédemment pour le séchage 22. En outre, le milieu de culture peut être renouvelé ou changé. Le séchage de la matrice poreuse 10 peut comprendre une aspiration de la solution ou du milieu cellulaire présent dans la matrice poreuse 10. Selon un exemple, lors du séchage, la matrice poreuse 10 peut en outre être déposée sur un support absorbant. La matrice poreuse 10 peut par exemple être déposée sur une compresse stérile, de préférence déposée sur un papier buvard stérile. De préférence, la matrice poreuse 10 est déposée sur le support absorbant après l'aspiration. L'aspiration permet de retirer la solution ou le milieu présent dans la matrice poreuse 10 de façon contrôlée et précise. Le dépôt sur le support absorbant permet de finaliser le séchage de la matrice poreuse 10 et ainsi d'améliorer encore l'adhésion cellulaire des cellules 11 ensemencées sur la matrice poreuse 10.

Les kératinocytes 1110 peuvent être ensemencés 26 par le dépôt 260 d'une suspension cellulaire, par exemple en goutte à goutte sur la surface 101 de la matrice poreuse 10, et notamment sur sa face supérieure 1010 selon l'exemple illustré en figure 4. De préférence, on évite le dépôt de la suspension cellulaire sur les faces latérales 1011 de la matrice poreuse 10 pour éviter la propagation des kératinocytes 1110 sur ces faces. Les kératinocytes 1110 peuvent être ensemencés à une densité cellulaire comprise entre 500 000 et 1 000 000 cellules/cm^2, voire sensiblement 800 000 cellules/cm^2. Notons à nouveau que selon le type de cellules biologiques ensemencées, la densité cellulaire peut être adaptée. Les kératinocytes 1110 ensemencés 26 restent à la surface extérieure 101 de la matrice poreuse 10 de par grâce à la présence de matrice extra cellulaire sécrétée par les fibroblastes et par la présence des fibroblastes eux-mêmes. Pour faciliter encore l'adhésion des kératinocytes 1110 ensemencées 26, le procédé peut en outre comprendre une étape d'adhésion des cellules 11, durant laquelle la matrice poreuse 10 est laissée pendant un temps déterminé, par exemple plusieurs minutes, voire une heure, et de préférence dans une atmosphère contenant 5 % en CO_2 et à 37°C.

Le procédé 2 comprend ensuite une croissance cellulaire 27 des kératinocytes 1110 ensemencés 26. Ainsi, les kératinocytes 1110 se multiplient par division cellulaire à la surface extérieure 101 de la matrice poreuse 10 jusqu'à la confluence 2702 des kératinocytes 1110, comme illustré par la figure 4.

Les kératinocytes 1110 en surface 101 de la matrice poreuse 10, forment ainsi un substitut épidermique équivalent à l'épiderme natif. L'on comprend qu'avec les

fibroblastes 1100, voire avec leur matrice extracellulaires 112, le biomatériau 1 peut former un substitut dermo-épidermique équivalent à la peau native.

Pour cela, la croissance cellulaire 27 des kératinocytes 1110 peut comprendre une première sous-croissance 270 durant laquelle la matrice poreuse 10 avec les cellules ensemencées 23, 26 est immergée dans un milieu de culture 3 configuré pour mimer l'environnement natif des kératinocytes 1110, par exemple un milieu appelé MC2, adapté du milieu « Green »tel que décrit ultérieurement. L'ajout du milieu de culture 3 est de préférence réalisé sans que le flux de milieu 3 incident ne touche directement la surface 101 de la matrice poreuse 10, de façon à ne pas perturber les kératinocytes 1110 adhérés, et jusqu'à recouvrir la matrice poreuse et les cellules, y compris les kératinocytes 1110. La matrice 10 étant poreuse, le milieu de culture 3 est ainsi réparti de façon homogène dans la matrice poreuse 10 pour faciliter la croissance cellulaire 27. En outre, durant la croissance cellulaire 26, le milieu de culture 3 peut en outre être renouvelé 2701, typiquement à une fréquence de plusieurs fois par semaine.

Suite à une première immersion dans un milieu de culture 3 exempt acide ascorbique et d'EGF, les renouvellements 2701 de milieu ultérieurs, peuvent être effectués avec un milieu de culture 3 comprenant de l'acide ascorbique et de l'EGF. Selon un exemple, le premier renouvellement 2701 de milieux a lieu 24 à 48h après l'ensemencement 26 des kératinocytes 1110.

Suite à la première sous-croissance, la croissance cellulaire 27 des kératinocytes 1110 peut comprendre une maturation 271 durant laquelle la matrice poreuse 10 est placée à une interface 5 entre de l'air 4 et un milieu de culture 3. Cette configuration permet d'induire une différenciation 2710 des kératinocytes 1110' pour former un épiderme complexe, voire complet, caractérisé par une pluri-stratification et une différenciation comme illustré en figure 5A. En outre, lors et suite à la maturation, il est possible d'observer l'expression de protéines ou lipides spécifiques liées à la différenciation des kératinocytes 1110, telles que la cytokératine 1 ou 10, la loricine, l'involucrine ou la filaggrine, ou à leur état prolifératif, telles que la cytokératine 5 ou 14, ki67. Le biomatériau 1 se rapproche encore d'une peau native, notamment en termes de diversité des cellules biologiques présentes dans le tissu, et de fonctions physiologiques. Plus particulièrement, les kératinocytes 1110' peuvent se différencier pour former au moins une partie, voire l'ensemble des couches de l'épiderme suivantes, données de l'intérieur vers l'extérieur :

- *stratum germinativum* : composée d'une assise unique de kératinocytes à la jonction avec le derme, ces cellules étant capables de proliférer,
- *stratum spinosum* : composée de 5 à 6 assises de kératinocytes polygonaux,

- *stratum granulosum* : composée de 2 à 4 couches de kératinocytes aplatis et fusiformes,
- *stratum corneum.*

Selon l'exemple illustré en figure 5A, la matrice poreuse 10 avec les cellules ensemencées 23, 26 peut être disposée sur un support absorbant 7, placé sur une grille 6, par exemple en métal inoxydable et stérilisable par autoclave. Un milieu de culture 3 adapté, désigné milieu Air/liquide, peut ensuite être ajouté de façon à affleurer le support absorbant 7, par exemple un papier buvard. Ainsi, la matrice poreuse est maintenue hydratée tout en permettant l'induction de la différenciation des kératinocytes 1110. De préférence, le niveau du milieu de culture 3 est ajusté de façon à éviter la présence de bulle sous la grille 6, voire entre le support absorbant 7 et la grille 6. En outre, durant la maturation 271, le milieu de culture Air/liquide, de préférence additionné uniquement de vitamine C, peut en outre être renouvelé 2711, typiquement à une fréquence de plusieurs fois par semaine.

À titre d'exemple, une chronologie des étapes du procédé 2 de fabrication est donnée en référence à la figure 7. Dans une première phase A, la matrice poreuse 10 peut être fournie 20, puis préparée par hydratation 21 et séchage 22. Les fibroblastes 1100 peuvent ensuite être ensemencés 23 à un jour désigné J0. Dans une deuxième phase B, la croissance cellulaire 24 des fibroblastes peut être réalisée, suite à quoi les kératinocytes 1110 peuvent être ensemencés 26, 14 jours à 21 jours après J0. Dans une troisième phase C, la première sous-croissance 270 des kératinocytes 1110 peut être réalisée. 21 à 28 jours après J0, la matrice poreuse 10 peut être disposée à l'interface 5 entre l'air et le milieu de culture 5 pour permettre la différenciation cellulaire 2710 dans une phase D, par exemple durant 7 à 21 jours. Notons que les durées indiquées peuvent varier selon les besoins et la quantité de cellules disponible.

De par les caractéristiques du procédé 2 décrites, on comprend que l'on peut obtenir le biomatériau 1 selon le premier aspect de l'invention, comprenant notamment au moins un type 110 de cellules biologiques 11 vivantes d'un tissu, disposé dans le volume intérieur 100. En alternative ou en complément, le type 110 de cellules biologiques 11 vivantes d'un tissu peuvent être disposées sur la surface 101 de la matrice poreuse 10. Notamment, la disposition des cellules 11 dans la matrice poreuse 10 peut dépendre de la nature de cellules biologiques11 ensemencées Dans le cas de la cornée, des cellules stromales de la cornée peuvent être déposées dans la matrice poreuse 10 pour former le stroma, et des kératocytes peuvent être déposées à la surface de la matrice poreuse 10 pour former l'épithélium de la cornée. Plus

particulièrement, le biomatériau 1 peut former un substitut dermo-épidermique d'une peau native, comme illustré en figure 5B.

Le biomatériau 1 peut notamment être utilisé pour une application *in vitro* de substances pharmaceutiques ou de substances cosmétiques. Lors du développement de l'invention, il a été mis en évidence que le procédé décrit permet d'obtenir le matériau 1 de façon reproductible. ailleurs, le biomatériau 1 peut être utilisé comme tissu modèle, par exemple pour la recherche fondamentale et dermatologique.

De par les caractéristiques précédemment décrites, le biomatériau 1 présente une activité régénératrice. Le biomatériau 1 est ainsi particulièrement adapté pour le traitement de lésions et/ou pour une greffe, par exemple sur la peau, sur l'œil ou sur un os.

Le biomatériau 1 pouvant former un substitut dermo-épidermique d'une peau native, obtenu de façon reproductible, le biomatériaux 1 est particulièrement adapté pour le test de nouveaux produits dans l'industrie pharmaceutique. Par exemple, les peaux reconstruites à partir des cellules de patient peuvent représenter un modèle alternatif pour la modélisation de maladies dermatologiques, telles que le vitiligo, le psoriasis, la dermatite atopique et le *Xeroderma Pigmentosum*, et pour le développement des molécules thérapeutiques.

Le biomatériau 1 formant un substitut dermo-épidermique d'une peau native, le biomatériau 1 peut notamment être utilisé pour :

- traiter des séquelles de brûlures,
- traiter une plaie cutanée aigue
- traiter une plaie cutanée chronique
- traiter des zones donneuses de peau une fois un prélèvement tissulaire effectué,
- réaliser une greffe cutanée, par exemple suite à une brûlure, un ulcère, un traumatisme de la peau
- traiter toute perte de substance dermique ou dermo-épidermique.

Le biomatériau 1 formant un substitut dermo-épidermique d'une peau native, est particulièrement adapté pour le traitement des plaies chroniques ou d'une brûlure car il minimise, voire évite, l'ajout d'un traumatisme d'origine iatrogène, lié au prélèvement de peau au niveau d'une zone donneuse. En outre, les cellules biologiques 11 pouvant être autologues, le biomatériau 1 minimise le risque de rejet immunitaire du biomatériau 1.

Pour obtenir les cellules autologues 11, une biopsie de peau peut être réalisée, sur une surface saine et réduite par rapport à la surface nécessaire pour le traitement

d'une plaie ou d'une brûlure. Les cellules 11 peuvent être multipliées par prolifération cellulaire dans le procédé de fabrication 2 afin d'obtenir une quantité suffisante pour permettre les croissances cellulaires 24 et/ou 27. Il est donc possible d'obtenir un biomatériau 1 présentant une surface adaptée à la surface de la zone lésée. Ainsi, le biomatériau 1 et son procédé de fabrication 2 permettent de minimiser les risques et traumatisme lié au prélèvement de peau chez une personne, par rapport aux solutions existantes.

En outre, le biomatériau 1 peut être utilisé comme substitut cutané pour le test *in vitro* de substances pharmaceutiques, par exemple de médicaments dermatologiques, et de produits dermo-cosmétiques. Les tests de l'efficacité et de l'innocuité des médicaments dermatologiques et des produits dermo-cométiques peuvent ainsi être effectués sur un biomatériau se rapprochant d'une peau native.

Des caractéristiques du biomatériau 1 ont été décrites lors de la description du procédé 2. Dans la suite, on détaille des caractéristiques additionnelles du biomatériau 1.

La matrice poreuse 10 du biomatériau 1 est maintenant décrite en détail. La matrice poreuse 10 peut être déformable élastiquement, par exemple sous la pression d'un doigt d'un utilisateur. Ainsi, l'utilisation du biomatériau 1 est facilitée, par exemple pour son application sur une zone lésée. Les propriétés de barrière du biomatériau 1 peuvent par exemple être améliorées.

La matrice poreuse 20 est résorbable. La matrice poreuse 20 peut en outre être biocompatible, notamment en étant constituée de matériaux biocompatibles. La matrice poreuse peut plus particulièrement être formée d'un matériau comprenant du collagène. Le collagène peut être du collagène bovin ou porcin de type I. Le matériau de la matrice poreuse 10 peut comprendre au moins 90%, de préférence au moins 95 %, et plus préférentiellement au moins 99 % en masse de collagène, par rapport à la masse sèche de la matrice poreuse 10. Ainsi, l'intégration dans l'organisme d'une partie du biomatériau 1, et plus particulièrement du tissu formé par les cellules biologiques 11 est facilitée.

En outre, le matériau de la matrice poreuse 10 peut être exempt de chitosan. Le chitosan présente une vitesse de dégradation dans un organisme inférieure à celle du collagène. Comme le biomatériau 1 comprend des cellules biologiques 11, il n'est pas nécessaire d'attendre que les cellules d'une personne colonisent le biomatériau suite à son implantation, par exemple par greffe, comme pour les solutions existantes. Dès lors, il est possible d'utiliser une matrice poreuse à base d'un matériau présentant une vitesse de dégradation plus rapide. Il peut être même avantageux d'accélérer la

dégradation de la matrice poreuse pour accélérer l'intégration du tissu formé par les cellules biologiques.

La matrice poreuse 10 peut être sous la forme d'un gel, et plus particulièrement d'une poudre ou d'une éponge. Ainsi, la matrice poreuse peut être mise en forme en deux, voire en trois dimensions selon les besoins. Selon un exemple, la matrice poreuse 10 est une éponge synthétique, et plus particulièrement une éponge hémostatique. Une éponge synthétique peut aisément être mise en forme par découpe et présente une dureté lui permettant de conserver la forme voulue, tout en restant suffisamment déformable pour pourvoir être appliquée sur un support, par exemple sur le corps d'une personne. Une éponge hémostatique est une éponge synthétique généralement composée de matériaux d'origine biologique, et résorbable par l'organisme. La porosité de ces éponges permet une grande absorption de fluide arrivant, typiquement jusqu'à 35 fois leur poids. Une éponge hémostatique se présente comme un matériau sec, souple, poreux et disponible commercialement sous diverses formes et marques commerciales. Ainsi, la matrice poreuse 10 peut être disponible commercialement et à un coût réduit.

Une éponge hémostatique permet d'absorber le sang issu du corps humain et animal, par exemple issu d'une plaie sur laquelle est déposé le biomatériau. Une éponge hémostatique peut absorber un poids en sang sensiblement supérieur ou égal à 10 fois, de préférence sensiblement supérieur ou égal à 20 fois, et plus préférentiellement sensiblement supérieur ou égal à 30 fois le poids de l'éponge avant absorption. L'éponge hémostatique produit ainsi une pression sur le site du saignement, induisant une agrégation plaquettaire et donc activant les voies de la coagulation de la fibrine pour atteindre une hémostase. On pourrait toutefois s'attendre à ce que le sang aspiré dégrade les cellules du biomatériau. Or, lors du développement de l'invention, il a été constaté de façon surprenante que l'aspiration du sang dans le biomatériau 1 ne nuisait pas aux cellules 11 ensemencées dans le biomatériau 1, notamment lorsque le biomatériau 1 comprend une matrice extracellulaire 112 sécrétées par les cellules 11.

La matrice poreuse 10 fournie 20 est de préférence stérile, afin d'éviter la prolifération d'organismes non désirés dans le biomatériau 1. Selon un exemple, la matrice poreuse 10 est un produit suivant les Bonnes Pratiques de Fabrication (abrégé BPF, et pouvant être traduit en anglais par *Good Manufacturing Practices*, abrégé GMP). Les bonnes pratiques de fabrication sont des textes réglementaires établis par des États, la Commission européenne ou l'Organisation mondiale de la santé, et s'appliquent notamment à la fabrication de médicaments à usage humain ou

vétérinaire. De façon équivalente, la matrice poreuse 10 est utilisable cliniquement sur un animal ou une personne, par exemple pour son application sur une plaie. Ainsi, le processus de mise sur le marché du biomatériau est simplifié.

Par exemple, la matrice poreuse 10 est une éponge hémostatique BPF choisie parmi les éponges hémostatiques des marques suivantes : Spongostan™, CuraSpon®, Gelfoam® et Surgifoam®.

À titre d'exemple, des caractérisations immunohistologiques par microscopie à fluorescence du biomatériau 1 selon un mode de réalisation sont illustrées par les figures 8 à 10. Pour ces caractérisations, des anticorps spécifiques à certaines structures cellulaires, et couplé à une sonde fluorescente sont appliquées sur le biomatériau 1.

La figure 8 représente une image de microscopie à fluorescence en coupe transversale du biomatériau, dans laquelle les noyaux 11' des cellules biologiques 11 et la cytokératine 14 1111 sont marqués. On peut observer la disposition des fibroblastes dans la matrice poreuse 10 et celles des kératinocytes au-dessus de la face supérieure 1010 de la matrice poreuse 10. Les kératinocytes différenciés 1110' présentent en outre un cytosquelette de cytokératine 1111.

La figure 9 représente une image de microscopie à fluorescence en coupe transversale du biomatériau 1, dans laquelle les noyaux 11' des cellules biologiques 11, la filaggrine 1120 et le collagène de type I 1121 sont marqués. On peut observer la matrice extracellulaire 112 sécrétée, par le marquage des protéines énoncées, respectivement en dessous et en dessus de la face supérieure 1010 de la matrice poreuse 10.

La figure 10 représente une image de microscopie à fluorescence en coupe transversale du biomatériau 1, dans laquelle les noyaux 11 des cellules biologiques, la fibrilline 1 1122 et l'élastine 1123 sont marqués. On peut observer la matrice extracellulaire 112 sécrétée par les fibroblastes 1100 dans la matrice poreuse 10, par le marquage des protéines énoncées.

Exemple de mode opératoire du procédé de fabrication du biomatériau

À titre d'exemple, un mode opératoire du procédé 2 de fabrication du biomatériau 1 est maintenant décrit selon un mode particulier de réalisation.

La composition des milieux de culture 3 utilisés est donnée dans les tableaux suivants. L'abréviation « qsp » signifie quantité suffisante pour le volume de solution voulue. Les pourcentages sont donnés en volume par rapport au volume de solution voulue.

Milieu de culture MC1

Tableau 1

Composants	Concentrations
Milieu DMEM/glutamax	qsp
Sérum de veau	5 %
Sérum de veau fœtal	5 %
EGF	10 ng/mL
Pénicilline	100 unité/mL
Streptomycine	100 µg/mL
Amphotéricine B	1 µg/mL
Vitamine C	82, 2µg/mL

Milieu culture MC2 adapté du Milieu GREEN

Tableau 2

Composants	Concentrations	Alternatives pour la clinique
Milieu DMEM/glutamax	qsp	
HAM F12	qsp	
Sérum de veau fœtal	10 %	
Hydrocortisone	0,4 µg/mL	
Insuline	5 µg/mL	Umuline® à 0,12 UI/ml
Choleratoxine	10^{-10} mol/L	Isuprel® à 0,4µg/ml
Adénine	24,3 µg/mL	
Tri Iodo Thyronine	2 nM	
EGF	10 ng/ml	
Amphotéricine B	100 unité/mL	
Pénicilline	100 µg/mL	
Streptomycine	1 µg/mL	
Vitamine C	82,2 µg/mL	

Notons qu'en vue d'une utilisation clinique, le milieu MC2 peut être adapté par l'homme du métier pour être compatible avec une utilisation clinique.

Milieu de culture A/L

Tableau 3

Composants	Concentrations
Milieu DMEM/glutamax	qsp
HAM F12	qsp
Albumine de sérum bovin (BSA)	8 mg/mL
Hydrocortisone	0,4 µg/mL
Insuline	5 µg/mL
Amphotéricine B	1 µg/mL
Pénicilline	100 unité/mL
Streptomycine	100 µg/mL
Vitamine C	82,2 µg/mL

La préparation des réactifs pour fabriquer les milieux de culture est maintenant décrite :

- préparation de la trio-iodo-L-thyronine (abrégé T3 dans la suite):
  - préparation d'une solution A à $2x10^{-4}$ M en pesant 13,6mg de T3, puis les dissoudre dans 1,5ml d'une solution d'hydroxyde de sodium NaOH à 0,02 mol/L, compléter à 100 mL avec de l'eau stérile,
  - préparation de la solution mère de $2x10^{-6}$ M en réalisant une dilution au $1/100^{ème}$ de la solution A,
  - filtrer sur un filtre de dimension 0,22 µm,
  - aliquoter en portions de 550µL et congeler à -20°C,
  - utiliser 500µl de la solution à $2x10^{-6}$M pour 500 mL de milieu MC2 pour obtenir une concentration finale $2x10^{-9}$ M,
- préparation de l'adénine :
  - dissoudre 0,486g d'adénine dans 3mL d'une solution de NaOH à 0,4 mol/L. Ajouter 10 mL d'eau stérile puis 10 mL d'une solution d'acide chlorhydrique HCl à 1 mol/L. Mélanger et compléter avec de l'eau stérile jusqu'à 200 mL,
  - filtrer sur un filtre de porosité 0,22µm,
  - aliquoter en portions de 5 mL et congeler à -20°C
  - utiliser 5 mL pour 500 m L de milieu pour obtenir une concentration finale à 24,3 µg/mL,
- préparation de l'EGF :

- préparer une solution à 10 µg/mL en reprenant 200 µg d'EGF lyophilisé par de l'eau stérile en qsp 20 mL,
- filtrer sur un filtre de porosité 0,22 µm,
- aliquoter en portions de 500µL et congeler à -20 °C,
- utiliser 500 µL pour 500 mL de milieu au moment de l'utilisation du milieu de culture, pour obtenir une concentration finale de 10 ng/mL,

- Préparation de la vitamine C :
  - peser 2,5g de vitamine C en poudre et les dissoudre progressivement dans 60,8 mL de milieu DMEM à 37°C pour obtenir une concentration de 41,1 mg/mL ou de façon équivalente 142 mmol/L,
  - filtrer sur un filtre de porosité 0,22 µm,
  - aliquoter en portions de 0,5ml et congeler à -20°C
  - Utilliser 200 µL dans 100mL de milieu pour obtenir une concentration finale de 82,2 µg/mL,
- préparation de la BSA :
  - peser 4 g de BSA et les dissoudre dans 20 mL de milieu DMEM à 37°C,
  - filtrer sur un filtre de porosité 0,22 µm,
  - utiliser les 20 mL pour 500 mL de milieu,
- préparation de l'hydrocortisone :
  - préparer une solution B à 5 mg/mL en diluant 25 mg dans 5 mL d'éthanol à 95%,
  - préparer une solution C à 200 µg/mL en prendre 0,4 mL de la solution B dans 9,6 mL (qsp 10mL),
  - aliquoter la solution C en portions de 1mL,
  - utiliser 1mL pour 500 mL de milieu, pour obtenir une concentration finale de 0,4 µg/mL,
- préparation de l'insuline :
  - re-suspendre 50 mg d'insuline avec 500 µL d'une solution d'HCL à 0,1 mol/L,
  - diluer la suspension obtenue dans de l'eau stérile qsp 10 mL, pour obtenir une concentration de 5 mg/mL,
  - filtrer sur un filtre de porosité 0,22 µm,
  - aliquoter en portions de 500 µL,
  - utiliser 500µL pour 500mL de milieu, pour obtenir une concentration finale de 5 µg/mL,
- préparation de la choleratoxine :

- re-suspendre 1 mg de choleratoxine dans 12 mL d'eau stérile pour obtenir une concentration de 10.10^{-7} mol/L,
- utiliser 500 µL de la solution à 10.10^{-7} pour 500 mL de milieu pour obtenir une concentration finale de 10^{-10} mol/L.

Les étapes du mode opératoire sont maintenant détaillées.

Pour l'extraction des fibroblastes et kératinocytes d'une biopsie cutanée d'un patient, la biopsie de peau est incubée durant 3 heures à 37°C dans une solution de dispase II stérile. Le derme et l'épiderme sont alors séparés à l'aide de pinces stériles puis rincés dans du PBS 1X stérile.

Pour l'extraction des fibroblastes, les morceaux de derme obtenus sont ensuite mis dans une solution stérile de collagénase A à 0,5 mg/mL, sous agitation, pendant 4 à 6 heures à 37°C. Puis la solution est filtrée sur un tamis de porosité 70 µm et centrifugée à 1200 rotations par minutes (abrégé rpm dans la suite) durant 5 à 10 minutes. Après re-suspension du culot, la numération et la viabilité cellulaire est évaluée. Les fibroblastes sont ensemencés à une densité de 8 000 cellules/cm^2.

Pour l'extraction des kératinocytes, les morceaux d'épiderme sont incubés dans de la trypsine-EDTA 0,25% à 37°C pendant 15 minutes. Après ajout de solution inhibitrice de trypsine, la suspension de kératinocytes est ensuite filtrée sur tamis de porosité 70 µm, centrifugée à 1200 rpm durant 5 à 10 minutes et le culot de cellules ainsi obtenu est re-suspendu dans du milieu de culture pour évaluation de la numération et de la viabilité. Les kératinocytes sont ensemencés à la densité de 8000 cellules/cm^2.

Les fibroblastes et les kératinocytes sont cultivés à 37°C en atmosphère humide avec 5% de CO_2, les milieux sont renouvelés 3 fois par semaine et les cellules sont passées à sub-confluence par l'action de la trypsine EDTA.

Pour la préparation de la matrice poreuse, la matrice poreuse est hydratée durant 6 à 48 heures préalablement à l'ensemencement des cellules par un bain PBS 1X dont le volume est suffisant pour recouvrir la face supérieur de la matrice. Le PBS peut être ensuite aspiré. Un volume de milieu MC1, sans antibiotiques, ni EGF, ni vitamine C, le volume étant suffisant pour immerger en recouvrant la face supérieure de la matrice, est ajouté afin d'équilibrer le pH et l'osmolarité des matrices. En alternative à ce mode de préparation préférentiel, on peut prévoir d'hydrater la matrice poreuse par un bain de 2 à 3 heures en PBS 1X.

Pour l'ensemencement des fibroblastes, les fibroblastes sont décollés et dissociés par action enzymatique et dénombrés. Une suspension cellulaire pour obtenir une densité cellulaire de $0.5.10^6$ cellules/cellules/cm^2 est préparée. Après avoir

aspiré le milieu de culture pour sécher la matrice poreuse, la suspension de fibroblastes est déposée gouttes à gouttes de façon homogène sur la face supérieure de la matrice poreuse. Les fibroblastes laissés à adhérer durant 1 heure à l'incubateur à 37°C et en atmosphère à 5% en CO_2.

Un volume suffisant pour recouvrir la face supérieure de la matrice poreuse de milieu MC1 avec antibiotiques mais sans EGF ni vitamine C est ajouté pour la croissance des fibroblastes. Le milieu est renouvelé 3 fois par semaine durant 2-3 semaines avec un milieu MC1 avec ajout d'antibiotiques, EGF et vitamine C.

Pour l'ensemencement des kératinocytes, les kératinocytes sont décollés par action enzymatique et dénombrés. Une suspension cellulaire à $0,5.10^6$ cellulescm²) est préparée. Après avoir aspiré le milieu de culture du substitut dermique formé, ce dernier peut être transféré sur une compresse stérile pour absorber le maximum d'humidité.

La suspension cellulaire de kératinocytes est déposée en goutte à goutte de façon homogène à la surface de la matrice en faisant attention que la suspension cellulaire ne déborde pas sur les faces latérales de la matrice poreuse. Les kératinocytes sont laissés à adhérer durant 1 heure à l'incubateur à 37°C et en atmosphère à 5% en CO_2.

Pour la première sous-croissance, un volume suffisant pour recouvrir la face supérieure de la matrice de milieu MC2 avec antibiotiques mais sans EGF ni vitamine C est ajouté sur la matrice très délicatement sans toucher les échantillons. Le milieu de culture est renouvelé 3 fois par semaine durant 1 semaine avec un milieu MC2 avec ajout d'antibiotiques, EGF et vitamine C.

Pour la maturation, la matrice poreuse est déposée après aspiration du milieu de culture et séchage sur une compresse stérile sur un papier buvard stérile lui-même posé sur une grille métallique. Le volume de milieu A/L supplémenté en antibiotiques et vitamine C est ajusté pour que celui-ci affleure le niveau du papier buvard en évitant la présence de bulle coincée sous la grille métallique. Le milieu est renouvelé 3 fois par semaine avec le milieu A/L contenant antibiotiques et vitamine C.

Au vu de la description qui précède, il apparaît clairement que l'invention propose un biomatériau se rapprochant davantage d'un tissu natif.

L'invention n'est pas limitée aux modes de réalisations précédemment décrits et s'étend à tous les modes de réalisation couverts par les revendications.

On peut notamment prévoir que les durées de croissance et/ou la composition des milieux soient adaptés pour d'autres types de cellules biologiques.

REFERENCES

1 Biomatériau
10 Matrice poreuse
100 Volume intérieur
101 Surface
1010 Face supérieure
1011 Face latérale
1012 Face inférieure
11 Cellules biologiques
11' Noyaux
110 Premier type
1100 Fibroblastes
111 Deuxième type
1110 Kératinocyte
1110' Kératinocyte différenciés
1111 Cytokératine 14
112 Matrice extracellulaire
1120 Filaggrine
1121 Collagène de type I
1122 Fibrilline
1123 Élastine
2 Procédé
20 Fourniture d'une matrice poreuse
21 Hydratation de la matrice poreuse
22 Séchage de la matrice poreuse
23 Premier ensemencement
230 Dépôt d'une suspension de cellules biologique
24 Première croissance cellulaire
240 Immersion de la matrice poreuse dans un milieu de culture
241 Synthèse de matrice extracellulaire
242 Renouvellement du milieu de culture
25 Séchage de la matrice poreuse
26 Deuxième ensemencement
260 Dépôt d'une suspension de cellules biologique
27 Deuxième croissance cellulaire

270 Première sous-croissance
2700 Immersion de la matrice poreuse dans un milieu de culture
2701 Renouvellement du milieu de culture
2702 Confluence des cellules biologiques
271 Maturation
2710 Différenciation cellulaire
2711 Renouvellement du milieu de culture
3 Milieu de culture
4 Air
5 Interface air/milieu de culture
6 Grille
7 Support poreux

REVENDICATIONS

1. Biomatériau (1) comprenant :
   - une matrice poreuse (10) résorbable formée d'un matériau comprenant du collagène, et présentant un volume intérieur (100) et une surface extérieure (101),
   - au moins un type (110) de cellules biologiques (11) vivantes d'un tissu, disposées dans le volume intérieur (100) et en alternative ou en complément sur la surface (101) de la matrice poreuse (10),

le biomatériau (1) étant caractérisé en ce que la matrice poreuse (10) est une éponge hémostatique.

2. Biomatériau (1) selon la revendication précédente dans lequel le matériau de la matrice poreuse (10) comprend au moins 90%, de préférence au moins 95 %, et plus préférentiellement au moins 99 % en masse de collagène, par rapport à la masse sèche de la matrice poreuse (10).

3. Biomatériau (1) selon l'une quelconque des revendications précédentes dans lequel le matériau de la matrice poreuse (10) est exempt de chitosan.

4. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel la matrice poreuse (10) est un produit suivant les Bonnes Pratiques de Fabrication (BPF).

5. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel la matrice poreuse (10) est apte à être utilisée cliniquement chez l'humain ou l'animal.

6. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel les cellules biologiques (11) comprennent des cellules choisies parmi des cellules de tissu conjonctif et des cellules épithéliales.

7. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel l'au moins un type (110) de cellules biologiques (11) est disposé au moins dans le volume intérieur (100) de la matrice poreuse (10), le biomatériau (1) comprenant en outre une matrice extracellulaire (112) dans ledit volume intérieur (100), la matrice extracellulaire (112) étant sécrétée par l'au moins un premier type (110) de cellules biologiques (11).

8. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel un premier type (110) de cellules biologiques (11) est disposé dans le volume intérieur (100) de la matrice poreuse (10), et le biomatériau (1) comprend au moins un deuxième type (111) de cellules biologiques (11) disposé sur la surface (101)

de la matrice poreuse (10), le deuxième type (111) de cellules biologiques (11) étant distinct du premier type (110) de cellules biologiques (11).

9. Biomatériau (1) selon la revendication précédente, dans lequel le premier type (110) de cellules biologiques (11) comprend des fibroblastes (1100), et le deuxième type (111) de cellules biologiques (11) comprend des kératinocytes (1110).

10. Biomatériau (1) selon l'une quelconque des revendications précédentes, dans lequel les cellules biologiques (11) sont choisies parmi des cellules dérivées de cultures de lignée cellulaire standard et des cellules isolées d'un échantillon biologique d'un organisme.

11. Procédé (2) de fabrication d'un biomatériau (1) selon l'une quelconque des revendications 1 à 10, comprenant :

- une fourniture (20) d'une matrice poreuse (10) résorbable formée d'un matériau comprenant du collagène, la matrice poreuse (10) étant une éponge hémostatique, et présentant un volume intérieur (100) et une surface extérieure (101), puis
- un ensemencement (23) de la matrice poreuse (10) par au moins un type (110) de cellules biologiques (11) vivantes d'un tissu, puis
- une croissance cellulaire (24) de l'au moins un type (110) de cellules biologiques (10).

12. Procédé (2) selon la revendication précédente, comprenant en outre une hydratation (20) de la matrice poreuse (10) de façon à équilibrer le pH et l'osmolarité de la matrice poreuse (10), préalablement à l'ensemencement (23).

13. Procédé (2) selon la revendication précédente, comprenant en outre un séchage (22) de la matrice poreuse (10) entre l'hydratation (21) de la matrice poreuse (10) et l'ensemencement (23) de la matrice poreuse (10).

14. Procédé (2) selon l'une quelconque des revendications 11 à 13, dans lequel l'ensemencement (23) de la matrice poreuse (10) est réalisé par dépôt (230) d'une suspension de l'au moins un type (110) de cellules biologiques (11) sur la matrice poreuse (10).

15. Procédé (2) selon l'une quelconque des revendications 11 à 14, comprenant en outre, après un premier ensemencement (23) par un premier type (110) de cellules biologiques (11), voire après le premier ensemencement (23) et une partie au moins d'une croissance cellulaire (24) du premier type (110) de cellules biologiques (11), un deuxième ensemencement (26) de la matrice poreuse (10) par au moins un deuxième type (111) de cellules biologiques (11), distinct du premier type (110) de cellules biologiques (11) ensemencées, puis une croissance cellulaire (27) de l'au

moins un deuxième type (111) de cellule biologiques (11).

16. Procédé (2) selon la revendication précédente, comprenant en outre un séchage (25) de la matrice poreuse (10) préalablement au deuxième ensemencement (26) de la matrice poreuse (10), le deuxième ensemencement (26) étant réalisé par dépôt (260) d'une suspension de l'au moins un deuxième type (111) de cellules biologiques (11) sur la matrice poreuse (10).

17. Procédé (2) selon l'une quelconque des revendications 11 à 16, dans lequel les cellules biologiques (11) comprennent des cellules choisies parmi des cellules de tissu conjonctif et des cellules épithéliales.

18. Procédé (2) selon la revendication précédente, dans lequel, lorsque les cellules biologiques (10) sont des cellules de tissu conjonctif, la croissance cellulaire (24) comprend une immersion (240) de la matrice poreuse (10) dans un milieu de culture (3).

19. Procédé (2) selon l'une quelconque des deux revendications précédentes, dans lequel, lorsque les cellules biologiques (10) sont des cellules de tissu épithélial, la croissance cellulaire (27) comprend une première sous-croissance (270) pendant laquelle la matrice poreuse est immergée (270a) dans un milieu de culture (3), jusqu'à une confluence des cellules biologiques (10) en surface de la matrice poreuse (10).

20. Procédé (2) selon la revendication précédente, dans lequel la croissance (27) comprend, suite à la première sous-croissance (270), une maturation (271) durant laquelle la matrice poreuse (10) est placée à une interface entre de l'air (4) et un milieu de culture (3).

21. Utilisation d'un biomatériau (1), le biomatériau (1) comprenant :

- une matrice poreuse (10) résorbable formée d'un matériau comprenant du collagène, la matrice poreuse (10) étant une éponge hémostatique, et présentant un volume intérieur (100) et une surface (101), et
- au moins un type (110) de cellules biologiques (11) vivantes d'un tissu, disposées dans le volume intérieur (100) et/ou sur la surface (101) de la matrice poreuse (10),

pour une application *in vitro* d'au moins une substance choisie parmi une substance pharmaceutique et une substance cosmétique.

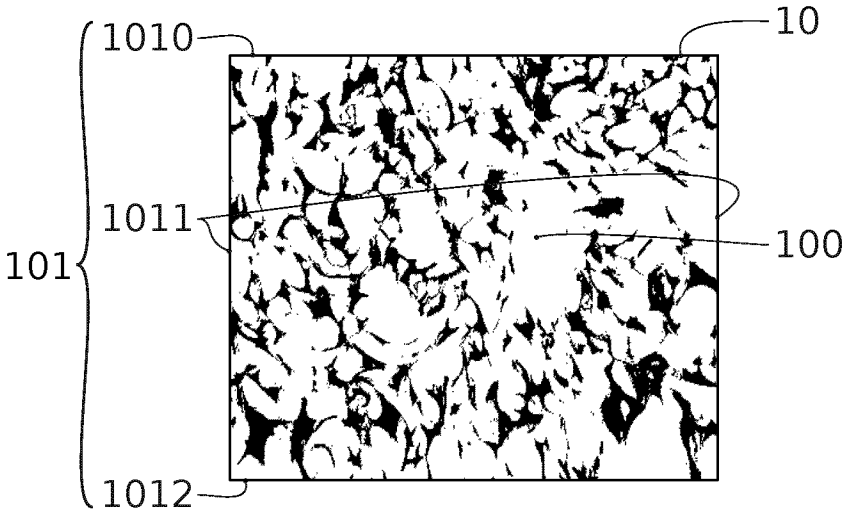


FIG. 1

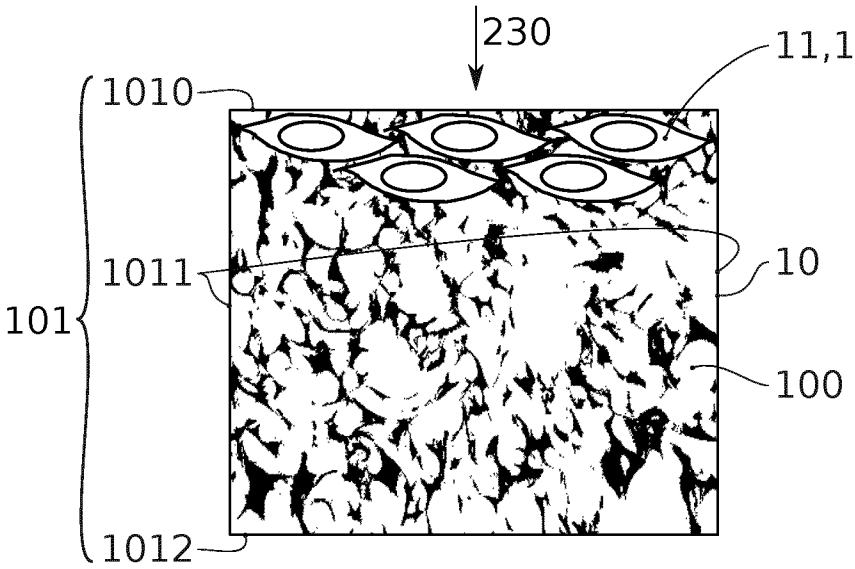


FIG. 2

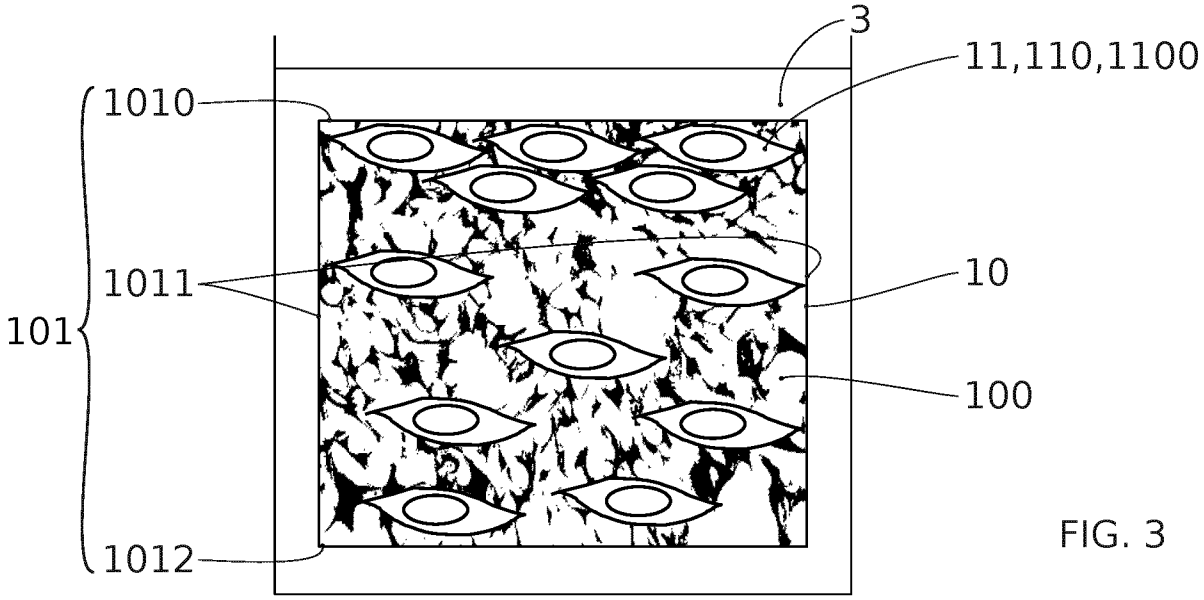


FIG. 3

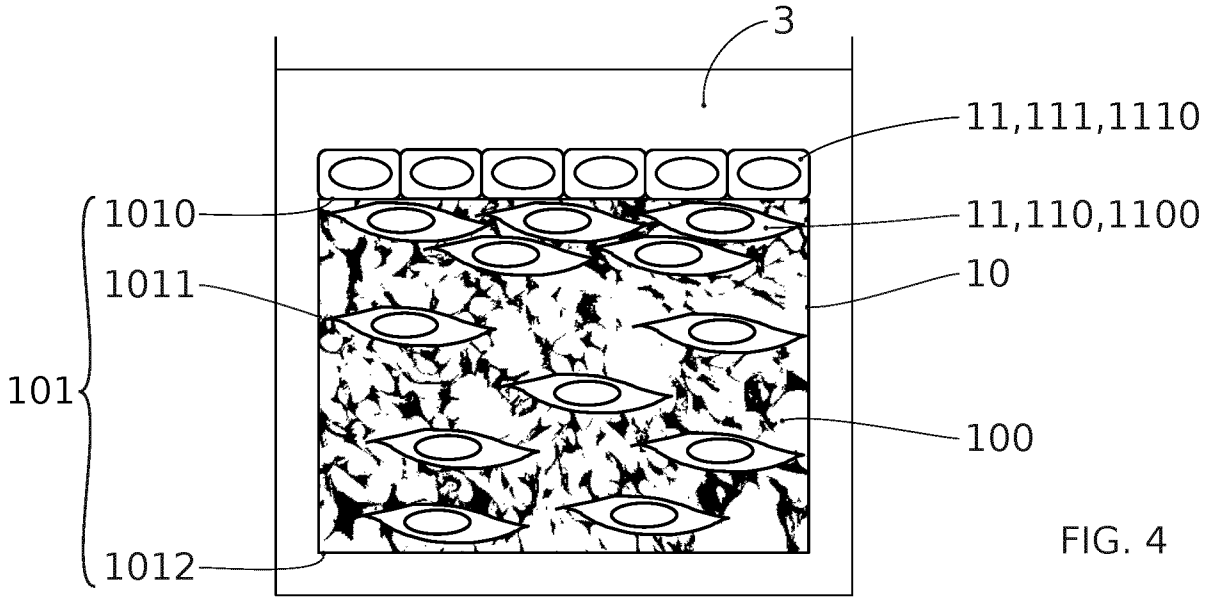


FIG. 4

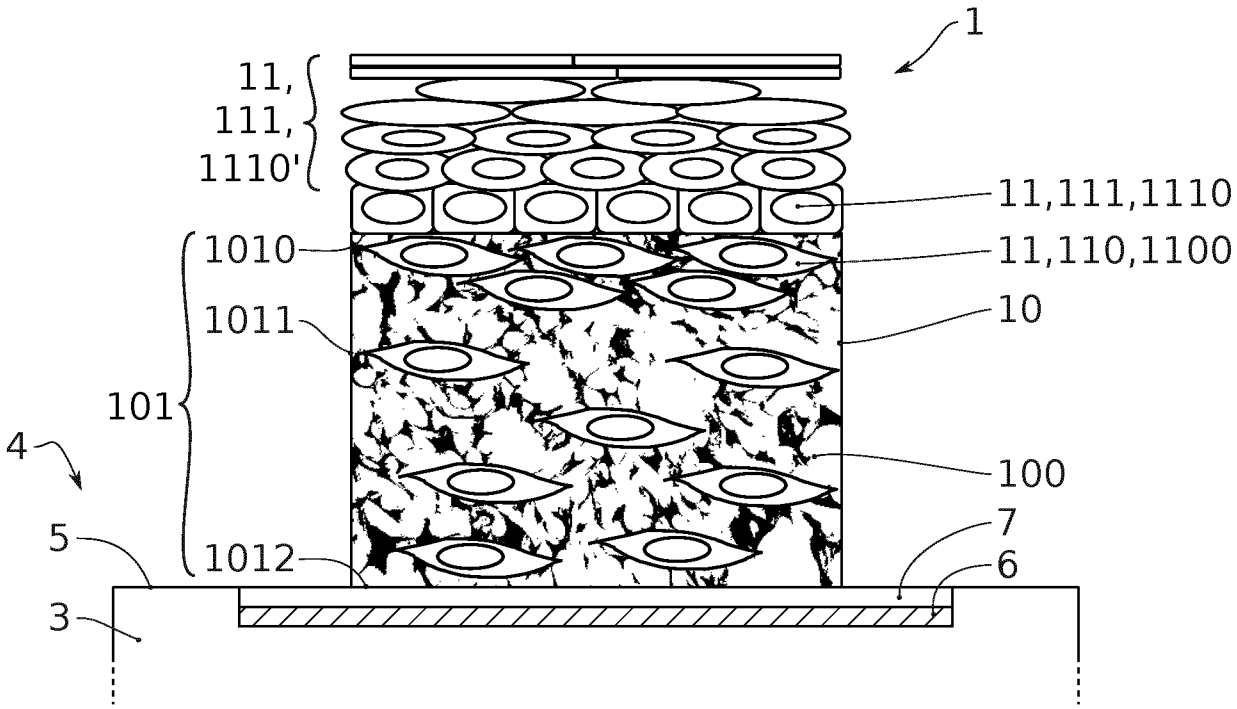

1
11,
111,
1110'
11,111,1110
1010
11,110,1100
1011
10
101
4
100
5
7
1012
6
3

FIG. 5A

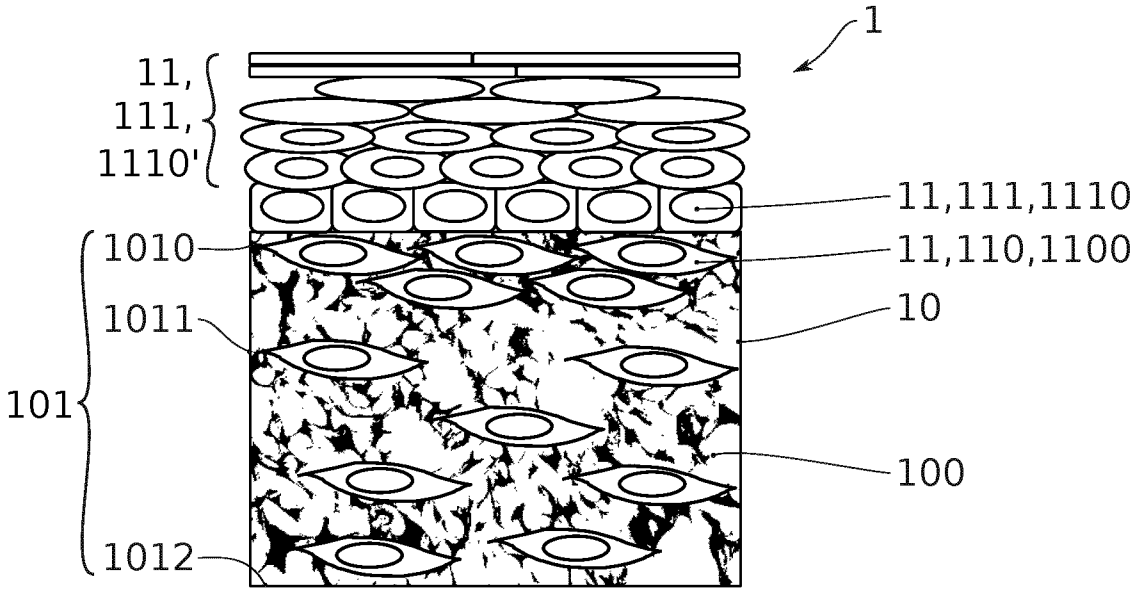

1
11,
111,
1110'
11,111,1110
1010
11,110,1100
1011
10
101
100
1012

FIG. 5B

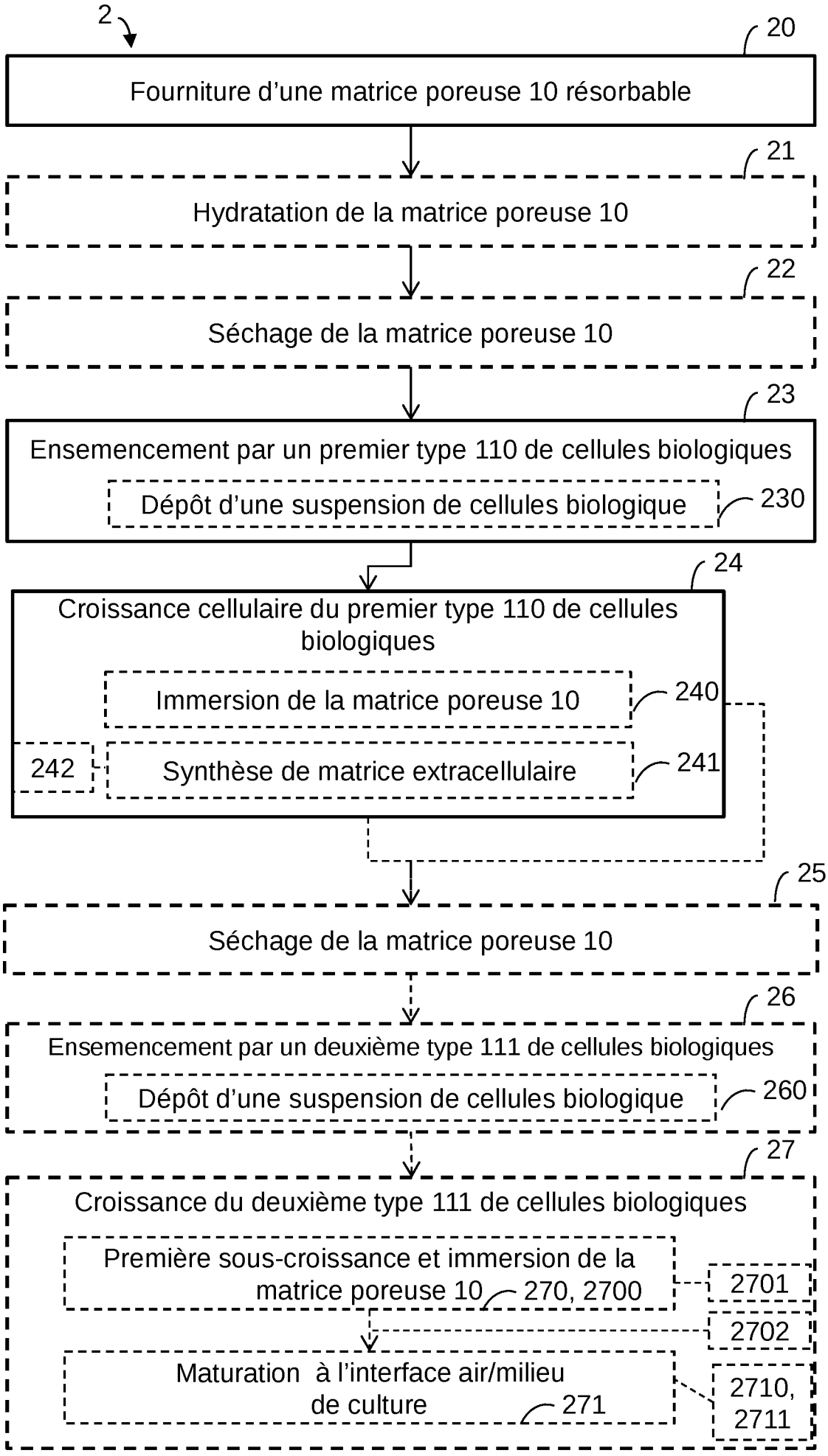

2
20
Fourniture d'une matrice poreuse 10 résorbable
21
Hydratation de la matrice poreuse 10
22
Séchage de la matrice poreuse 10
23
Ensemencement par un premier type 110 de cellules biologiques
Dépôt d'une suspension de cellules biologique
230
24
Croissance cellulaire du premier type 110 de cellules biologiques
Immersion de la matrice poreuse 10
240
242
Synthèse de matrice extracellulaire
241
25
Séchage de la matrice poreuse 10
26
Ensemencement par un deuxième type 111 de cellules biologiques
Dépôt d'une suspension de cellules biologique
260
27
Croissance du deuxième type 111 de cellules biologiques
Première sous-croissance et immersion de la matrice poreuse 10
270, 2700
2701
2702
Maturation à l'interface air/milieu de culture
271
2710, 2711

FIG. 6

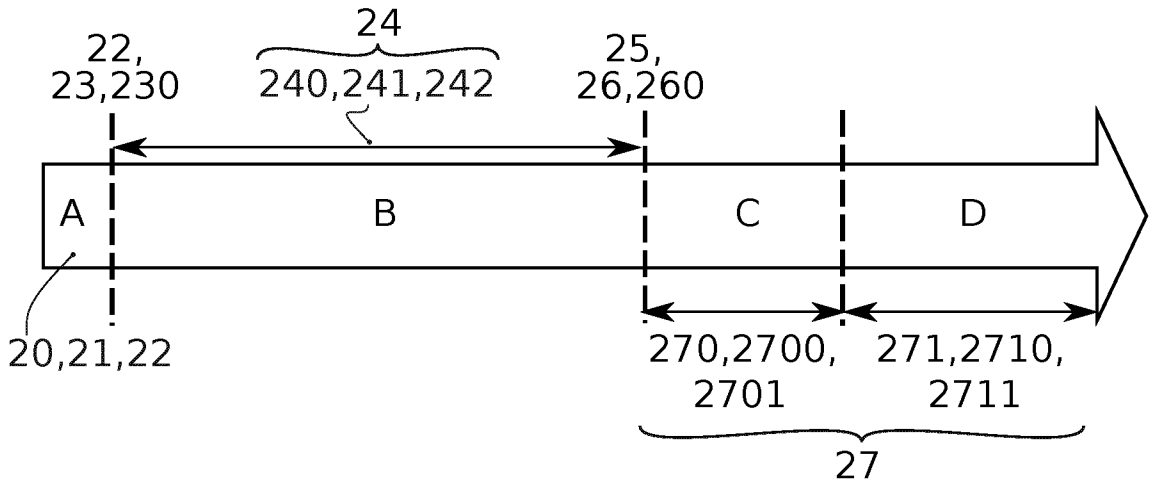

24
22,
23,230
240,241,242
25,
26,260
A
B
C
D
20,21,22
270,2700,
2701
271,2710,
2711
27

FIG. 7

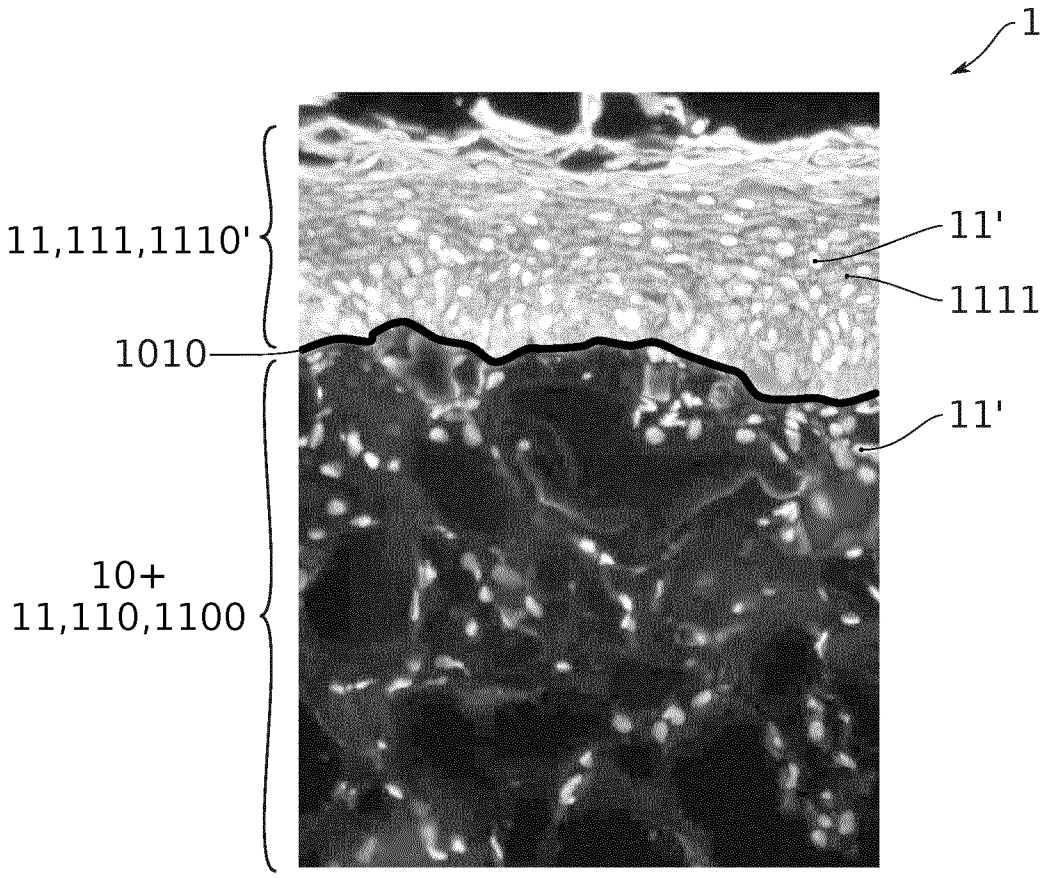

1
11,111,1110'
11'
1111
1010
11'
10+
11,110,1100

FIG. 8

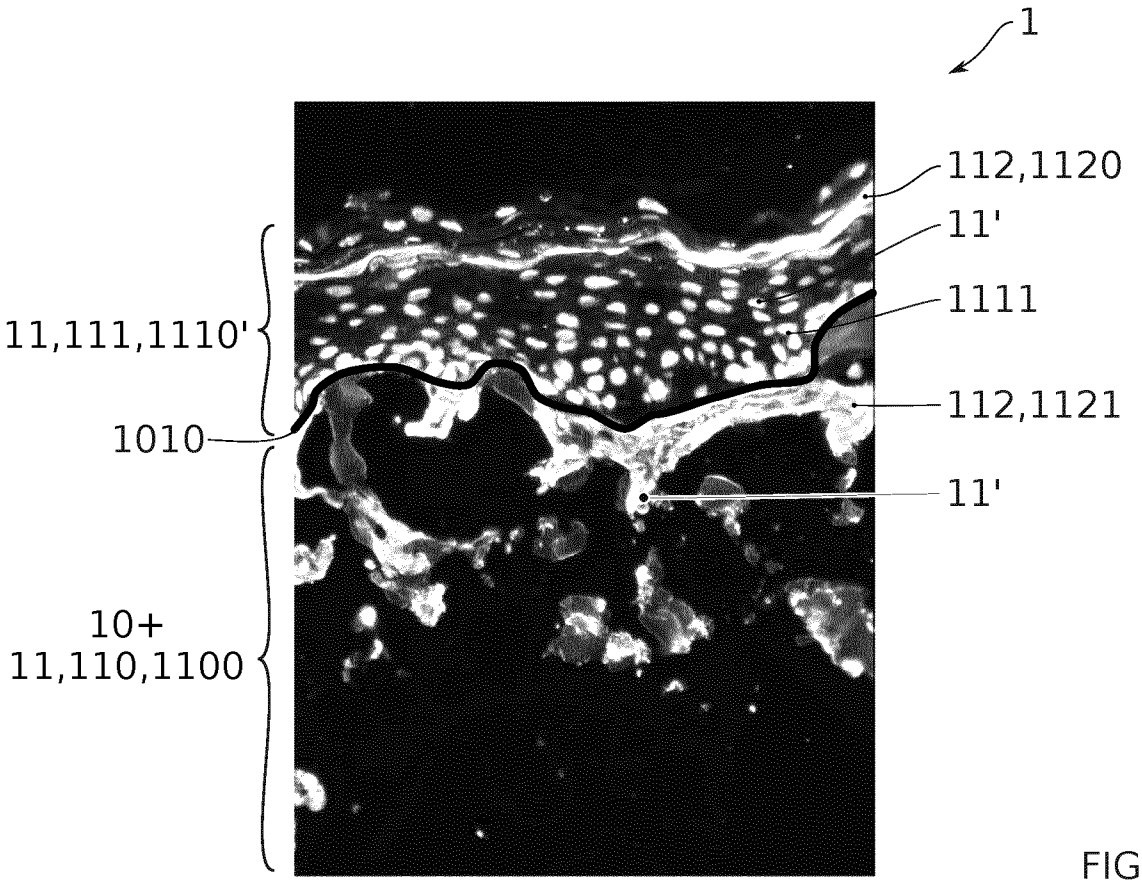

1
112,1120
11'
1111
112,1121
11'
11,111,1110'
1010
10+
11,110,1100

FIG. 9

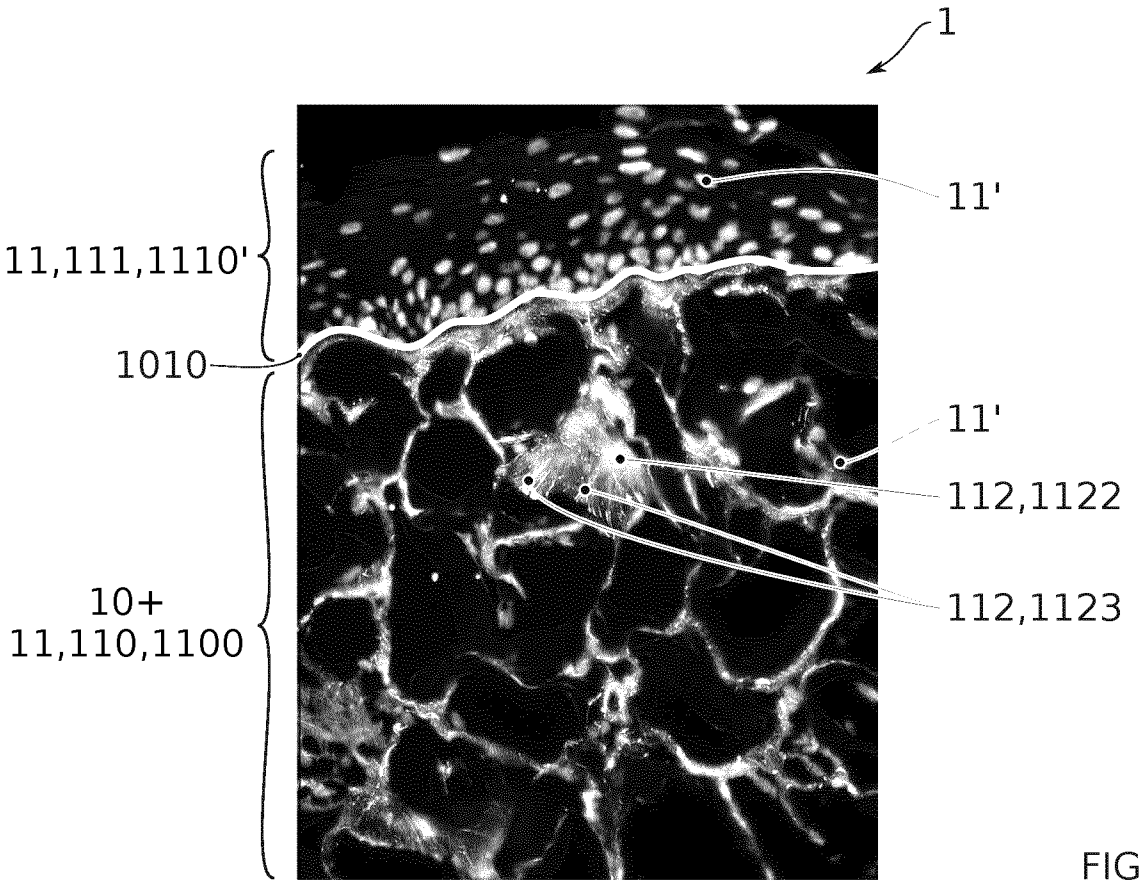

1
11'
11,111,1110'
1010
11'
112,1122
112,1123
10+
11,110,1100

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2021/058151

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/36(2015.01)i; ***A61K 35/33***(2015.01)i; ***A61L 27/24***(2006.01)i; ***A61P 17/02***(2006.01)i; ***A61K 8/98***(2006.01)i; ***A61L 24/00***(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61L; A61K; A61P; A61Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2809313 A1 (COLETICA [FR]) 30 November 2001 (2001-11-30)	1-11,14,15,17-21
Y	page 5, lines 7-32; example 14 page 8, lines 25-27 page 9, lines 10-11	12,13,16
X	US 5282859 A (EISENBERG MARK [AU]) 01 February 1994 (1994-02-01)	1-11,14,15,17-21
Y	column 5, lines 25-38; example 1 column 6, line 27 - line 38	12,13,16
X	US 2006135921 A1 (WIERCINSKI ROBERT A [US] ET AL) 22 June 2006 (2006-06-22)	1-11,14,15,17-20
Y	paragraph [0186]; figures 10,13; example 8	12,13,16
X	US 6153292 A (BELL EUGENE [US] ET AL) 28 November 2000 (2000-11-28)	1-11,14,15,17-20
Y	column 3, lines 40-60; claims 1,8,9,13	12,13,16
X	US 5945101 A (BERG RICHARD A [US] ET AL) 31 August 1999 (1999-08-31) column 2, lines 29-48; claim 1; example 8 column 5, line 37 - column 6, line 18	1-11,14,15,17-20

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2021	**28 May 2021**
Name and mailing address of the ISA/EP **European Patent Office** **p.b. 5818, Patentlaan 2, 2280 HV Rijswijk** **Netherlands** Telephone No. **(+31-70)340-2040** Facsimile No. **(+31-70)340-3016**	Authorized officer **Bochelen, Damien** Telephone No.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAISSIERE G ET AL. "COMPARATIVE ANALYSIS OF DIFFERENT COLLAGEN-BASED BIOMATERIALS AS SCAFFOLDS FOR LONG-TERM CULTURE OF HUMAN FIBROBLASTS" *MEDICAL AND BIOLOGICAL ENGINEERING AND COMPUTING, SPRINGER, HEILDELBERG, DE,* Vol. 38, No. 2, 01 March 2000 (2000-03-01), pages 205-210 DOI: 10.1007/BF02344778 ISSN: 0140-0118, XP000914401 page 206	1-7,10,11,14,17,18

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/EP2021/058151

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
FR	2809313	A1	30 November 2001	NONE			
US	5282859	A	01 February 1994	AT	161408	T	15 January 1998
				AU	632693	B2	07 January 1993
				BR	9106354	A	27 April 1993
				CA	2080693	A1	25 October 1991
				DE	69128530	T2	20 August 1998
				DK	0526550	T3	31 August 1998
				EP	0526550	A1	10 February 1993
				ES	2111566	T3	16 March 1998
				FI	924773	A	21 October 1992
				GR	3026463	T3	30 June 1998
				JP	H0747043	B2	24 May 1995
				JP	H05506169	A	16 September 1993
				RU	2135191	C1	27 August 1999
				TW	225986	B	01 July 1994
				US	RE35399	E	10 December 1996
				US	5282859	A	01 February 1994
				US	6039760	A	21 March 2000
				WO	9116010	A1	31 October 1991
US	2006135921	A1	22 June 2006	EP	1615676	A2	18 January 2006
				US	2006135921	A1	22 June 2006
				WO	2005004928	A2	20 January 2005
US	6153292	A	28 November 2000	AT	247452	T	15 September 2003
				AU	727696	B2	21 December 2000
				CA	2278497	A1	28 May 1998
				DE	69724275	T2	24 June 2004
				EP	0946127	A2	06 October 1999
				JP	2001510358	A	31 July 2001
				US	5891558	A	06 April 1999
				US	6153292	A	28 November 2000
				WO	9822154	A2	28 May 1998
US	5945101	A	31 August 1999	CA	2119064	A1	18 September 1994
				US	5755814	A	26 May 1998
				US	5888248	A	30 March 1999
				US	5945101	A	31 August 1999

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale n°
PCT/EP2021/058151

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
INV. A61K35/36 A61K35/33 A61L27/24 A61P17/02 A61K8/98
A61L24/00
ADD.
Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
A61L A61K A61P A61Q

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie*	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	FR 2 809 313 A1 (COLETICA [FR])	1-11,14,
	30 novembre 2001 (2001-11-30)	15,17-21
Y	page 5, lignes 7-32; exemple 14	12,13,16
	page 8, lignes 25-27	
	page 9, lignes 10-11	

X	US 5 282 859 A (EISENBERG MARK [AU])	1-11,14,
	1 février 1994 (1994-02-01)	15,17-21
Y	colonne 5, lignes 25-38; exemple 1	12,13,16
	colonne 6, ligne 27 - ligne 38	

X	US 2006/135921 A1 (WIERCINSKI ROBERT A	1-11,14,
	[US] ET AL) 22 juin 2006 (2006-06-22)	15,17-20
Y	alinéa [0186]; figures 10,13; exemple 8	12,13,16

	-/--	

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
18 mai 2021	28/05/2021

Nom et adresse postale de l'administration chargée de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bochelen, Damien

1

C(suite). DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie*	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X Y	US 6 153 292 A (BELL EUGENE [US] ET AL) 28 novembre 2000 (2000-11-28) colonne 3, lignes 40-60; revendications 1,8,9,13 -----	1-11,14, 15,17-20 12,13,16
X	US 5 945 101 A (BERG RICHARD A [US] ET AL) 31 août 1999 (1999-08-31) colonne 2, lignes 29-48; revendication 1; exemple 8 colonne 5, ligne 37 - colonne 6, ligne 18 -----	1-11,14, 15,17-20
X	VAISSIERE G ET AL: "COMPARATIVE ANALYSIS OF DIFFERENT COLLAGEN-BASED BIOMATERIALS AS SCAFFOLDS FOR LONG-TERM CULTURE OF HUMAN FIBROBLASTS", MEDICAL AND BIOLOGICAL ENGINEERING AND COMPUTING, SPRINGER, HEILDELBERG, DE, vol. 38, no. 2, 1 mars 2000 (2000-03-01), pages 205-210, XP000914401, ISSN: 0140-0118, DOI: 10.1007/BF02344778 page 206 -----	1-7,10, 11,14, 17,18

1

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Demande internationale n°
PCT/EP2021/058151

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
FR 2809313 A1	30-11-2001	AUCUN	
US 5282859 A	01-02-1994	AT 161408 T	15-01-1998
		AU 632693 B2	07-01-1993
		BR 9106354 A	27-04-1993
		CA 2080693 A1	25-10-1991
		DE 69128530 T2	20-08-1998
		DK 0526550 T3	31-08-1998
		EP 0526550 A1	10-02-1993
		ES 2111566 T3	16-03-1998
		FI 924773 A	21-10-1992
		GR 3026463 T3	30-06-1998
		JP H0747043 B2	24-05-1995
		JP H05506169 A	16-09-1993
		RU 2135191 C1	27-08-1999
		TW 225986 B	01-07-1994
		US RE35399 E	10-12-1996
		US 5282859 A	01-02-1994
		US 6039760 A	21-03-2000
		WO 9116010 A1	31-10-1991
US 2006135921 A1	22-06-2006	EP 1615676 A2	18-01-2006
		US 2006135921 A1	22-06-2006
		WO 2005004928 A2	20-01-2005
US 6153292 A	28-11-2000	AT 247452 T	15-09-2003
		AU 727696 B2	21-12-2000
		CA 2278497 A1	28-05-1998
		DE 69724275 T2	24-06-2004
		EP 0946127 A2	06-10-1999
		JP 2001510358 A	31-07-2001
		US 5891558 A	06-04-1999
		US 6153292 A	28-11-2000
		WO 9822154 A2	28-05-1998
US 5945101 A	31-08-1999	CA 2119064 A1	18-09-1994
		US 5755814 A	26-05-1998
		US 5888248 A	30-03-1999
		US 5945101 A	31-08-1999

CHAPTER 18

Elaboration of a Good Manufacturing Product Skin Substitute from an Absorbable Gelatin Sponge: A Preclinical Study on Large Animal

Alexandre G. Lellouch
Lara Jobeili
Naji Fatima
Laurent A. Lantieri
Walid Rachidi

ABSTRACT

Extensive skin defects caused by burns (thermal, chemical, electrical), trauma, and other aetiologies (ulcers, etc.) constitute a severe public health problem. It can induce a loss of motor and aesthetic function causing difficulties in social reintegration. The "gold standard" in skin coverage remains the skin autograft (split thickness skin graft (STSG)). Morbidity and the small number of donor sites remain the limiting factor of this procedure. Over the past 50 years, many teams have tried to design a dermal-epidermal skin substitute (DESS) to avoid inducing additional sequelae on the patient (donor site). Many difficulties have arisen with, among others: the technical difficulty of setting-up this biomaterial, the need to use autologous cells (to avoid immune rejection) and perfect coordination between clinicians and scientists. Our team at CEA/UGA has recently manufacture a DESS (International Publication number WO 2021/198177 A1). The particularity of this project is that our DESS was carried out by a multidisciplinary team associating clinicians (plastic surgeon) and scientists. In addition, all the components of this DESS are GMP (Good Manufacturing Practice). The expected benefit is to provide a better healing process and a restauration of the skin structure. This study evaluates this DESS on a large animal model (minipig) of skin graft.

Objective
This research project consists of testing a dermal-epidermal skin substitute (DESS) to replace current skin autograft techniques.

Main evaluation criteria:
The viability of the dermo-epidermal substitute / engraftment (photo)

Secondary evaluation criteria:
- Animal welfare (pain)
- Signs of infection, inflammation
- Immune rejection (histology)

Figure 1. Protocol patented 2020: « Biomaterial comprising a porous resorbable matrix and associated manufacturing method" Application No. PCT/EP2021/058151 International Publication number WO 2021/198177 A1

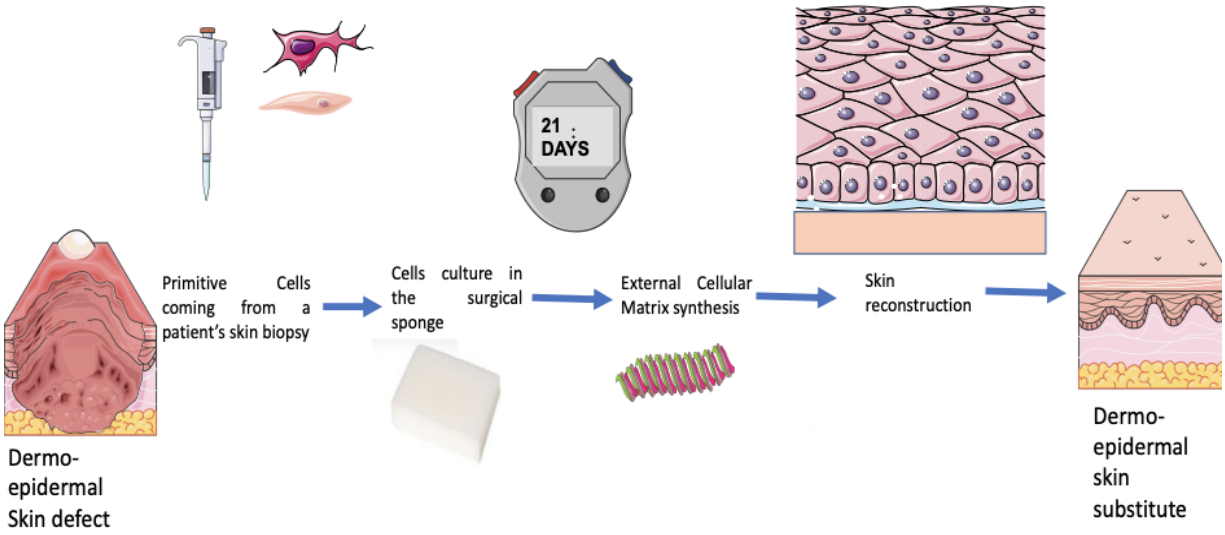


Figure 2. (*Left*) DESS macroscopic aspect (right) X20 magnification H&E histology examination showing an epidermis (above the black dotted line) and a dermis (below the black dotted line). The DESS preparation protocol is described in Chapter 17.

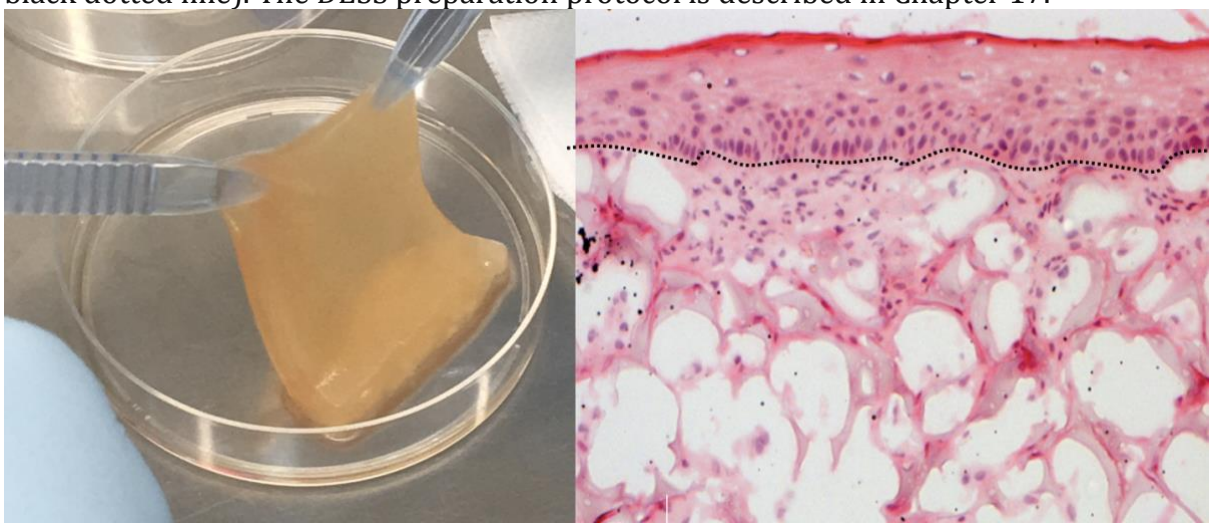
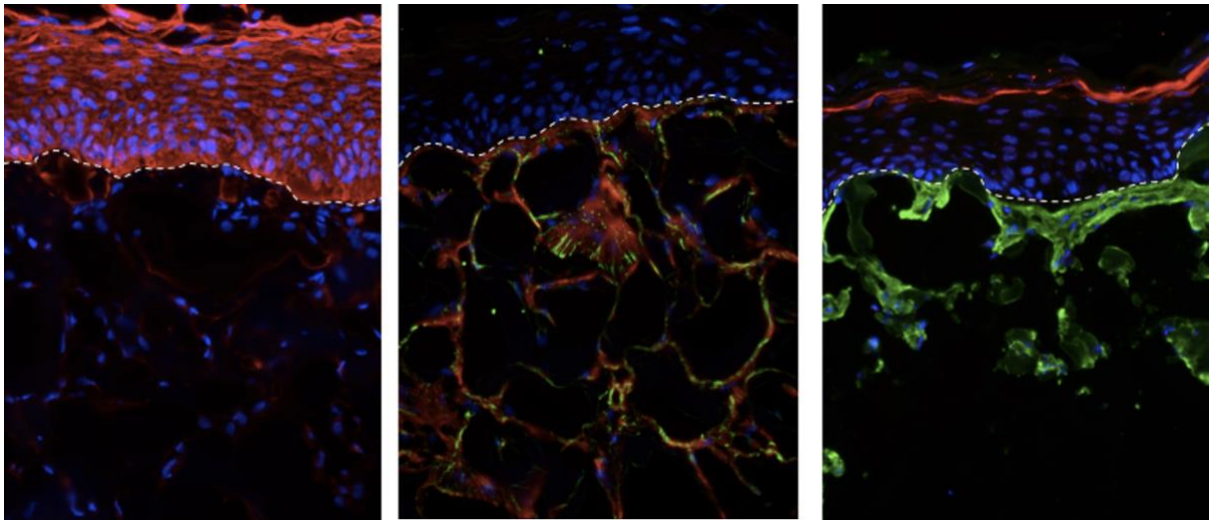


Figure 3. Immunohistochemistry (left) The cytokeratin 14 (red) represents the keratinocyte layer and is responsible of epidermis thickness. (middle) The fibrillin (red) and elastin (green) are part of the dermis component. ((right) The collagen 1 (green), predominant in the natural dermis is well represented in our DESS. The red line represents the filaggrin component.



Experimental design :

The "demande d'autorisation de projet utilisant des animaux à des fins scientifiques (APAFIS) » was approved in August 2020. After a quarantine period, the animals will be trained for two weeks to be handled by the laboratory personnel. They will be housed throughout their stay at the animal facility (Clinatec) in individual courtyards with perforated walls, allowing visual and olfactory contact between them. The surgery was performed (Operator: Alexandre Lellouch) under general anaesthesia under optimal health security conditions in the operating room, with strict asepsis and adequate analgesic coverage. All skin substitutes were prepared by Lara Jobeili. A Fentanyl patch was maintained for 3 days to avoid postoperative pain, then analgesics will be continued, if necessary, especially when performing weekly skin biopsies (buprenorphine). Oral antibiotic prophylaxis was administered daily, and the animals isolated to limit the risk of infection induced by their immunosuppression.

Statistical approach:

Thirty years of the team's experience shows that 5 animals are necessary to demonstrate a significant difference (non-parametric statistical tests, with or without paired series for comparisons between animals or scarring observed on the same pig). This number of 5 animals per experimental group allows the detection of an RH of 9.5 with 80% power and alpha = 0.05. However, we would like to adopt a 20% margin for potential complications and provide for a sixth pig in case the results observed on one of the five are uninterpretable and it is necessary to replace it (bad engraftment or rejection of a plugin for example). The maximum number of animals used would therefore be 6 pigs, but the experiment will be stopped before the use of the 6th minipig if the grafts are taken as expected.

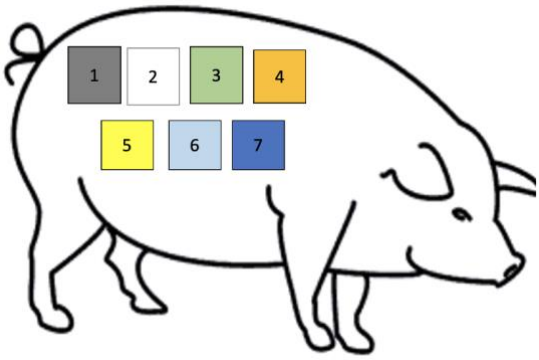
Human cells isolation for DESS preparation:

Discarded skin specimens from patients with abdominoplasty (tummy tuck) will be collected for cell culture. Skin specimens will be decontaminated by 0,4% betadine® over 15 mins followed by several washes in PBS (Gibco 14190144) and Penicilline/Streptomycine 10% (Life Technologies ref 1507-063) and a final wash in Penicilline/streptomycine 100%. Small pieces will be cut using scalpel and incubate during 3hrs at 37°C in a solution of sterile dispase II at 10mg/mL (Gibco 17105041). Dermis and epidermis parts are then separated using sterile forceps and washed with sterile PBS 1X. For fibroblasts isolation, dermis samples are incubated in a sterile solution of collagenase A at 0,5 mg/ml (Sigma 101035780001) under agitation during 4 to 6 hours at 37°C. The digest is finally filtered on a 70µm sieve and centrifuge during 5min at 2000rpm. The cell pellet is suspended; the cells are counted using trypan blue for cell viability evaluation. Fibroblasts are seeded at 8 000 cells /cm^2 in a DMEM medium containing 10% SVF and 1% P/S and medium is changed 3 times per week. For keratinocytes isolation, epidermis samples are incubated in a sterile solution of EDTA-Trypsine 0.25% during 15min at 37°C and suspended using a pipette. Cells are filtered on a 70µm sieve and centrifuged for 5 mins at 2000rpm. The cell pellet is suspended; the cells are stained with trypan blue for cell viability counting. Keratinocytes are seeded at 8 000 cells /cm^2 in KSFM medium (+BPE 25µg/mL + EGF 0,9ng/mL) and 1% P/S and medium is changed 3 times a week. Cells are cultivated at 37°C / 5%CO2 and amplified after detachment using EDTA-Trypsine 0.25% (Gibco 25300054) since 80% of confluency. For 3D reconstructed skin models, fibroblasts are cultivated and amplified in DMEM+10% SVF for one week. They are detached using EDTA-Trypsine and counted using trypan blue technique and the cell density must be 400 000cells/20µl. Then the gel mixture is prepared using 86% of NaCL/HEPES, 8% of fibrinogen, 1.4% of CaCl2, 1% of aprotinine and 1.6% of thrombin. The gel is poured into a transparent insert of culture Transwell (Ref3460 from Corning). After 1 hour, a medium composed of DMEM + 5% SVF is added. At the first medium changing 82.2µg/ml ascorbic acid (Sigma A8960) and 10ng/ml of EGF (R&D 263-EG-200) is added until the end of the immerged culture. After 12 days of dermal equivalent culture, pre amplified keratinocytes are seeded on the top of the dermal equivalent to 45 000 cells/dermis in 200µl of Green medium. After five additional days of culture, the skin samples are raised at the interface air/liquid. The medium used needs to allow maturation of the skin and is composed of 66% DMEM and 33% of HAM F12 containing 8mg/ml of BSA (Sigma A2153-50G), 0.4µg/ml of hydrocortisone (Sigma H0888) and 0.12UI/ml of Insuline (Sigma i6634). After a total of 30 days of culture, skin samples are detached from the insert and fixed for histology analysis.

Figure 4. Cells culture plan :

	For 1 pig (3cm X 3cm sample)	**Cells of interest**				Culture timing (pre op)
	CONDITIONS	**Fibroblastes**	**ASC**	**Keratinocytes**	**hdmecs**	
1	Positive control: split thickness skin graft (autograft)	//	//	//	//	//
2	Negative control: porous resorbable matrix (spongostan®)	//	//	//	//	48h hydrations
3	Negative control: area left by healing with secondary intention	//	//	//	//	//
4	Biological dressing with ASC (Adipose-derived Stem Cells)	9 M	//	//	//	15 days
5	Dermal equivalent with dermal fibroblasts	//	9 M	//	//	15 days
6	Dermo-epidermal equivalent (with fibroblasts and keratinocytes) (derme + épiderme)	9 M	//	9M	//	28 days
7	Vascularized dermo-epidermal equivalent (with fibroblasts, keratinocytes, and Human Dermal Microvascular Endothelial Cells).	9 M	//	9M	2 M	28 days
	TOTAL	27 M	9 M	18 M	2 M	

Figure 5. Each pig will present seven skin defects to limit the number of pigs used:



Controls:
1 Positive control: split thickness skin graft (autograft) seven
2 Negative control: porous resorbable matrix (spongostan®)
3 Negative control: area left by healing with secondary intention

Experimental grafts:
4 Biological dressing with ASC (Adipose-derived Stem Cells)
5 Dermal equivalent with dermal fibroblasts
6 Dermo-epidermal equivalent (with fibroblasts and keratinocytes)
7 Vascularized dermo-epidermal equivalent (with fibroblasts, keratinocytes, and Human Dermal Microvascular Endothelial Cells).

Skin Graft Harvest Procedure (control + allograft – bank skin)
The goal is to test a new dermo-epidermal skin substitute. In this protocol we used the same surgical techniques as performed in humans. For each minipig, the procedure will consist of several phases during the experiment (duration = one month). The Göttingen minipigs came from Ellegaard breeding which guarantees their IOPS health status and handling from birth. In addition, during the two weeks quarantine, the animals were accustomed to the presence of the experimenters and to vigilant manipulations in the laboratory to avoid any subsequent stress. Swine were anesthetized with I.M. 2 mg/kg telazol (tiletamine HCl and zolazepam HCl, Zoetis Inc., Kalamazoo, MI) and brought to the operating room for orotracheal intubation. Anesthesia was maintained using 2% isoflurane and oxygen. Skin surfaces were disinfected before surgery with chlorhexidine acetate (NolvasanR Surgical Scrub, Fort Dodge Animal Health, Fort Dodge, IA) and povidone-iodine, 10% (Betadine Solution, Purdue Products, L.P., Stamford, CT). The animals were then draped, leaving the right side of the dorsum exposed. Split-thickness skin grafts, measuring approximately 5 × 5 cm each, were harvested between the scapula and inferior margin of the lowermost rib from each animal using an air-driven Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ) with the depth set to 0.056 cm (0.022 inches). First, two strips of desepidermization (1 strip: 4cm (width) x 16 cm (length), the other strip: 4cm (width) x 12 cm (length)) will then be made on his back on both sides using the electric dermatome (depth of 0.2 mm). These lesions represent 112 cm^2 which are approximately 1% of the body surface area of the minipigs (total skin surface area is estimated at 1.11 m2 for a 40 kg minipigs, evaluated according to the Bollen formula BSA = 700xBW0.75 [with BSA: body surface area in cm^2, and BW: live weight in kg] adapted

Elaboration of a Good Manufacturing Product Skin Substitute from an Absorbable Gelatin Sponge: A Preclinical Study on Large Animal

to the Göttingen minipig of this size according to Ellegaard). Hemostasis of the resulting wound bed was achieved with gauze soaked in saline and epinephrine (1 ml, 1:1000).

Skin Graft Placement and Wound Dressings

All skin grafts were fenestrated using a 15 (size) blade to prevent the development of hematoma or seroma and sutured in place using 3/0 nylon. Overlying pressure dressings consisted of XEROFORM Petrolatum Gauze (Medtronic), Telfa™ nonadhesive dressing (Covidien, Minneapolis, MN), sterile dry gauze and maintained in place with Tegaderm™ (3M, St. Paul, MN). Recipients were then dressed with cotton jackets to reduce interference with the grafts. Transdermal fentanyl patches were applied for post-operative analgesia. Immunosuppression regimen was administered during this experiment.

Figure 6. Experimental timeline

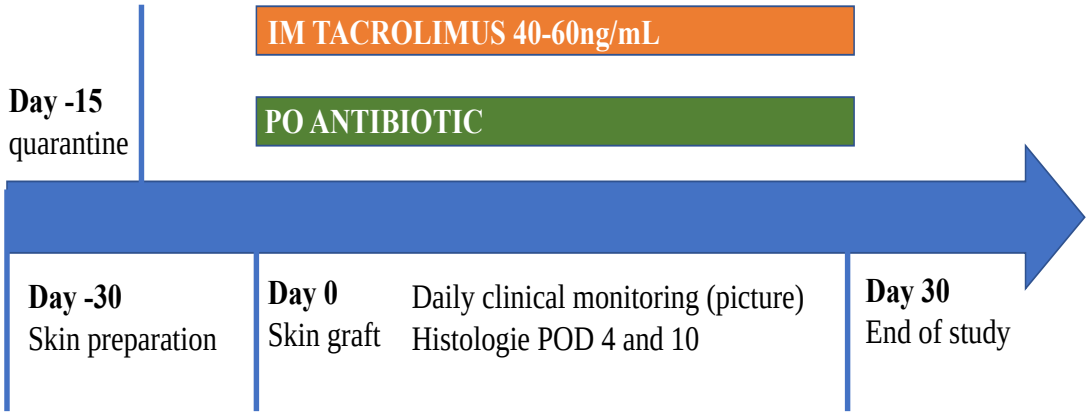


Figure 7. Surgical procedure: Split Thickness Skin Graft (numbers correspond to Figure 4 and 5)

From surgery, the animals will be placed on immunosuppressive regimen for all duration of the experiment. They will remain under antibiotics to avoid any infection during this period.

- **Antibiotic:** IM of cefazolin at 40 mg / kg at the time of surgery, and then PO Enrofloxacin at 2.5 mg / kg / day i.e. 1 ml / 10 kg of Baytril 2.5% for 30 days.

- Immunosuppression: treatment with high dose Tacrolimus FK506® (IM at 0.1 to 0.2 mg / kg), once a day. Tacrolimus level range expected: 40 to 60 ng/L (5 mg solution / mL, i.e. an injection volume of 0.8 to 1.6 mL for a 40 kg animal).

The animals will then be anaesthetised:

- Several times the first week for tacrolemia control on POD2, 4, +/- 6 depending on the level of tacrolemia (objective 40-60ng / ml), blood test on the dorsal face of the ear, if not possible on the lower or upper limbs.
- once a week on POD8, POD15, POD22 and POD 29 for clinical examination with quantification of healing (photo), blood test <4mL for CBC and biochemical assessment) and performing a punch biopsy (0.4cm in diameter).

After the last samples on D30, the animals were euthanized before they wake up.

Figure 8. Tacrolimus level for the 5 experimental animals

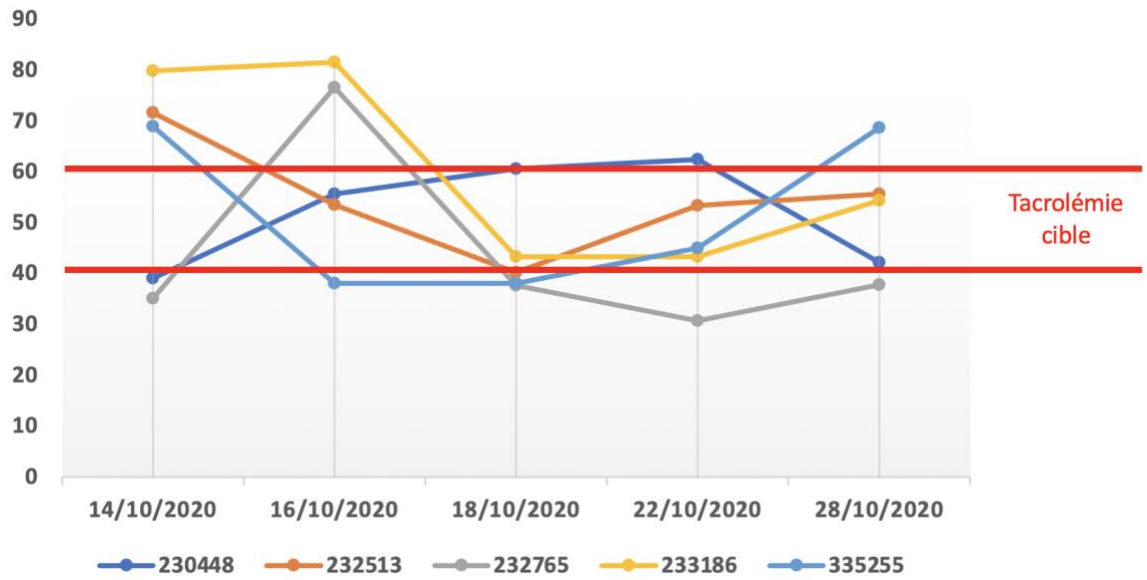
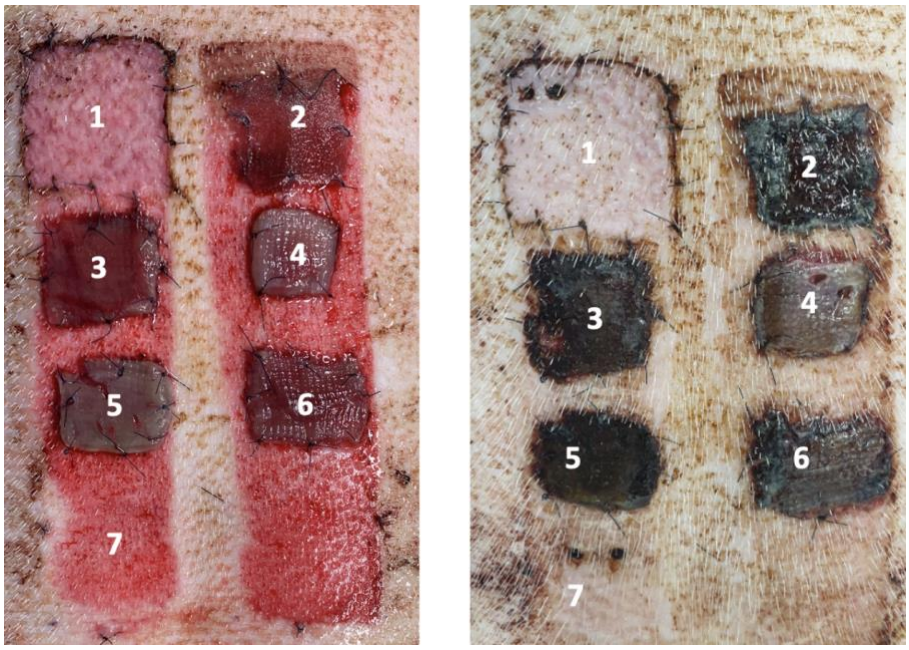
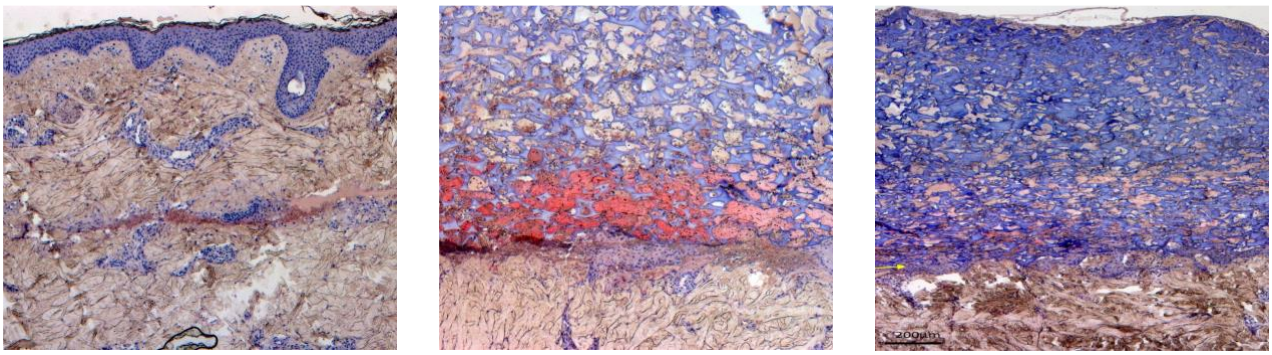


Figure 9. (Left) POD4 (right) POD10



The autologous skin (1) is viable at POD 10 demonstrating a good engraftment and a proper surgical technique. All the other condition tested (2,3,4,5, 6) failed due to a haemorrhagic infiltration.

Figure 10. Histology examination at POD 4
(left): condition 1 STSG is fully engrafted on POD4 testifying the good surgical technique.
(middle): condition 5: massive infiltration of red blood cells in the sponge preventing good engraftment. The sponge was also too porous.
(right): Condition 6: less haemorrhagic compared to condition 2 but the porosity did not allow good engraftment



CONCLUSION

This first experiment using our patented DESS on large animals was a failure for different reasons:

- The high porosity of the biomaterials renders the engraftment impossible due to massive haemorrhagic infiltration. For the next experiment we will change our protocol to produce an impermeable dermis layer (increasing the duration of external cellular matrix synthesis).
- The high thickness of our biomaterial does not allow an efficient angiogenesis formation. We decided to use the 0.2mm sponge (previously 1cm) for our next experiment.

LIST OF PUBLICATIONS AND PATENTS

PART A

Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL Jr. Mixed chimerism based regimens in VCA. Curr Transpl Rep 2016;3(4):390-4. DOI 10.1007/s40472-016-0124-7

Lellouch AG, Ng ZY, Rosales IA, Schol IM, Leonard DA, Gama AR, Colvin RB, Lantieri LA, Randolph MA, Cetrulo CL Application of the Delayed Tolerance Induction Protocol to Vascularized Composite Allografts in Non-Human Primates Plast Reconstr Surg. 2020 Apr;145(4):757e768e.doi:10.1097/PRS.0000000000006676.PMID:32221215DOI:10.1097/PRS.0000000000006676

Ng ZY, **Lellouch AG**, Rosales IA, Geoghegan L, Gama AR, Colvin RB, Lantieri LA, Randolph MA, Cetrulo CL Jr Graft Vasculopathy of Vascularized Composite Allografts In Humans: A Literature Review. Transpl Int. 2019 Mar 4. doi: 10.1111/tri.13421

Gama AR, Ng ZY, Shanmugarajah K, Mastroianni M, Randolph MA, **Lellouch AG**, Kohn J, Cetrulo CL Local Immunosuppression for Vascularized Composite Allografts: Application of Topical FK506-TyroSpheres in a Non-Human Primate Model Model J Burn Care Res. 2020 Apr 30;iraa062. doi: 10.1093/jbcr/iraa062. PMID: 32352521

Lellouch AG, Taveau CB, Andrews AR, Molde J, Ng ZY, Tratnig-Frankl P, Rosales IA, Goutard M, Lupon E. Lantieri L, Colvin RB, Randolph MA, Kohn J, Cetrulo CL, Local FK506 implants in non-human primates to prevent early acute rejection in vascularized composite allograft. Ann Transl Med 2021. doi: 10.21037/atm-21-313

Lellouch AG; Saviane G, Burlage L, Schol IM, Laurent LA, Randolph MA, Benichou G, Cetrulo CL, Curtis L. Tolerance Induction of Vascularized Composite Allografts Across a Class I Barrier in Swine Plastic and Reconstructive Surgery – Global Open: April 2019 - Volume 7 - Issue 4S - p 48–49doi: 10.1097/01.GOX.0000558342.57607.80

Ng ZY, **Lellouch, AG**, Defazio, MW, Powell H, Leonard, DA, Heroux, ZW, Kurtz, JM, Cetrulo CL Abstract: Analysis of Acute Skin Rejection in Non-Human Primate Models of Face and Hand Allotransplantation Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 53-54. Published online 2016 Sep 16. doi: 10.1097/01.GOX.0000502935.06726.2a

Ng ZY, **Lellouch AG**, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Abstract: Immunomodulation in Vascularized Composite Allostransplantation – Preliminary Results in a Non-human Primate Model with Tocilizimab. Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 54-55. Published online 2016 Sep 20. doi: 10.1097/01.GOX.0000502936.44843.57

Andrews AR, Tratnig-Frankl P, Taveau CB, Jonczyk MM, Lantieri L, Randolph MA, **Lellouch AG**, Benichou G, Cetrulo CL Tolerance Induction Protocol For VCA Across Full Mismatch Barriers In MHC Miniature Swine, Plast Reconstr Surg Glob Open. 2020 Apr; 8(4 Suppl): 33-33. Published online 2020 May 13. doi: 10.1097/01.GOX.0000667264.18161.d9 PMCID: PMC7224917

PART B

Lellouch AG, Lantieri L A Second chance at life Cambridge Quarterly of Healthcare Ethics (2019), Cambridge University Press 2019. doi:10.1017/S0963180119000380

Ng ZY, **Lellouch AG**, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL Jr. Vascularized composite allotransplantation - an emerging concept for burn reconstruction. J Burn Care Res. doi: 10.1097/BCR.0000000000000532. PMID: 28328655

Cetrulo CL Jr, Li K, Salinas H, Treiser M, Schol IM, McGovern F, Barrisford G, Feldman A, Grant M, Tanrikut C, Lee J, Ehrlichman R, Holzer PW, Choy G, Liu R, Rosales IA, Colvin RB, Ng ZY, **Lellouch AG**, Kurtz JM, Austen WG Jr, Winograd JM, Bojovic B, Eberlin KR, Ko DS. Penis transplantation: first U.S. experience. Annals of Surgery. 2016 PMID: 28509699

Ng ZY, Cetrulo CL, Lhuaire M, Lantieri L, **Lellouch AG** Comment" Full Facial Allotransplantation including the Temporo-Mandibular Joints: A Radiological and Anatomical cadaveric Study" Plast Reconstr Surg. 2020 Sep;146(3):635-636. doi: 10.1097/PRS.0000000000007128.PMID: 32842113

Lantieri L, Cholley B, Lemogne C, Guillemain R, Ortonne N, M Grimbert P, Thervet E, **Lellouch AG** First human facial retransplantation: 30-months follow-up Lancet. 2020 Nov 28;396(10264):1758-1765. doi: 10.1016/S0140-6736(20)32438-7. PMID: 33248497

Goutard M, **Lellouch AG**, Dussol B, Lantieri L Facial Trauma 10 years After a Face Transplantation Plast Reconstr Surg. Global Open Vol. 9, No. 5, May 2021 DOI: 10.1097/GOX.0000000000003575

Lupon E, Cetrulo CL, Goutard M, Taveau CB, Grolleau JL, Lantieri LA, **Lellouch AG** Comment on "First Russian Experience of Composite Facial Tissue Allotransplantation" Plastic and Reconstructive Surgery - Global Open: April 23, 2020 - Volume Latest Articles - Issue - doi: 10.1097/GOX.0000000000002772

Lupon E, Cetrulo CL, Lantieri L, **Lellouch AG,** "Comment on: Vascularized Composite Allotransplantation in a Post-COVID-19 Pandemic World." Plast Reconstr Surg. 2021 Jul 6. doi: 10.1097/PRS.0000000000008134. PMID: 34228010

Jonczyk M, Schol IM, Tratnig-Frankl P, **Lellouch AG**, Dicken S.C. Ko, Cetrulo CL 3281 Management of Acute Rejection in Penile Allotransplantation J Clin Transl Sci. 2019 Mar; 3(Suppl 1): 15–16. Published online 2019 Mar 27. doi: 10.1017/cts.2019.38

PART C

Taveau CB, **Lellouch AG**, Chin LY, Mamane O, Tratnig-Frankl P, Lantieri LA, Randolph MA, Uygun K, Cetrulo CL, Parekkadan B, "In Vivo Activity of Genetically-Modified Cells Pre-Seeded in Vascularized Composite Allografts" Transplant Proc. 2021 May 10:S0041-1345(21)00230-X. doi: 10.1016/j.transproceed.2021.02.028. PMID: 33985799

Goutard, M., Randolph, M.A., Taveau, C., Lupon, E., Lantieri, L., Uygun, K., Cetrulo Jr., C.L., **Lellouch, A.G**. Partial Heterotopic Hindlimb Transplantation Model in Rats. J. Vis. Exp. 2021, e62586, doi:10.3791/62586 (2021).

Burlage LC, **Lellouch AG**, Tessier SN, Pendexter CA, Randolph MA, Porte RJ, Lantieri LA, Cetrulo CL, Uygun K Optimization of Subnormothermic Machine Perfusion for Ex Situ Preservation of Vascularized Composite Allograts (in press Journal of Surgical Research)

Lellouch AG, Negin K, Ng ZY, Mert S, Geerts S, Uygun K, Cetrulo CL Abstract: Ex-vivo Subnormothermic Oxygenated Machine Perfusion of Swine Forelimbs Enables Prolonged Graft Preservation Prior to Transplantation Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 55-56. Published online 2016 Sep 16. doi: 10.1097/01.GOX.0000502937.52467.dc

Burlage LC, **Lellouch AG**, Saviane G, Randolph MA, Lantieri L, Porte RJ, Tessier SN, Uygun K, Cetrulo CL First Vascularized Composite Allotransplantations in Rats after 6 Hours of Ex Vivosubnormothermic Machine Perfusion Using an Hemoglobin Oxygen Carrier: A Proof of Concept StudyJournal of Burn Care & Research, Volume 40, Issue Supplement_1, April 2019, Pages S133–S134, https://doi.org/10.1093/jbcr/irz013.226

Brevet MGH/HARVARD: **Lellouch AG**, Cetrulo CL, Tessier S, Burlage L, De Vries R, Toner M, Uygun K Preservation of vascularized composite allografts: Strategy to homogenously load VCAs with CPAs. Application No. PCT/US2020/016840 International Publication number : WO 2020/163500 A1

PART D

Holzer PW, **Lellouch AG**, Moulton K, Zhu L, Ng ZY, Overschmidt B, Gama AR, Leto Barone A, Rosales I, Monroy R, Cetrulo CL, Jr. Clinical Impact of Cryopreservation on Split Thickness Skin Grafts in the Porcine Model *Journal of Burn Care & Research*, J Burn Care Res. 2020 Feb 19;41(2):306-316. doi: 10.1093/jbcr/irz145.

Lupon E, **Lellouch AG**, Acun A, Andrews AR, Oganesyan R, Goutard M, Taveau CB, Lantieri LA, Cetrulo CL, Uygun BE Engineering Vascularized Composite Allografts Using Natural Scaffolds: A Systematic Review. .Tissue Eng Part B Rev. 2021 Jul 9. doi: 10.1089/ten.TEB.2021.0102.PMID: 34238047

Oganesyan RV, **Lellouch AG**, Acun A, Lupon E, Corentin B. Taveau CB, Burlage LC, Lantieri LA, Randolph MA, Cetrulo CL, Uygun BE Establishment of an acellular nipple scaffold in a swine model for pre-clinical application (accepted with minor edits in PRS)

Duisit J, Maistriaux L, Bertheuil N, **Lellouch AG** Engineering vascularized composite tissues by perfusion Decellularization/Recellularization: Review Current Transplantation Reports, (2021), 1-13 10.1007/s40472-021-00317-2

Cetrulo CL, Wilson AS, Matar A, Torabi R, Pakyari MS, Elise Lupon E, Goutard M, **Lellouch AG**, "Two Decades of Experience in Clinical Reconstructive Transplantation - Lessons Learned" Taylor and Francis Special Edition: Reconstructive Transplantation and

Regenerative Medicine – The Emerging Interface. 2020" DOI: 10.1201/9780429260179-2

Brevet UGA/CEA : **Lellouch AG**, Jobeili L, Lantieri L, Rachidi W« Biomatériau comprenant une matrice poreuse résorbable et procédé de fabrication associé" Application No. PCT/EP2021/058151 International Publication number WO 2021/198177 A1

LIST OF FUNDING AND AWARDS

PART A

(***2016***) Medical Z: Zagame Award 10k€

(***2016***) ARS grant 20k€ and (2017) ARS grant 20k€

(***2016***) Best Abstract ASPS meeting (Awarded Outstanding Paper Presentation in Research & Technology Track)

(***2017***) W81XWH- 16-1-0702 (Cetrulo) 2017-2019 DoD/RTR Optimization of Delayed Tolerance Induction in Swine: A Clinically-Relevant Protocol for Immunosuppression-Free Vascularized Composite Allotransplantation 500k dollars

(***2017***) W81XWH-15-1-0281 (Cetrulo) 2017-2019 DoD/RTR Local Tacrolimus (FK506) Delivery for Prevention of Acute Rejection in the Non-Human Primate Delayed Mixed Chimerism Vascularized Composite Allograft Tolerance Induction Protocol 2.5 millions dollars

(***2017***) 85230-BOS-14 (Cetrulo) 2017-2019) Shriners Hospital for Children-Boston 1 million dollars Immunology of Hand and Face Transplantation for Burns

(***2017***) Nominee at Surgical Academy in Paris for the "Prix du forum de la recherche chirurgical 2016, dotation BROTHIER"

(***2017***) TTS-IVCA Travel Award (co-author) Toward Tolerance of VCA.
Development of clinical Mixed Chimerism-Based Protocols in swine

(***2018***) 85103-BOS-18 (Cetrulo) Shriners Hospital for Children-Boston 1 million dollars Role of the Thymus in Tolerance of Vascularized Composite Allotransplantation

(***2020***) TTS-IVCA Travel Award (co-author) Local FK506 Implant Technology in VCA – Successful Bridge to Delayed Mixed Chimerism

(***2021***) Promoted **Lecturer on Surgery at Harvard Medical School** (Faculty position)

PART B

(***2019***) Burroughs Wellcome Fund Trainee Travel Award (co-author) Management of Acute Rejection in Penile Allotransplantation

(***2021***) Harvard Medical School News Clips, December 2nd, 2020 for the First human facial retransplantation: 30-months follow-up Lancet. 2020 Nov 28;396(10264):1758-1765. doi: 10.1016/S0140-6736(20)32438-7. PMID: 33248497

PART C

(***2017***) W81XWH-17-1-0680 (Uygun) 2017-2019 DoD / RTR / 1 million dollars Development of a Supercooled Limb Preservation Protocol Role: Postdoctoral Research Fellow

(**2019**) RT180063 (Cetrulo/ Uygun) 2019-2022 Grant12736118 Investigator-Initiated Research Award - Multiple PI Option / 1.5 million Supercooled ex vivo Porcine VCA preservation to extend the timeline between procurement and transplantation and enable tolerance induction to eliminate immunotherapy needs and risks Role : Postdoc Research Fellow

(***2021***) NIH-NIBIB National Institute of Biomedical Imaging and Bioengineering (Parekkadan / Uygun/ Cetrulo) Grant238672 1.5 million ADVANCE Genetic-engineered control of the immunogeneic state of vascular composite allografts during preservation PROPOSAL NUMBER: 2020A009250 AWARD NUMBER: 1R01EB028782-01A1 Role: Co-investigator / Lecturer

(***2021***) Best Abstract Award " Smartphone-based DIY home microsurgical training with 3D printed microvascular clamps and konjac flour noodles" 15th Congress of the International Society for Experimental Microsurgery (ISEM)

PART D

(***2019***) International mobility grant (Bourse de "mobilité international") EDISCE / UGA

(***2020***) (Cetrulo/Uygun) 2020/2023
Shriners Hospital for Children-Boston 700k dollars Recellularization of vascularized engineered scaffolds for facial reconstruction

<u>Number of resident in plastic surgery supervised (in the setting of a Master of Science: 10)</u>

(***2018***) Scholarship Hospital of Paris (AP-HP) (Corentin Taveau)

(***2019***) Scholarship Hospital of Paris (AP-HP) (Elise Lupon and Marion Goutard)

(***2019***) Bourse de la vocation Fondation Marcel Bleustein-Blanchet (Elise Lupon)

(***2020***) Scholarship Hospital of Paris (AP-HP) (Golda Romano and Victor Pozzo (AIHP))

(***2020***) "Bourse des Gueules cassées" Grant 35k€ x 3 (Golda Romano, Pierre Tawa and Victor Pozzo (105k€).

(***2021***) ARTHUR SACHS SCHOLARSHIP (Claire Guinier) , "Bourse des Gueules cassées" Grant 35k€ x 2 (Eloi Clermont de Tonnerre et Yanis Berkane)

HARVARD MEDICAL SCHOOL

MARS Medical Area Reporting S

Job Summary

Alexandre Lellouch

Harvard ID

51171349

Active/Faculty

Prime Location

Massachusetts General Hospital

100170

Surgery-Massachusetts General

Job History

Job Select	HMS Indicator	Dept id	Department	Title	Start Date	End Date	Last Job Change
	P	100170	Surgery-Massachusetts General Hospital	Research Fellow in Surgery (EXT)	11/01/2015	06/30/2021	
		100170	Surgery-Massachusetts General Hospital	Lecturer on Surgery, Part-time	07/01/2021	06/30/2022	

1 - 2

release 1.0 Set Screen Reader Mode On

LIST OF COMMUNICATIONS

PART A

Ng ZY, **Lellouch AG**, Defazio MW, Powell H, Leonard DA, Heroux ZW, Kurtz JM, Cetrulo CL Jr. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation (Shortlisted for Joseph E. Murray, M.D. Award). 57th Annual meeting of the New England Society of Plastic and Reconstructive Surgeons, Bretton Woods, NH; 11 June 2016

Lellouch AG, Ng ZY, Rosales IA, Colvin RB, Gama A, Schol IM, Geoghegan L, Kurtz JM, Cetrulo CL Jr. Delayed tolerance induction protocol for vascularized composite allografts in non-human primates: the immunomodulatory effect of donor bone marrow transplantation does not prevent the development of chronic rejection in the absence of durable mixed chimerism. 62nd Annual meeting of the Plastic Surgery Research Council, Durham, NC; 5 May 2017

Lellouch AG, Ng ZY, Leonard DA, Powell HA, Defazio MW, Heroux ZH, Schol IM, Gama AR, Randolph MA, Kurtz JM, Sachs DH, Cetrulo CL Towards a preclinical large animal tolerance protocol for vascularized composite allotransplantation in swine, AFIRM meeting 2017

Molde J, Merolli A, Steele JA, **Lellouch AG**, Schol I, Cetrulo CL,Kohn J Preventing Transplant Rejection Through Localized Immunosuppression.. Rutgers Biomedical Engineering Department Annual Symposium. Piscataway, NJ. Dec. 13, 2018.

Ng ZY, **Lellouch AG**, Defazio MW, Heroux ZW, Schol I, Kurtz JM, Cetrulo CL, 2552: Toward tolerance of facial allotransplantation: Preliminary results in a non-human primate model with tocilizumab Vascularized Composite Allotransplantation, 3:1-2, 12-12, DOI:10.1080/23723505.2016.12329882552:

Ng ZY, Defazio MW, Powell H, Leonard DA, Heroux ZW, **Lellouch AG**, Schol I, Kurtz JM, Cetrulo, CL 2551: Acute skin rejection in non-human primate models of face and hand allotransplantation: Before and after tolerance induction Vascularized Composite Allotransplantation, 3:1-2, 10-10, DOI: 10.1080/23723505.2016.1232984

Lellouch AG, Schol IM, Gama AR, Ng ZY, Randolph MA, Kurtz JM, Cetrulo CL. Induction of Tolerance to Vascularized Composite Allografts across MHC Barriers in Swine Orthopaedic Research Society, ORS, 2018 Annual Meeting in New Orleans, Louisiana

Ng ZY, **Lellouch AG**, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Immunomodulation in vascularized composite allotransplantation – preliminary results in a non-human primate model with tocilizumab. American Society of Plastic Surgeons Annual meeting, Los Angeles, CA; 24 September 2016 (Awarded Outstanding Paper Presentation in Research & Technology Track)

Ng ZY, **Lellouch, AG**, Defazio, MW, Powell H, Leonard, DA, Heroux, ZW, Kurtz, JM, Cetrulo CL Abstract: Analysis of Acute Skin Rejection in Non-Human Primate Models of Face and

Hand Allotransplantation Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 53-54. Published online 2016 Sep 16. doi: 10.1097/01.GOX.0000502935.06726.2a

Lellouch AG, Taveau CB, Andrews AR, Molde J, Ng ZY, Tratnig-Frankl P, Randolph MA, Kohn J, Cetrulo CL Local Fk506 Implant Technology In Vca - Successful Bridge To Delayed Mixed Chimerism Protocol" , Plast Reconstr Surg Glob Open. 2020 Apr; 8(4 Suppl): 33-34. Published online 2020 May 13. doi: 10.1097/01.GOX.0000667268.21091.d8 PMCID: PMC7224883

Ng ZY, **Lellouch AG**, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Abstract: Immunomodulation in Vascularized Composite Allostransplantation – Preliminary Results in a Non-human Primate Model with Tocilizimab. Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 54-55. Published online 2016 Sep 20. doi: 10.1097/01.GOX.0000502936.44843.57

Lellouch AG, Ng ZY, Rosales IA, Colvin RB, Gama AR, Schol IM, Geoghegan L, Kurtz JM, Cetrulo CL Abstract 47: Delayed Tolerance Induction Protocol for Vascularized Composite Allografts in Non-Human Primates: The Immunomodulatory Effect of Donor Bone Marrow Transplantation Does Not Prevent the Development of Chronic Rejection in the Absence of Durable Mixed Chimerism Plast Reconstr Surg Glob Open. 2017 Apr; 5(4 Suppl): 36-37. Published online 2017 May 1. doi: 10.1097/01.GOX.0000516567.40845.df

Lellouch AG, Saviane G, Burlage LC, IMSchol, Lantieri LA, Randolph MA, Benichou G, Cetrulo CL Jr Long-term tolerance to VCA across a Class I Barrier in swine employing a clinically applicable protocol with CTLA4-Ig (Belatacept®) and Anti-Il6R (Tociluzimab®) American Society for Reconstructive Transplantation 6th Biennial Meeting, November 15-17, 2018 at the Drake Hotel in Chicago, Illinois

Molde J, Dube K, Iovine C, Merolli A, Ortiz O, Steele J, **Lellouch AG**, Ng ZY, Schol IM, Cetrulo CL Jr., Joachim Kohn. Degradable Devices for Localized Delivery of Immunosuppressants to Prevent Transplant Rejection. SFB Annual Meeting, 2018 April 11-14; Atlanta GA.

Lellouch AG; Saviane G, Burlage L, Schol IM, Laurent LA, Randolph MA, Benichou G, Cetrulo CL, Curtis L. Tolerance Induction of Vascularized Composite Allografts Across a Class I Barrier in Swine Plastic and Reconstructive Surgery – Global Open: April 2019 - Volume 7 - Issue 4S - p 48–49doi: 10.1097/01.GOX.0000558342.57607.80

Lellouch AG, Saviane G, Overschmidt B, Burlage LC, Randolph M, Benichou G, Cetrulo CL Induction of Tolerance to Vascularized Composite Allografts Across MHC Class I Barriers in Swine: A Clinically Relevant Protocol Using CTLA4-Ig and Anti-Il6R Military Health System Research Symposium - Army, 2018

Saviane G, **Lellouch AG**, Andrews A, Lantieri L, Randolph M, Benichou G, Cetrulo CL Expansion of Regulatory T and B Cells In Swine Rendered Tolerant of Allogeneic Vascularized Composite Allografts Via Mixed Chimerism Am J Transplant. 2019 at the 2019 American Transplant Congress (ATC) at the John B. Hynes Convention Center, Boston, MA

Molde J, Merolli A, Steele JAM ,Lima MRN, **Lellouch AG**, Pratts SG, Cetrulo CL, Kohn J. Localized tacrolimus delivery for transplant rejection prevention via tyrosphere-loaded rapidly soluble films Transactions of the Annual Meeting of the Society for Biomaterials and the Annual International Biomaterials SymposiumVolume 40, 2019, Page 51042nd Society for Biomaterials Annual Meeting and Exposition 2019: The Pinnacle of Biomaterials Innovation and Excellence; Seattle; United States; 3 April 2019 through 6 April 2019; Code 147567

PART B

Lellouch AG, Ng ZY, Drijkoningen T, Chang IA, Sachs DH, Cetrulo CL Jr. Vascularized composite allotransplantation – an emerging concept for burn reconstruction. 49th Annual meeting of the American Burn Association, Boston, MA; 23 March 2017
Lellouch AG, Taveau CB, Cetrulo CL Lantieri L, Management Of Ischemia Duration In The First FVCA Re-transplantation: The Use Of A Novel Marine Oxygen Carrier M101, 65thAnnual Meeting of the Research Council, May 28-31, 2020 at the Omni King Edward Hotel in Toronto, Canada.

Lhuaire M, **Lellouch AG** , Athlani L, Taveau CB , Ilyes Khalifa I , Hivelin M, Lantieri L Bases anatomiques de l'allotransplantation d'avant-bras : nécessité d'un retour aux fondamentaux, dissections cadavériques et tableau noir 102e Congrès de l'Association des Morphologistes 2020

Cetrulo CL Jr, Li K, Salinas H, Treiser M, Schol IM, McGovern F, Barrisford G, Feldman A, Grant M, Tanrikut C, Lee J, Ehrlichman R, Holzer PW, Choy G, Liu R, Rosales IA, Colvin RB, Ng ZY, **Lellouch AG**, Kurtz JM, Austen WG Jr, Winograd JM, Bojovic B, Eberlin KR, Ko DS. Penis transplantation: first U.S. experience. AAPS 2017

PART C

Lellouch AG, Karimian N, Ng ZY, Mert S, Geerts S, Uygun K, Cetrulo CL Jr. Ex-vivo subnormothermic oxygenated machine perfusion of swine forelimbs enables prolonged graft preservation prior to transplantation. American Society of Plastic Surgeons Annual meeting, Los Angeles, CA; 24 September 2016

Lellouch AG, Negin K, Ng ZY, Mert S, Geerts S, Uygun K, Cetrulo CL Abstract: Ex-vivo Subnormothermic Oxygenated Machine Perfusion of Swine Forelimbs Enables Prolonged Graft Preservation Prior to Transplantation Plast Reconstr Surg Glob Open. 2016 Sep; 4(9 Suppl): 55-56. Published online 2016 Sep 16. doi: 10.1097/01.GOX.0000502937.52467.dc

Lellouch AG, Tessier SN, Cronin SE, Schol IM, Pendexter CA,Randolph MA, Lantieri LA, Uygun K, Cetrulo CL, Optimization of ex-vivo subnormothermic oxygenated machine perfusion in vascularized composite allograft on rat to prolong preservation duration Journal of burn care & research: official publication of the American Burn Association Apr 2018 Chicago

Burlage LC, **Lellouch AG**, Saviane G, Randolph MA, Lantieri L, Porte RJ, Tessier SN, Uygun K, Cetrulo CL First Vascularized Composite Allotransplantations in Rats after 6 Hours of

Ex Vivosubnormothermic Machine Perfusion Using an Hemoglobin Oxygen Carrier: A Proof of Concept StudyJournal of Burn Care & Research, Volume 40, Issue Supplement_1, April 2019, Pages S133–S134, https://doi.org/10.1093/jbcr/irz013.226

Burlage LC, **Lellouch AG**, Pendexter CA, Mamane ON, Lantieri LA, Randolph MA, Robert J. Porte RJ, Tessier SN, Cetrulo CL, Uygun K "First Successful 24 Hours Preservation in Vascularized Composite Allograft Using a Subzero Non-Freezing Protocol " podium presentation at the American Society for Reconstructive Microsurgery Annual Meeting, February 1 - 5, 2019 at the JW Marriott Desert Springs in Palm Desert, California.

Burlage LC, **Lellouch AG**, Saviane G, Randolph MA, Lantieri LA, Porte RJ, Tessier SN, Uygun K, Cetrulo CL First Vascularized Composite Allotransplantations in Rats after 6 Hours of Ex Vivosubnormothermic Machine Perfusion Using an Hemoglobin Oxygen Carrier: A Proof of Concept Study *Journal of Burn Care & Research*, Volume 40, Issue Supplement_1, April 2019, Pages S133–S134, https://doi.org/10.1093/jbcr/irz013.226

Goutard M, Dr Vries, **Lellouch AG**, E. Lupon, Pendexter C., Tessier SN, Randolph MA, Lantieri L, Cetrulo CL, Uygun K Recovery after extended static cold storage preservation using subnormothermic machine perfusion in VCA ATC 2021 Virtual connect June 5-9, 2021

Goutard M, **Lellouch AG**, Lupon E, Pendexter C, Randolph MA, Lantieri L, Cetrulo CL, Uygun K, Effect Of Static Cold Storage On Vascularized Composite Allotransplantation On Rodent PSRC 66th Annual meeting May 20-23, 2021 Hilon Cleveland Downtown Cleveland, OH

Ng ZY, Honeyman CS, **Lellouch AG**, Ankur P, Papavassiliou T Smartphone-based DIY home microsurgical training with 3D printed microvascular clamps and konjac flour noodles" 15th Congress of the International Society for Experimental Microsurgery (ISEM) (Best Abstract Award)

Burlage L, **Lellouch AG**, Tessier S, Pendexter C, Cronin S., Schol M, Randolph M, Porte R, Lantieri L, Cetrulo C, Uygun K Ex-Vivo Subnormothermic Oxygenated Machine Perfusion of Rodent Hindlimb: Feasibility Study to Elongate Preservation Time of Vascularized Composite Allograft Am J Transplant. 2017;17 (suppl 3).

Burlage LC, **Lellouch AG**, Saviane G, Randolph MA, Lantieri L, Porte RJ, Tessier SN, PhD, K. Uygun K, Cetrulo CL 312 First Vascularized Composite Allotransplantations in Rats after 6 Hours of Ex Vivosubnormothermic Machine Perfusion Using an Hemoglobin Oxygen Carrier: A Proof of Concept Study *Journal of Burn Care & Research*, Volume 40, Issue Supplement_1, April 2019, Pages S133–S134, https://doi.org/10.1093/jbcr/irz013.226

Goutard M, tawa P, Andrews AR, De Vries RJ, **Lellouch AG**, Romano G, Pozzo V, Pendexter C,… Randolph MA, Cetrulo CL, Uygun K"A Simplified Perfusion Protocol for 24-Hr Vca Ex Vivo Preservation in a Swine Limb Transplantation Model", has been accepted for podium presentation at the American Society for Reconstructive Microsurgery Annual Meeting, January 14-18, 2022 at the Omni La Costa Resort in Carlsbad, California.

Taveau CB, **Lellouch AG**, Chin LY, Mamane O, Tratnig-Frankl P, Andrews AR,. Lantieri LA, Randolph MA, Uygun K, Parekkadan B, Cetrulo CL Biosensor Cells Use In A Heterotopic Hind Limb Transplantation In Rat: Proof-of-concept Study 65thAnnual Meeting of the Research Council, May 28-31, 2020 at the Omni King Edward Hotel in Toronto, Canada.

PART D

Holzer P, **Lellouch AG**, Leto Barone A, Overschmidt B, Gama AR, Mackenzie T, Zhu L, Monroy R, Cetrulo CL Clinical Impact of cryopreservation on split thickness skin grafts in the porcine model American Burn Association 2019, Las Vegas *Journal of Burn Care & Research*, Volume 40, Issue Supplement_1, 9 March 2019, Pages S238–S239, https://doi.org/10.1093/jbcr/irz013.414

Lellouch AG, Guyette JP, Bernhard J, Cetrulo CL, ; Goverman J, Creation of a bioengineered, porcine, full-thickness skin graft with a perfusable vascular pedicle Poster, The Future of Regenerative Medicine. May 2017, Teaneck, NJ.

Lupon E, Acun A, Taveau C, **Lellouch AG**, Goutard M, Oganesyan R, Lantieri L, Grolleau JL, Uygun B, Cetrulo CL Decellularization protocol for porcine Fasciocutaneaousflap: an update. The Virginia Society of Plastic Surgeron « VASPS » (virtual). Oral presentation, the 24th October 2020

Lupon E, Acun A, Goutard M, **Lellouch AG**, Oganesyan R, Andrews AR, Lantieri L, Randolph MA, Cetrulo CL., Uygun B QS9: Decellularization Of Vascularized Composite Allografts In The Rat Plast Reconstr Surg Glob Open. 2021 Jul; 9(7 Suppl): 29-30. Published online 2021 Jul 26. doi: 10.1097/01.GOX.0000770084.89448.e2 PMCID: PMC8312819

Pozzo V, Acun A, Lupon E, Oganesyan R, Romano G, Goutard M, Andrews AR, Tawa P, Randolph MA, **Lellouch AG**, Cetrulo CL, Basak E. Uygun BE Reendothelialization of decellularized swine fasciocutaneous flap: a proof-of-concept study IXA-CTRMS 2021 Virtual Congress, September 23-25, 2021

LIST OF CONTRIBUTING AUTHORS

Walid Rachidi
SyMMES/CIBEST, Université Grenoble Alpes
UMR 5819 UGA-CNRS-CEA,
Grenoble, France.
BIG-BGE, Université Grenoble Alpes, CEA, Inserm, U1038.

Laurent A. Lantieri, MD, PhD, Professor of Plastic Surgery
Department of Plastic and Reconstructive Surgery
European George Pompidou Hospital
Paris, France

Curtis L. Cetrulo, Jr., MD, FACS, FAAP
Department of Plastic and Reconstructive Surgery
Center for Transplantation Sciences
Vascularized Composite Tissue Allotransplantation Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Korkut Uygun, PhD
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Basak Uygun, PhD
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Mark A. Randolph
Center for Transplantation Sciences
Vascularized Composite Tissue Allotransplantation Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America
Marion Goutard, MD
Department of Plastic and Reconstructive Surgery
European George Pompidou Hospital
Paris, France

Alec R Andrews, BS
Department of Plastic and Reconstructive Surgery

Center for Transplantation Sciences
Vascularized Composite Tissue Allotransplantation Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Elise Lupon, MD
Department of Plastic and Reconstructive Surgery
European George Pompidou Hospital
Paris, France

Corentin Taveau, MD
Department of Plastic and Reconstructive Surgery
European George Pompidou Hospital
Paris, France

Ruben V Oganesyan, MD
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Lara Jobeili, PhD
SyMMES/CIBEST, Université Grenoble Alpes
UMR 5819 UGA-CNRS-CEA,
Grenoble, France.
BIG-BGE, Université Grenoble Alpes, CEA, Inserm, U1038.

Fatima Naji
SyMMES/CIBEST, Université Grenoble Alpes
UMR 5819 UGA-CNRS-CEA,
Grenoble, France.
BIG-BGE, Université Grenoble Alpes, CEA, Inserm, U1038.

Zhi Yang Ng, MD
Mountbatten Department of Plastic Surgery
Queen Alexandra Hospital, Portsmouth, England
United Kingdom

Gaelle G.A. Saviane
Center for Transplantation Sciences
Vascularized Composite Tissue Allotransplantation Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Joachim Kohn, PhD
Department of Chemistry and Chemical Biology
Rutgers University, 123 Bevier Road, Piscataway

NJ, 08854, USA.
Shannon N. Tessier, PhD
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Olivia Mamane
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Robert J. Porte, MD, PhD, Professor of Surgery
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

ACKNOWLEDGEMENTS

Elodie, we got married 8 years ago, time flies! I could write a book to describe how great you are. I am so blessed to be with such an extraordinary wife. Thanks to you, I wake up every day at 4:40AM knowing it will be a good day because you are close to me. You still impress me how you handle so many things and especially keeping a great mood ☺. We are a strong team, and we face all the challenges together. Thank you for the hours spent in formatting this thesis! You know you can count on me anytime. Love you honey.

Kids Liam, Mila, Jonas and Naomi you guys are the meaning of my life. I am so grateful to have such amazing kids. Mom and I will do our best to raise you in the right direction and give you all the tools to lead a happy and peaceful life. Love you kids

My parents and my sister Elsa, thank you for believing in me and supporting me. I am so lucky to have you. We share so many great memories and looking forward to many great more together. Love you dearly. A tender thought to my grandfather **Jules Cohen** and my grandmother **Lucette Cohen** who supported and encouraged me. You will stay in my heart for life.

Michel and Eva, David and Hanna, you gave me my greatest gift – Elodie. You welcomed me as a son and brother into your family right away. Thank you for your endless support and enthusiasm through this adventure. So blessed to be part of your family. Love.

Pr. Walid Rachidi, I was introduced to you by Pr. Paul-Henri Romeo during a zoom call at CEA. I immediately felt your expertise in tissue engineering and your great spirit. I was so honoured to become your PhD student! Your vision of research and industry push me to think differently for the development of new technologies and how to go from bench to bedside. This work started with you is just the beginning of a long way. I am confident that we have great things to accomplish in a near future!

Pr. Laurent A. Lantieri, where should I start? I met you when I was a 4th year med student (clerkship). I observed a full-face transplant, and I was so amazed! I saw surgery through a whole new light. It truly inspired me to become a plastic surgeon. Years after years I focused my energy and motivation to learn the "Vascularized Composite Allotransplantation" (VCA) world. The more I learned, the more I understood where I can be useful in this area. You are a pioneer of VCA, and to say I learned a lot from you, is an understatement. You gave me the opportunity to participate in the first face re-transplantation, recently published in the Lancet journal. I saw the challenges of VCA through the patient follow-up. This work showed me why we need research to keep going with this amazing field. Thanks to you I spent 2 years at Massachusetts General Hospital (MGH / Harvard) and 4 others between Paris and Boston. Thanks to this unique pathway, I was able to keep both my research and clinical activities. Thank you again for your strong support. You can count on me, and on your staff to continue making progress on this field and help patient with complex defect untreatable with autologous surgery.

Dr. Curtis L. Cetrulo, my American mentor! I remember the first interview I got with you! I was supposed to spend one year in your lab and look at us 6 years later! I really enjoy doing research with you. Your kindness and guidance help me with daily

challenges. You are a source of inspiration for multitasking! As you said, "be well organized". Thank you for promoting me in the lab and let me be the link between Paris and Boston. The dynamic is now well established between our 2 prestigious institutions. I love my life between France and Boston, I will do my best to maintain this lifestyle as long as possible because we have so many things to get done! This is my American dream ☺

Dr. David Sachs you are a hero! You have an exceptional career, family, … You speak 5 languages. I remember during the morning rounds when you discussed with all the international fellows in French, German, Spanish, such a wonderful souvenir! You still help extract the best of me. You are a true inspiration and role model…

Dr. Korkut Uygun It's been already 5 years since our first perfusion! We applied almost every year for VCA perfusion grant, and we got 2 from the Department of Defense of the United States! I am so glad to be working with you. Your humility and your vision to move forward is key for the success of our projects. Thank you for making life easy for our French fellows, they all are so grateful. I hope to continue working together as long as possible.

Dr. Basak Uygun! Almost 3 years since our first meeting to start the decellularization project! Every single month I stopped by your office to apply for grants, and in January 2020, we made it ☺. You never give up with our group despite the unsuccessful grants. Thank for believing in us and I am so happy we managed to sort out few papers together. I really believe that this technology is crucial to understand the mechanism of tissues recellularization. We need to be the first team to transplant a recellularized face !

Pr. Mark A. Randolph You are the guru of the lab ! Always there to help and to guide. If this project between France and the US work, it is thanks to you and always making sure everything is moving in the right direction. I pray every day to keep you as long as possible in the laboratory. Seeing your long career, you are a true model of righteousness. Hope to follow in your footsteps. Thank you for your strong support Pr Randolph !

Dr. Gilles Benichou Thank you so much for helping me with the fundamental side of my work. You have the hard skills in immunology that I would never have. I take advantage of every meeting to improve my knowledge in immunology. J'espère que l'on pourra collaborer le plus longtemps possible !

Alec R Andrews Everyone likes you, and you know why? Because you are the nicest person in this planet. You deal with so many problems every day…not many could handle it like you! You are strong, and always here to help you colleagues. Be sure, I will never let you down buddy !

Pr. Joachim Kohn It was a great pleasure working with you through the RTR 2 project. I liked all the phone calls with Curt, with your crystal-clear explanations. You are looking for the most accurate answer everytime. I hope you will enjoy your retirement (but I don't believe someone like you can be retired…).

Dr. Marion Goutard you are so amazing! You are very talented, and I am very proud of what you have done in the USA. You have a great potential and hope you will keep this flame active as long as possible! Now I count on you in France ☺.

Dr. Elise Lupon you never give up and you don't take no for an answer. Keep up the hardwork, set up your life anywhere in the globe. I am sure if you continue, you will be unstoppable! Run Forest Run !

Dr. Corentin Taveau you are so funny my friend. I really enjoyed my time in research and clinics with you. But most importantly you are very skilled, and I can count on you in tough situation. Stay tuned !

Dr. Ruben V Oganesyan It was so nice working with you. You are so disciplined and learn very fast in the lab. If you don't know something, you will work as hell to solve the problem. You are working hard to find a spot in the USA, but you need to realize, USA is so lucky to have someone like you!

Dr. Zhi Yang Ng Thank you for helping me since day 1 in research! You are as crazy as me. I know I can reach out to you anytime for anything. We share so many things together in research, clinics, … I am so happy you matched at the prestigious Oxford University. They need people like you to get things done. We stay in touch!

Dr. Lara Jobeili Thank you Lara for helping me remotely! You were my hands in Grenoble ☺. Your expertise in skin engineering is a gift and I am so blessed that our way crossed. Hope to see you soon

Dr. Naji Fatima Thank you for your help pursuing the skin project and I hope to keep going our collaboration !

Gaelle G.A. Saviane You are the most organized technician I have ever met. Thanks to you we learned a lot from our tolerant pigs. I wish you the best in France.

Dr. Shannon N. Tessier Thank you for the help and guidance for our French fellows. Your knowledge in perfusion system is indescribable!

Beth Avraham community in Boston: Would like to thank all of you for your kindness and your hospitality! You are part of my family now! The Danan, Kamoun, Mamane, Sellam, Simnegar, Taieb family…

Young Israel of Harrison: you accepted me from day 1! You are a strong and smart community and a great source of inspiration! Love you guys

Would like to thank the board of examiners for analyzing this work: **Pr Vayssade Muriel, Pr Lamartine Jerome, Pr Fortunel Nicolas, Giot Jean-Philippe**