LOCAL IMMUNOSUPPRESSION FOR LONG-TERM MAINTENANCE OF VASCULARIZED COMPOSITE ALLOTRANSPLANTATION

The Evaluation of a Drug Delivery System in a Clinically Relevant Large Animal Model

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1 ABSTRACT

Background: Vascularized composite allotransplantation (VCA), like hand or face transplantations, enable the replacement and reconstruction of different traumatic tissue loss. However, for VCA as a non-life-saving procedure, immunosuppressive therapy is inevitable and entails some side effects that prevent its widespread clinical use. Therefore, logistic and matching problems, as well as the optimization of immunosuppression protocols must be considered as a goal to overcome this hurdle. An innovative strategy based on the anatomy and function should be the solution. Through a specific drug delivery system (DDS) a local and site-specific administration of an immunosuppressive drug is to be made possible. The efficiency and mechanism of DDS will now be tested and investigated in the context of rejection reactions in a major large animal model for VCA.

Methods: In pigs with at least one SLA mismatch, an osteomyocutaneous flap allograft will be performed to assess the hypothesis in a relevant VCA model. The pigs are randomly sorted into one of the following groups: I. Control group without treatment, II. Systemic treatment with Tacrolimus and III. Triglycerol-Monostearate with Tacrolimus (TGMS-TAC). The animals are regularly observed for a fixed period of 90 days and blood and skin samples are collected for a complete analysis. Furthermore, peripheral blood mononuclear cells (PBMC) are analyzed with FACS for the comparison of the immune response. If rejection (grade III-IV) is detected in the pigs, they must be euthanized before the end point.

Results: Group I. pigs were rejected as expected between post-operative day (POD) 7 and 9. The median survival time (MST) in the group II. was 37 days, more than a week below group III., which reached 46 days. Blood and skin concentrations of Tacrolimus reach maximum values of 55 ng/ml respectively 80 ng/g at POD7 with a significant decrease thereafter, which is related to the TGMS-TAC released dose of the drug. The Tacrolimus skin level in the graft and contralateral side show also peak values at POD7. PBMC dynamics and values of the individual groups show an insignificant advantage for group III.

Conclusion: Injection of the enzyme-dependent TGMS-TAC hydrogel causes graft survival compared to the control group. In the long term, the difference to Group II is still insufficiently significant, which is why some forms of improvement and adjustment are still necessary.

2 ACKNOWLEDGMENTS

I wish to express my sincere thanks to Prof. Dr. Robert Rieben from the Department of Biomedical Research at the University of Bern for giving me the opportunity to do my bachelor internship and thesis with his group in his laboratory. His commitment and helpfulness are greatly appreciated. The enthusiasm for science is clearly visible in him, as well as in everyone in the group. I am also grateful to Isabel Arenas, who always had an open ear for my questions. I am indebted to her for sharing her expertise and the valuable guidance extended to me. She introduced me excellently to her project, was always patient with me and inspired me a lot. Lastly, I express my sense of gratitude to all lab members who supported me through this venture.

3 VASCULARIZED COMPOSITE ALLOTRANSPLANTATION

Vascularized composite allotransplantation (VCA) is an increasingly performed reconstructive medical procedure to restore appearance, anatomy and function in patients who suffer major tissue loss and where there is no possibility for conventional reconstruction with autologous tissues and prostheses.[1–3] However, since VCA consists of complex tissue types such as skin, muscle, bone, nerves and blood vessels, there will be immunological rejections after transplantation. Following the discovery of cyclosporine and more generally of highly effective immunosuppressive drugs, both solid organ transplantation (SOT) and VCA have developed rapidly.[4] VCA is of course also very important for the aesthetic aspect of the patients and the related psychological and social implications of such injuries with tissue loss. Especially for people who are strongly bound to physical activities, VCA can function as a suitable solution for returning to normal everyday life.[5] Given the very encouraging immunological and functional results observed after upper extremity and facial transplantation over the last two decades, new potential for VCA, such as urogenital including penile and uterine transplantation and abdominal walls, has emerged.[6,7] As VCA has developed more and in recent years, a standardization and regulation of the procedure has become inevitable. An agreement was reached which states that the newly adapted definitions, such as to be vascularized and requires blood flow by surgical connection of blood vessels to function after transplantation, containing multiple tissue types, minimally manipulated and some more are under directive 2010/53/EU of the European Parliament and of the Council on quality and safety standards of human organs intended for transplantation.[8]

VCA allografts have the important advantage of direct monitoring of the graft, which may explain the high diagnosis rates of acute rejection. However, the field is at a disadvantage in that there are no assays for transplant rejection as are used for surveillance for SOT's. The principal difference between VCA and SOT is that for successful outcomes a vigorous physical therapy is required and that in most cases these procedures are not lifesaving. Currently, over 130 hand and face transplants have been performed worldwide and most patients enjoy good function of the transplants and are able to use their hands in everyday life.[9]

In earlier VCA transplantations, little attention was paid to matching for human leukocyte antigen (HLA) in order to find suitable donors. This was based on the urgency

of the process and the small number of donors with the appropriate characteristics, such as suitable skin color, gender or age. For example, they make sure that the age difference between donor and recipient is not more than ten years. The experience with SOT, considering that donor allocation is organized nationally and internationally, makes HLA matching indispensable when it comes to VCA. For example, there is a definite risk if a donor has an HLA allele, resulting in an unacceptable level of donor-specific antibodies in the VCA recipient and endangering long-term graft survival.[5,10] The latest review by Bonastre *et al.* identified a link between the number of acute rejection episodes and the number of HLA mismatches by recording 68 rejection episodes in 28 recipients of hand transplants from 1999 to 2011.[10] The potential impact of tissue antigen expression levels on transplant outcome has been described by understanding the circulation of HLA-specific antibodies and the ability to measure the cellular expression of HLA antigens, and is proving to be very important in assessing the risk of transplantation.[11] This results in several aspects that must be taken into account when allocating VCA. In transplantation, specifically also in VCA, time is the most valuable resource and the tolerable ischemia time and ischemia reperfusion injuries must take into account.[5] Today's standards do not allow to preserve tissue for more than a few hours and therefore represent a technological bottleneck for the widespread application of VCA. New methods using machine perfusion or cryobiology are well underway and may give us the opportunity to overcome these hurdles and further increase the clinical potential of VCA.[12] Nevertheless, the cold ischemia is harmful to the organs, because over time the amount of oxygen is no longer sufficient and ischemic cell injuries are triggered by ATP depletion, impairment of mitochondrial respiratory function and acidosis by glycolysis.[13] In addition, cell injury and harmful signaling pathways can occur even after blood flow has been restored, which can also result in ishemia-reperfusion injury (IRI).[14]

In any case, improvements and refinements in preservative methods are required in order to make the most valuable resource in VCA transplantation, namely time and the resulting opportunities for general improvement in treatment, even more available.[15] There are some aspects which have to be considered for VCA allocation and therefore some criteria have been established:

- Donor selection is based on skin color, gender, age, ABO group and HLA matching.
- VCAs should only be undertaken among ABO-identical or ABO-compatible donors and recipients.
- The national priority is set for hypersensitised and sensitised recipients, urgent cases and combined face and arm transplants.
- If there is no national priority, the allocation within a region/province has priority. In the case of local allocation, the transplant team is free to choose the most appropriate recipient.
- For national allocations, priorities are defined as (1) age difference of less than 10 years between donor and recipient, and (2) time on the waiting list.[5]

The fact that VCA is not used worldwide is mainly due to two defined reasons, which are logistical and matching problems on the one hand and the optimization and minimization of immunosuppression protocols/therapies on the other hand. Regarding immunosuppression, an advanced understanding of alloimmunity associated with SOT has paved the way for VCA.[16,17] But science in the field of VCA is on the verge of overcoming these hurdles. Apart from the problems that still remain to be solved, VCA is applicable in itself and success is determined by the control of the alloimmune response but also by the speed and quality of nerve regeneration. After transplantation, the recipient's peripheral nerve endings must recover into the graft to innervate the transplanted tissue such as muscle and skin. Only through this process the recipient can maintain the motor control and sensory abilities of the graft. Nerve regeneration will continue to have a major impact on the widespread application of VCA and again, strategies have to be optimized to understand the pathophysiological mechanisms, including stem cell based therapies to overcome this hurdle.[12] As mentioned above, the logistical problems are dealt with by the new preservation methods. The awareness of HLA has now received much more focus on solid organ transplantation but also on VCA. In a report by Chandraker *et al.* (2014) on the management of antibody-mediated rejection in a presensitized face allograft recipient, evidence of rejection was found by the fifth postoperative day (POD). The causes were the correlating increase in HLA specific antibodies of the donor (DSA) and an accumulation of C4d in the allograft, reflecting the relevance of

compatibility in VCA.[18] Later, the patient also had T cell mediated rejection episodes, which could be treated. However, it was found that the expansion of T follicular helper and memory B cell populations was associated with increased DSA during rejection. This and other cases in the review by Daniel J. Wilks *et al.* show how important HLA compatibility is for VCA. New techniques such as limb perfusion, protocol development and the expansion of the donor pool should not actually make it possible to perform transplants across the antibody barriers, and yet it is likely that antibody compatibility will have to be addressed using desensitization approaches.[19] A further hurdle for the widespread application of VCA is lifelong multidrug immunosuppression, which ensures allograft survival, treats and prevents acute rejection. This hurdle must be overcome with targeted immunosuppressive therapy using innovative drug delivery systems (DDS), to increase therapeutic efficiency while reducing systemic toxicity to the lowest possible level. Due to its good accessibility, VCA offers a unique opportunity for site-specific administration of immunosuppressive drugs directly into the graft to improve patient compliance and reduce the intensity and occurrence of acute or even chronic rejections.[12]

4 IMMUNOSUPPRESSIVE THERAPY FOR VCA

To ensure long-term graft survival, a lifelong immunosuppressive therapy for VCA patients is absolutely indispensable. Several different drugs are available for therapy, such as Tacrolimus, Mycophenolate Mofetil, Rapamycin or a combination of these and in some cases with additional substances such as Ciclosporin and Corticosteroids.[2,10,20] It is generally known that a systemic therapy with the above mentioned drugs can be associated with transplant survival. One could show reduced acute rejection rates after kidney transplantations with a high dosage of Tacrolimus und this led to the assumption that maintenance of a high-dose immunosuppressive therapy could lead to long-term success. However, the fact of the side effects does not allow a continuous increase of the systemic dosage.[21,22] In addition, the daily intake and the accumulating long-term impacts of the drugs represent a very heavy burden for the patients.[23] From the data of the IRHCT registry it is also known that patient compliance is decisive for the prevention of acute rejection episodes.[24] Therefore, a novel drug delivery strategy is absolutely necessary to avoid the daily administration of drugs and the urgency for systemic immunosuppression. [23]

In the beginning of VCA, it was especially the experience gained from SOT that led to improved outcomes in patients. In the meantime, researchers have started to develop VCA-specific strategies for the problems of composite grafts. They are aware that immunosuppression is obviously one of the most pressing challenges.[25] The long-term adverse effects currently posed the barrier to the widespread use of VCA.[24,26] Nevertheless, VCA is comparatively accessible for local drug delivery, which is the main focus of immunosuppressive therapy. Such site-specific immunosuppression could reduce the systemic exposure, the frequency and intensity of graft rejections, the resulting risks and the dosage that would be necessary to prevent rejection.[23,27,28] Currently there is no standardized treatment, as strategies for DDS should always be developed based on the anatomy and function of the organs involved.[29,30] It is already possible to develop good approaches because the advantageous property of the visibility of the grafts is used to detect and observe rejections more efficiently and quickly. By using targeted immunosuppression and an according drug delivery system, the aim is to reach a sufficient dosage locally at the transplant side to trigger the effect where it is needed. In this way, a low concentration of the drugs in the blood is achieved, which at the same time reduces systemic toxicity and important immune cells remain, such as the regulatory T cells (T_{Reg}), whose suppressive effect could further promote VCA therapy.[29,31]

There are different approaches for a promising DDS, such as topical applications, intra-graft injections of Tacrolimus (TAC), or biodegradable discs with TAC loaded microspheres and some more. These are being investigated and developed in studies worldwide.[32–35] The focus in this thesis is on a very specific strategy, which is composed of a Triglycerol Monostearate (TGMS) hydrogel loaded with the immunosuppressive TAC. The TGMS hydrogel loaded with TAC (TGMS-TAC) is injected subcutaneously into the VCA graft. This novel DDS for local immunosuppression is designed to reduce the systemic immunosuppressive associated side effects by reducing the total amount of drugs taken without losing the prevention of rejection.[34]

The hydrogel is biocompatible, biodegradable and is generally considered to be safe by the US Food and Drug Administration. In addition, the hydrogel targeted at the graft is dependent on the time of inflammation and releases TAC "on demand" exactly when and also where it is needed. It is expected that with TGMS-TAC a high concentration of TAC can be achieved in the transplant and at the same time a low

concentration in the blood compared to the daily dose of TAC, which should significantly reduce the off-target effects and nephrotoxicity. In addition, we are also interested in the dynamics of chimerism and different immune cells, such as effector T cells (T_{Eff}) and T_{Reg}.[34,35] The hydrogel is formed from low molecular weight amphiphilic gelators with enzymatic bonds, which in response to an inflammatory rejection episode ensures sustained release of the encapsulated therapeutically relevant immunosuppressive drug doses. Due to the immune response triggered by an inflammation, upregulated proteolytic enzymes can decompose the injected self-assembled hydrogel and the drug will be released (Fig 1).[34,36,37]

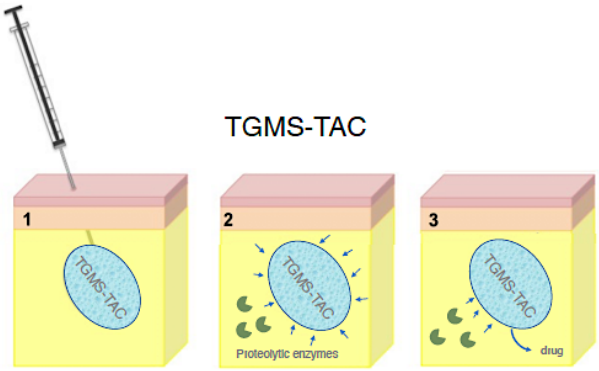

Figure 1 *TGMS self-assemble into a hydrogel loaded with TAC and disassemble in an enzyme-responsive aspect in presence of proteolytic enzymes which are present in inflammatory conditions and lead to the release of the drug. Adapted from Gajanayake et al. Sci Transl Med. 2014*[34,38]

The amphiphilic TGMS has a polyhydroxyl sugar head group to form a hydrogen bonding network that renders self-organized TGMS nanofibers water-soluble and includes a polymethylene hydrocarbon chain for efficient encapsulation of hydrophobic molecules using Van der Waals forces. In supplement, TGMS has an ester bond that enables cleavage by esterases and matrix metalproteinases (MMPs) present during inflammatory conditions like acute rejection in VCA (Fig.2A).

The stability of the TGMS-TAC at higher temperatures has been measured and it has been found that there is a transformation of the gel phase to the liquid phase at temperatures of 55° to 60°C and this leads us to assume that it remains in the gel phase at physiological temperatures.[34] The expression and concentration of the enzymes correlates with the degree of inflammation and this could be shown by measuring the release of the encapsulated drug.[39] TGMS-TAC hydrogel was incubated in PBS at 37°C with lipase (esterase), MMP-2 and MMP-9 and after regular intervals samples were collected and the release of Tacrolimus was quantified by high-performance liquid chromatography (HPLC). The results showed a release of the drug

dependent on the initiation of lipase and MMP enzymes, whereas the PBS control showed no release (Fig. 2C).[37]

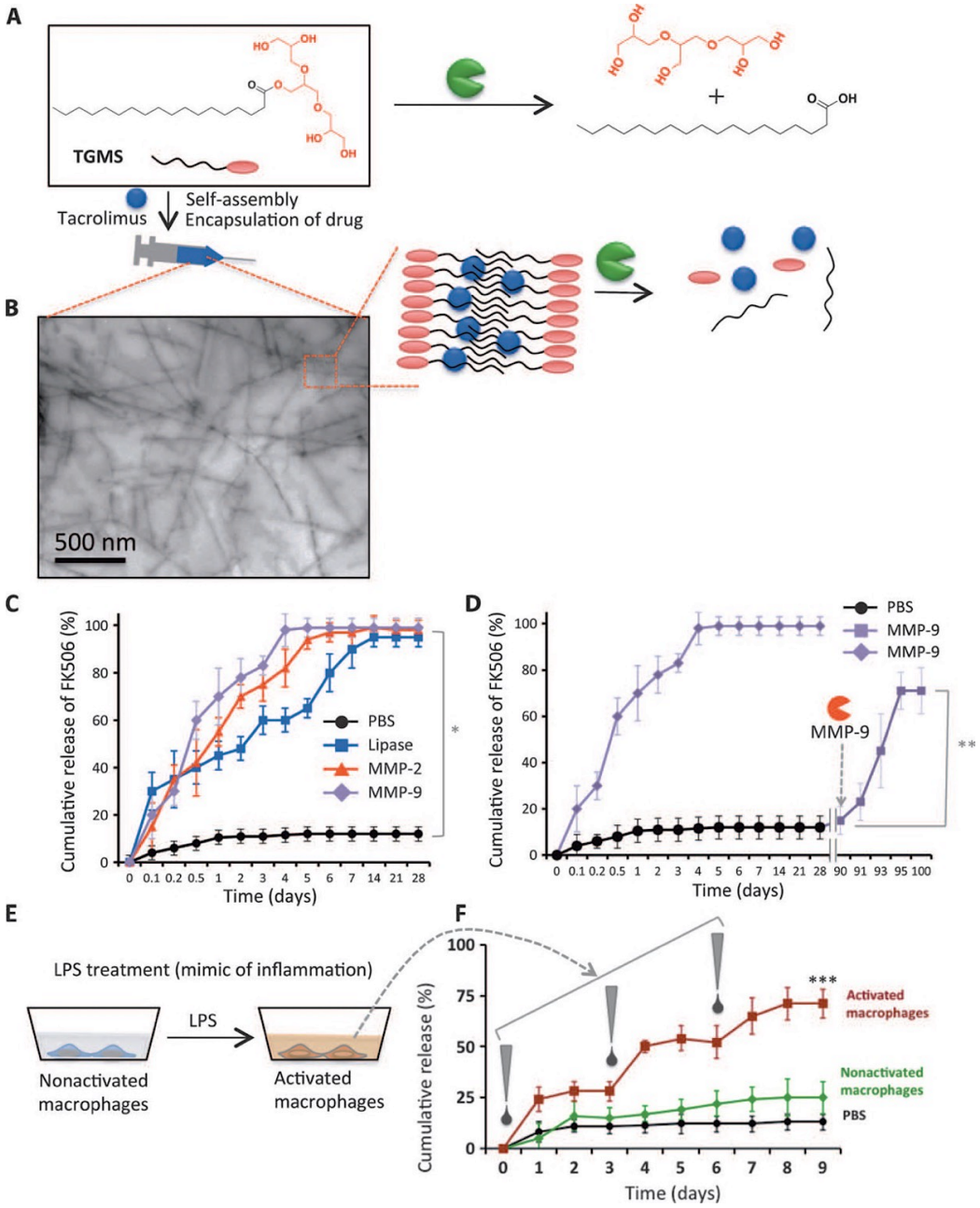

Figure 2 The composition of the TGMS hydrogel with Tacrolimus and the enzyme dependent release. (**A**) Schematic set-up of the TGMS self-assembly and the encapsulation of Tacrolimus. (**B**) Transmission electron micrograph of TGMS-TAC hydrogel. (**C** and **D**) Release of Tacrolimus by the proteolytic enzymes. The hydrogels incubated in PBS remain hydrolytically stable for at least 3 months and then by introducing proteolytic enzymes (lipase, MMP-2 and MMP-9) the release is triggered. (**E**) Schematic LPS activation of macrophages to simulate inflammatory conditions. (**F**) Cell culture supernatants of activated macrophages stimulate the release of the drug when added to TGMS-TAC, where the supernatant of non-activated macrophages and it PBS do not.[34]

In another study a clearly increased release of Tacrolimus in the blood of LPS challenged rats compared to the unchallenged ones could be shown and thus also the connection of the dependency of the inflammatory condition for the release of the

drug could be confirmed.[40] With the stable gels in PBS it could be shown that the presence of the enzymes was necessary to enable a release, after MMP-9 was added in the preincubation of TGMS-TAC for 90 days in PBS (Fig. 2D). In addition to the enzymes, macrophages activated by LPS for an inflammatory condition were used to confirm the release. And also here a release of tacrolimus was achieved with the activated macrophages by adding macrophages to the TGMS-TAC in a three-day interval and in comparison the controls with non-activated macrophages and PBS showed no release (Fig. 2E and 2F).[34,37]

At present, mainly studies with TGMS-TAC in rat models have been tested and good results have been achieved. For example, a single dose of TAC (7 mg TAC in 1 ml TGMS gel) allowed a transplant survival of more than 100 days without additional systemic immunosuppression.[34] In another experiment rats with a high-dose Tacrolimus injection at POD1 showed a transplant median survival time (MST) of 152.5 days, whereas half of the rats reached even more than 200 days without further additional immunosuppression.[41] So the first important stages are well behind us, now they have to be able to reproduce these results in large animals in a further step before clinical trials can establish. The risks, such as acute rejection episodes and systemic toxicity, which this drug delivery system is intended to reduce, remain for the time being. The aim is also to improve patient compliance. The skin is the most immunogenic component of VCA and therefore it is very important to understand the rejection reaction itself and the mechanisms behind it in order to develop and hopefully use an innovative and successful drug delivery system for localized immunosuppressive therapy for VCA.

5 THE IMMUNOLOGICAL MECHANISM OF VCA RISKS

Since the beginning of VCA, over 200 transplantations have been performed with improved surgical and immunological results. Nevertheless, rejection remains an integral part of VCA and not all cellular details have yet been clarified regarding the correct dosage in immunosuppression and, in general, the different mechanisms that play an important role.[16,42] The antibody-mediated rejection is mediated by the humoral level of donor specific antibodies (DSA) of B lymphocytes.[24] The sensitization that is present in recipient candidates is one of the biggest hurdles in VCA. Many patients often receive numerous blood transfusions and/or skin grafts,

resulting in sensitization and the presence of DSA capable of cross-reacting with HLA antigens of the transplanted origin. The activated B cells lead to antibody production and their reaction with cells of blood vessels. The resulting blood clots thus prevent the supply of the graft, which leads to immediate rejection.[43] Chronic rejection is the main cause of late graft loss and is mainly due to an alloimmune response of the recipient to the allograft.[44] In general, however, few cases of both forms have been reported and therefore the focus is not very much on these types of rejection. While "replacing like with like", the allogenic characteristics of transplanted tissues in VCA transplantation lead to immunological challenges that must be kept under monitoring at all times to ensure successful therapy. Approximately 85% of all VCA patients experience an acute rejection episode in the first year and 50% are burdened with multiple episodes. These rejection episodes are much more frequent compared to SOT, which is 10% in kidney transplantation, and this may be due to the highly immunogenic component of the skin, which plays a much more significant role in VCA than in SOT as it serves both as an immunogenic target and as a means of monitoring.[24,45–47] The fact that immunosuppressive drugs can be used has had a decisive influence on the development of VCA.[24] The practice of hand transplantation is nevertheless surrounded by the limitations of only supposed improvements in limb function and the requirement of chronic administration of immunosuppressive agents to prevent rejection, which can cause an accumulation of side effects and complications such as diabetes mellitus, nephrotoxicity, osteonecrosis, leukopenia, opportunistic infections and malignancies.[20,24,26,48] For many specialists, the key challenge is to find a balance between the rejection episodes caused by inadequate immunosuppression and the complications of the drugs in order to be able to adjust them for each patient. It has also been observed that a change in the anti-rejection cocktail has led to rejection and/or graft loss in patients. The general problems associated with immunosuppressive therapy can be divided into two groups: Those which are caused by the drugs toxicity and those which are due to low immunity. Such an attenuation of your own immune system makes you very susceptible to bacterial, fungal or viral infections and in addition, tumor surveillance during therapy is certainly not at its peak. VCA patients have been diagnosed with dysplastic and neoplastic transformations, like posttransplant lymphoproliferative disorder (PTLD) or marginal zone lymphoma and in such a case, one would have to

discontinue immunosuppression in order to treat the cancer, which would again result in VCA-related complications that should not be underestimated.[16,49,50]

The immune system exists to protect the body from foreign material, such as infectious microorganisms or mutated cells, and eliminate it. VCA is about the same response, which in the context is called alloresponse, and that arises from the interplay of innate and adaptive immunity.[51] An important component of the immune system are the peripheral blood mononuclear cells (PBMCs), which includes T cells, B cells, monocytes, macrophages and dendritic cells.[52] These blood cells play an important role in the immune response in that they ensure the aforementioned protection of the body. Therefore, the isolation of PBMCs and their evaluation of the dynamics and occurrence is essential for scientists and clinicians working in the field of immunology or in our case of VCA.[53]

The most common and best understood mechanism of rejection, namely cell-mediated rejection, is based on this mentioned interplay. This form is often also known as acute rejection and is based on direct and indirect pathways of antigen recognition with consecutive T cell priming.[54] This is mainly due to the perivascular CD3/CD4-positive infiltrators. In acute cell-mediated rejection, the T_{Eff} perform their function mainly through two mechanisms. First, CD8 positive T cells mediate cytolysis after binding the peptides to the major histocompatibility complex (MHC) class I molecule and second, CD4 positive effector T cells respond to the binding of antigens to class II MHC molecules.[55] This is the most pronounced form of cell-mediated rejection which leads to the activation of alloreactive T cells, that in turn quickly divide and differentiate into effector T cells. Once effector T cells reach the transplanted tissue, they steer an extensive graft destruction program based on secreted cytokines, which leads to an increase in inflammatory cell response and apoptosis of graft cells through the release of $CD8^+$ cytotoxins.[54] And of course lymphocyte infiltration can be well detected by the upregulation of some lymphocyte adhesion markers like ICAM-1, VCAM-1 or E-Selection.[16] Endothelial cells (ECs) are the primary target of T_{Eff} cells because they have a high concentration of class I and II HLA molecules compared to other tissue cells and additionally also due to the presence of the costimulatory molecules and receptors required for T cell recruitment and activation.[56,57] It is assumed that donor T_{Eff} are mainly responsible for vascular damage and for triggering acute cell-mediated rejection. Therefore, the use of

immunosuppressive drugs such as calcineurin inhibitors, mTOR inhibitors, Mycophenolate Mofetil (MMF) or steroids, which influence the function of T cells, is essential to control acute rejections.[58]

The innate immune response is mediated by some specific leukocytes, such as macrophages, dendritic cells (DCs) and neutrophils or by the complement system. These cells and molecules form a pre-set immune system that reacts to "danger". Such danger signals can be triggered by cell death, allergic insults, IRI, surgical trauma or brain death of the donor and lead to activation of the innate immune system.[59] Invariant pattern recognition receptors on innate leukocytes recognize so-called damage-associated molecular pattern molecules (DAMPs) on the allograft, which have been altered by the inflammatory processes of the transplantation. This leads to activation of the inflammasome and the upregulation of inflammatory response genes, which in turn leads to an increase in chemokines and P-selectin and the recruitment of activated lymphocytes. In addition, recruited macrophages secrete inflammatory cytokines, such as interleukin (IL-1) and (IL-6) which enhance the immune response against the graft and activate and recruit antigen-presenting cells (APCs).[60–62] A large group of soluble extracellular proteins or glycoproteins, namely cytokines, are the major intracellular regulators and mobilizers. They are considered crucial for innate and adaptive inflammatory reactions, cell growth and differentiation, cell death and developmental and repair processes. Their secretion by basically every nuclear cell type is usually an inducible response to harmful stimuli. In addition, cytokines establish a connection between organ systems and provide molecular clues for the maintenance of physiological stability.[63,64] Damage-associated inflammation, as occurs in transplantation, is mainly mediated by cytokines which regulate the recruitment of macrophages and lymphocytes.[65] VCA rejections are mainly governed by the high expression of INFγ within the allograft, possibly by upregulating IL-18 and triggering a pathway of cytokines that eventually leads to recruitment and activation of T cells and NK cells.[66] For this reason, the evaluation of cytokine events and movement is also of certain importance when considering the connection to VCA rejection reactions. A discussion of the immune mechanisms of VCA rejection is not complete without considering the importance of the skin. Usually the skin is also the first and sometimes the only component in VCA that experiences rejection and controlling rejection of the skin is the most challenging

part anyway. This is mainly due to the peculiarity of keratinocytes compared to other components of VCA, which allows an expression of HLA when stimulated by an inflammation. Figure 3 provides a summary of some aspects of skin immunology that may be relevant for transplant rejection.[67]

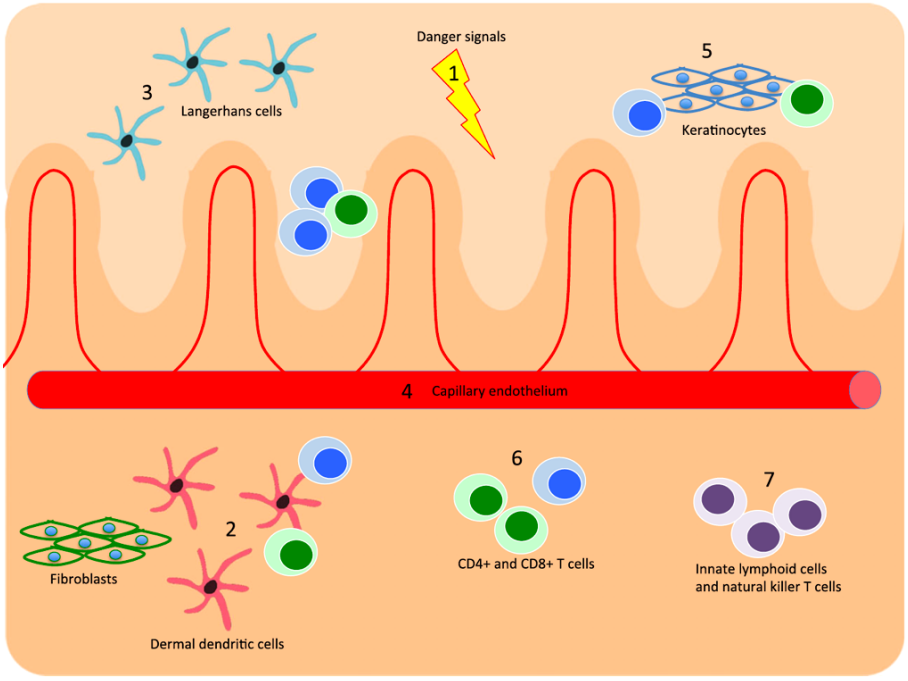

Figure 3 The skin contains an autonomous immune system, which is able to activate the host's immune system. This microenvironment contains some elements that can influence the susceptibility to rejection reactions. (**1**) Different danger signals activate innate cells via toll-like receptors and other pattern recognition receptors. (**2**) Once activated, innate cells such as fibroblasts secrete inflammatory cytokines such as IFN-α, TNF or IL-6 to activate professional antigen-presenting cells (e.g. dendritic cells) that present T-cell antigens. The activated dendritic cells in turn can produce chemokines to stimulate the recruitment of lymphocytes from peripheral blood. (**3**) Langerhans cells can also produce inflammatory cytokines to support the activation of dendritic cells or can present antigens themselves. (**4**) Activated endothelium can express human leukocyte antigens (HLA) class I and II and recruit resting memory cells. In addition, the expression of lymphocyte adhesion molecules enables the transcellular migration of circulating immune cells. (**5**) Keratinocytes stimulated with pro-inflammatory cytokines can also present antigens to T cells via HLA class I and II. (**6**) Effector and memory cells, which migrate in the dermis and epidermis and can cause damage. (**7**) The bridge between the innate and adaptive immune system is formed by the innate lymphatic cells and the natural T-killer cells, which are able to activate dendritic cells or to be activated themselves and to recognize certain foreign antigens via T-cell receptors.[67]

ECs also have the special property that they can activate resting memory T cells, which fibroblasts or epithelial cells cannot do, although they also express MHC molecules.[68] Circulation from the skin to the lymph nodes and back from these memory T cells is considered to be the cause of the development of so-called tertiary lymphatic organs (TLO) during rejection episodes. It is suggested that these TLO are the local sides for T-cell activation and alloantibody production.[69] In addition, the skin has a high number of APCs, including specialized Langerhans cells, which may also influence the recruitment and activation of lymphocytes. There is also evidence that they play a crucial role in immune regulation.[70]

One would instinctively think that the cells that infiltrate the allograft, especially during rejection episodes, come primarily from the recipient. A study by Lian *et al.* concluded that this not may be the case in VCA allografts.[71] Findings have shown that the skin accommodate twice as many T_{Eff} as are present in the peripheral blood and it can be assumed that the donor's skin still has a large number of donor T cells after transplantation.[72] This has been documented by over 100 biopsies of face transplants during acute rejections, where mainly original donor lymphocytes related to the injuries were found.[47,71] The authors assumed a response of the existing donor lymphocytes in the skin allograft against the T cells and APCs of the recipient, which migrate into the graft. In this instance, it could indeed be an influx of recipient regulatory T cells that express skin homing receptors (CLA+) and control the donor-specific «graft versus host» response in VCA patients. This hypothesis is supported by the fact that all human $CD4^+$ T_{Reg} in peripheral blood also express the chemokine receptor CCR4 and 80% of them also express CLA, which affects cell migration. From these observations it can be concluded that the donor T cell response may migrate on the skin of the recipient and cause any injury or disease.[73,74] In summary, the properties of the skin are a major factor in the recruitment and activation of T cells, which causes rejection reactions. One can see that it has many components that have to be taken into account when considering the risks of VCA. Even if one focuses on the most immunogenic component, namely the skin, one also has many particular elements that play an important role in the rejection.

6 THE IMMUNSUPPRESSOR – TACROLIMUS (FK506)

For transplants, be it SOT or VCA, immunosuppressors are essential because they have the ability to prevent graft rejection. This is also the case for the immunosuppressor Tacrolimus, which we use in our project with the porcine model to test site-specific immunosuppression for long-term maintenance of vascularized composite allotransplantation. Tacrolimus is a calcineurin inhibitor and is applied in almost all immunosuppressive cures for VCA.[30]

The main function of the drug is to inhibit T cell activation by switching off the transcription of early genes that code for cytokines, which are responsible for coordinating the various cells of the immune response. It appears to be distal to the cell membrane receptors and the known second messengers, but proximal to the late

signaling events such as the transcriptional activation of earlier genes. These findings led to the assumption that the drug can be used as a probe for the effect of certain critical intracellular communication pathways. When it was found that the concentration that completely blocked T cell activation was not toxic to cells and did not prevent the proliferation of other cells, it was considered a useful immunosuppressant.[76]

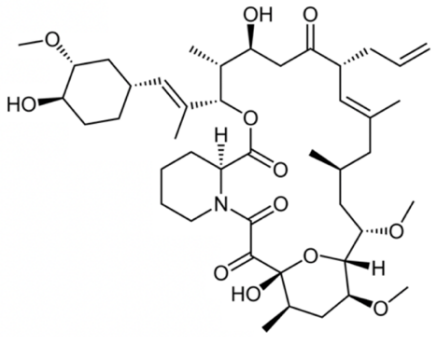

Figure 4 Molecular structure of Tacrolimus[75]

The intracellular FK506 binding protein 12 (FKBP12), a so-called immunophilin, is identical to a cis-trans peptidyl-prolyl isomerase and by binding of FK506 the activity of the isomerase is blocked.[77,78] The Ca^{2+}- and calmodulin-dependent protein phosphatase, calcineurin, has the ability to dephosphorylate a synthetic peptide from the regulatory subunit of the cAMP kinase as long as the complex of immunophilin and Tacrolimus does not block this. Thus, FK506 can prevent dephosphorylation of nuclear factor of activated T cells (NF-AT), which are responsible for the transcription of T cell activating cytokines like IL-2 or IL-4.[79,80] NF-AT is a protein complex of at least two subunits, one T-cell specific and restricted to the cytoplasm, while the other is ubiquitous, quickly induced by the activators of protein kinase C (PKC) and is predominantly nuclear. An increase in intracellular Ca^{2+} concentration leads to nuclear translocation of the cytosolic subunit and the composition of a functional transcription factor and furthermore to the increase of the phosphatase activity of the calcineurin. Dephosphorylation also seems to contribute to the translocation of NF-AT_c and thus the importance of FK506 in this context is well-established.[76,82,83] The addition of FK506 in this system blocks the translocation of NF-AT_c and NF-AT_n cannot serve as a transcription factor for IL-2, which is necessary for full T cell function and activation.[81]

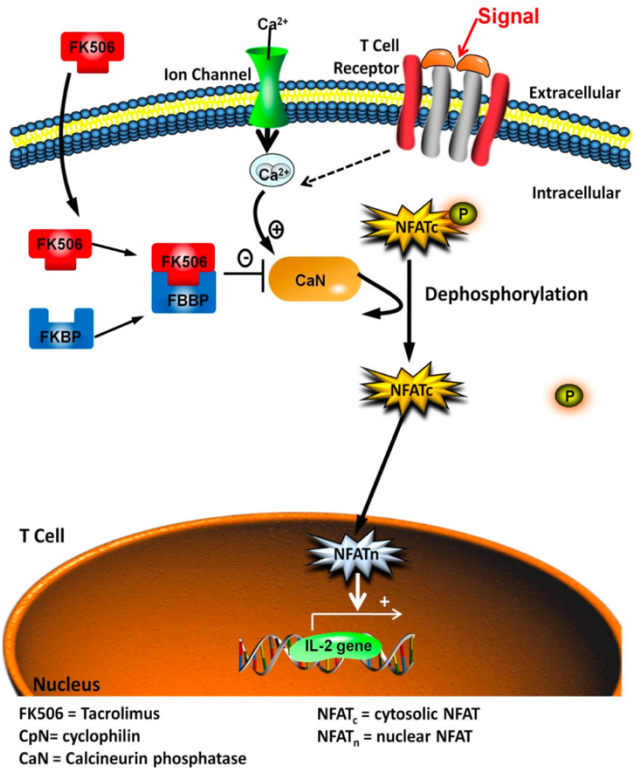

Figure 5 Immunosuppressive mechanism of action of Tacrolimus (FK506). In the cytoplasm of T cells Tacrolimus binds the immunophilin FK506 binding protein (FKBP) and the developed complex binds again the enzyme calcineurin (CaN). As a result, phosphorylation of the cytosolic component of the nuclear factor of activated T cells (NF-AT_c) is inhibited. This blocks the transport of NF-AT_c into the nucleus, which prevents the binding of NF-AT_n to the promoter for the interleukin 2 gene and thus the T-cells can no longer produce IL-2, which in the example of inflammation would be necessary for total T-cell activation.[81]

7 *IN VIVO* VCA MODEL WITH PORCINE

Before the TGMS-TAC drug delivery system for site-specific immunosuppressive therapy for VCA can be granted unrestricted approval worldwide, it must undergo several stages of experimental research. The first two steps were *in vitro* tests and then *in vivo* experiments with small animals such as rats. The last pre-clinical step is also in the field of in vivo experiments, this time with large animals. Pigs have been chosen in this case because they are relatively docile and economically not too expensive. In addition, they have a similar anatomy and tissue composition as humans, which makes them an optimal model for VCA. Their immune response is also very similar to that of humans, which complements each other well to achieve the goal of the study.[84]

8 AIM OF THE STUDY

A challenging and clinically relevant porcine model is used to demonstrate the possibilities of preventing VCA rejection by site-specific immunosuppression. This includes the evaluation of the efficiency of local immunosuppression, which should allow long-term graft survival using the TGMS-TAC treatment. But also, the characterization of the mechanism of action of immunosuppression by analyzing the systemic and tissue-specific levels of the drug and their off-target toxicity. In relation to the immune response of the recipient to the graft, the third specific aim is to assess the capacity of site-specific immunosuppression to induce immunoregulation in a clinically relevant VCA model with continuous TGMS-TAC injections.

9 MATERIALS AND METHODS

9.1 ANIMALS

For the project, pigs bred in Switzerland are used that weigh between 18-30 kg and are about three months old. The pigs are selected according to their swine leukocyte antigen (SLA) in such a way that there is at least one complete mismatch between donor and recipient in order to implement the project in extremely challenging conditions.[38] The MHC-defined heterotrophic porcine hind limb transplantation model will be used as a basis for present and future immunomodulatory strategies aiming at the induction of immunotolerance to VCA to expand the clinical application. Only characteristic inbred pigs bred for homozygosity at the SLA locus and specifically used for transplantation-related experiments and studies will be used.[85]

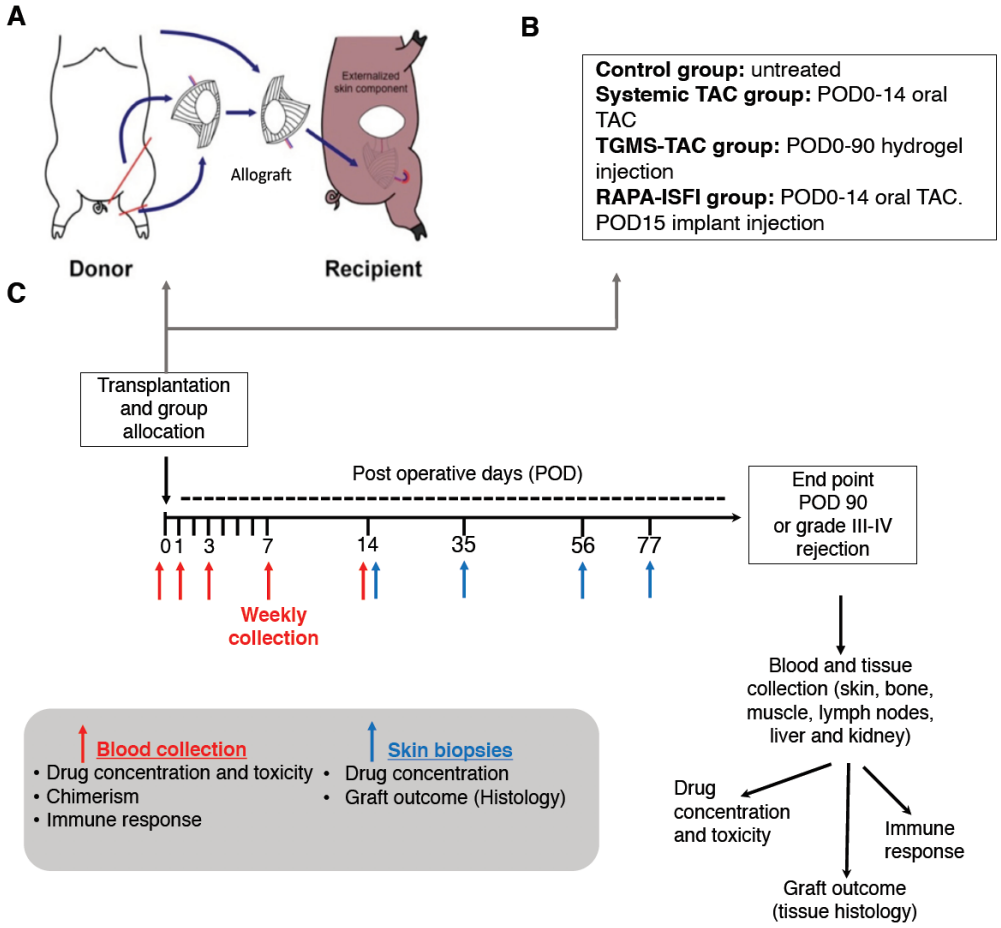

Figure 6 Experimental design (**A**)[86] Schematic representation of an osteomyocutaneous alloflap in a pig heterotrophic hind limb transplantation. (**B**) treatment groups of the project and (**C**) the timeline of the recipients after surgery.[38]

9.2 HIND LIMB TRANSPLANTATION

A vascularized osteomycutaneous flap is raised on the basis of femoral vessels (Fig 6A). The flap holds intact vascularized bone marrow in the distal femur and proximal tibia. The anteromedial femoral skin is also included in the graft to allow immune monitoring using the most immunogenic component of VCA. The dorsolateral positioning facilitates the clinical examination and also keeps the skin of the allograft relatively clean.[87] From each donor always two recipients receive an allograft. To minimize the time of anesthesia and ischemia during the operation, the pigs are prepared simultaneously on three different tables.[86] The transplantations are performed according to the VCA Project Surgical Protocol based on the publications of Ibrahim *et al.* and Chuang MS *et al.*[86,88] General balanced anesthesia was provided by Propofol and Ketamine and in addition recipients and donors are treated with prophylactic antibiotics during the procedure. The procedure is of course carried out under sterile conditions and with sterile instruments. The donor limb is transplanted on the contralateral side of the recipient and after the surgery and harvesting the collection of samples the donor is immediately euthanized and confirmed by the veterinary team. Arterial and venous anastomoses are performed on the recipient to restore the blood supply to the allograft before it gets sutured up. In addition, wound catheters for the administration of local anesthesia and a central venous port-a catheter will be placed in the posterior neck region to facilitate postoperative blood.

9.3 TREATMENTGROUPS & TGMS-TAC PREPARATION

For the project, after the surgery the recipients are randomly assigned to one of the four treatment groups (Fig 6B). There is the control group, which does not receive any treatment, so that you always have the comparison of the results of the strategies and methods that you want to test in the experiment. The systemic TAC group is listed on the basis of formality to show that it is effective including the associated side effects and off-target toxicity. In this group the pigs receive a dose of 1 mg/kg Tacrolimus. The treatment will be stopped after two weeks, because the effect and efficiency of Tacrolimus is already known. The TGMS-TAC group aims to identify the tolerable TAC doses that lead to long-term graft survival. To date, two doses have been investigated (49 mg and 93 mg per 500 g) and the range for the tolerable dose is expected to be in between.[84] Our pigs are treated with a dose of 70 mg/500g. The hydrogel is injected locally at POD0 just after transplantation at the allograft for

a 90-day treatment. The pigs are either euthanized at the end point (POD90), at a grade III or IV rejection of the Banff classification (Table 1) or if vascular complications/failures occur. In the last group with RAPA-ISFI, the pigs are first treated with oral TAC for 14 days and then the implant is injected, which ensures a 90-day release of Rapamycin. This group is not relevant in this thesis and will therefore not be in focus, as the project has not yet reached that stage.

Table 1 The Banff VCA working classification system.[89]

Grade	**Inflammatory infiltrate**	**Involvement of epithelium (epidermis or adnexal)**
0 (no rejection)	None/rare	None
I (mild rejection)	Mild perivascular	None
II (moderate rejection)	Moderate to severe perivascular	Mild (limited to spongiosis or lymphocytic exocytosis)
III (severe rejection)	Dense	Apoptosis, dyskeratosis, and/or keratinolysis
IV (acute necrotizing rejection)	Frank necrosis of the epidermis or its structures	

The TGMS-TAC hydrogel consists of the following:

- 10% TGMS (e.g. for 3.5 ml gel 350 mg TGMS is used)
- 20% DMSO (e.g. for 3.5 ml gel 700 µl DMSO is used)
- TAC dose (e.g 7mg/ml Tacrolimus)
- The rest is H_2O (e.g. for 3.5 ml gel 2.8 ml H_2O is used)

In a first step, the TGMS is weighed and heated in the glass vial. Tacrolimus is also weighed on a piece of paper and added to the bottle. In addition, the DMSO is added and it is heated again under the hood. The vial can be stirred well without the gel sticking to the walls. Once the mixture becomes clear, you can put it aside until it forms a gel. To add the water, you first have to melt the gel again and slowly add all the water over the wall. If you want to monitor the gel with LiCor imager, you have to add the NIR dye in this step as well. In the meantime, it should always be heated well until it has melted well. Then when everything is ready, you can go in with a syringe and take it without making bubbles. If bubbles appear, you can jerk it firmly until it reaches the exit of the syringe where you can carefully blow them out.
For 2-3 days it must leave undisturbed in the dark. Finally, the gel is checked for purity with the LONZA traditional gel cloth assay whether it can be used in animals.

Table 2 Final group selection for the project.

Day of surgery	Donor/ Recipi-ent	Mother	Pig ID	Treatment group	Final fol-low-up	Date of Eu-thanasia
15.10.19	D1	2	6470	NA	Donor	15.10.19
	R1	3	6482	Model/Control	Euthanasia POD26	10.11.19
	R2	4	6498	Model	Euthanasia POD0	15.10.19
17.10.19	D2	2	6471	NA	Donor	17.10.19
	R3	3	6483	Control	Euthanasia POD7	24.10.19
	R4	3	6484	Control	Euthanasia POD7	24.10.19
12.11.19	D3	1	6605	NA	Donor	12.11.19
	R5	2	6596	Control	Euthanasia POD9	21.11.19
	R6	3	6623	Model	Euthanasia POD0	12.11.19
14.11.19	D4	4	6576	NA	Donor	14.11.19
	R7	3	6615	Model	Euthanasia POD0	14.11.19
	R8	2	6597	Control	Euthanasia POD8	22.11.19
21.01.20	D5	4	6928	NA	Donor	21.01.20
	R9	3	6918	Systemic	Euthanasia POD48	09.03.20
	R10	2	6933	TGMS-TAC	Euthanasia POD51	12.03.20
23.01.20	D6	4	6925	NA	Donor	23.01.20
	R11	3	6913	TGMS-TAC	Euthanasia POD39	02.03.20
	R12	3	6916	Systemic	Euthanasia POD55	18.03.20
18.02.20	D7	5	7034	NA	Donor	18.02.20
	R13	4	7023	TGMS-TAC	Euthanasia POD34	23.03.20
	R14	1	6971	Systemic	Euthanasia POD10	28.02.20
20.02.20	D8	3	6973	NA	Donor	20.02.20
	R15	5	7033	TGMS-TAC	Euthanasia POD60	20.04.20
	R16	2	6974/ 6574	Systemic	Euthanasia POD27	18.03.20

9.4 MACROSCOPIC AND MICROSCOPIC ASSESSMENT

To assess graft survival and off-target toxicity, blood and skin samples are collected at different time points (Fig 6C). The first samples are collected during the operation, where tissue and blood samples are collected from the donor and the first blood samples from the recipient (Table 3).

Table 3 Sample collection of the donor during surgery (A) and of the recipient at endpoint (B). The tissue samples are used for isolation from the associated cell types and are also preserved with OCT, Snap Frozen and formaldehyde.

Blood samples	
Arterial blood (A and B)	In cloth activator for serum In L-Heparin for plasma In EDTA for PBMC isolation
Tissue samples	
Lymph Node from harvested limb (A)	Digestion for LNSC
Lymph Node (B)	Transplanted and contralateral side
Spleen (A and B)	Isolation of Splenocytes
Thymus (A and B)	Isolation of Thymocytes
Skin (A and B)	For all the layers of the skin/ transplanted and contralateral side
Muscle (A and B)	Cross-section and longitudinal section of muscle fibers/ transplanted and contralateral side
Liver (B)	
Kidney (B)	
Suture (B)	Medial and lateral side

With the macroscopic assessment, visual observation of the allograft and subsequent classification of rejections can evaluate graft survival according to Banff (Table 1). Microscopic evaluation is then mainly based on blood and tissue samples. The blood samples are used to examine plasma and serum on the one hand and PBMCs and drug concentrations on the other. This step is very important to determine the immune system responsible for a rejection reaction. In particular, the plasma can be used to determine complement and cytokine activation and to incorporate the data into the immune system rejection context. With the PBMCs one can determine the dynamics of myeloid cells and subpopulations.

The tissue samples are then mainly required for histopathological evaluation, immune staining and also for determining the concentration of drugs. To enable the individual components for the analysis of the project, specifically developed protocols are used for this purpose. The basis of the different steps usually remains the same, but sometimes some procedures are adapted due to experience or due to suggestions from colleagues.

9.4.1 ISOLATION OF PBMCS

The collected arterial blood is used for the isolation of PBMCs. The EDTA tubes are centrifuged at 1000G for 15 minutes at room temperature to separate the plasma. As long as the tubes are closed and must be outside the hood due to a process, work is always carried out under sterile conditions. The cellular portion is then diluted (1:1) with PBS + 2%FBS and mixed by inverting. A new tube of density gradient Lymphoprep is then prepared, again with a dilution of about 1:1 with the blood, tending to be less of the density gradient (4 ml Lymphoprep per 6 ml of diluted blood). This step must be carried out carefully so that the two layers do not mix (Fig 7) before centrifuging again at 400G for 20 minutes at room temperature with an acceleration of 2 and a braking off.

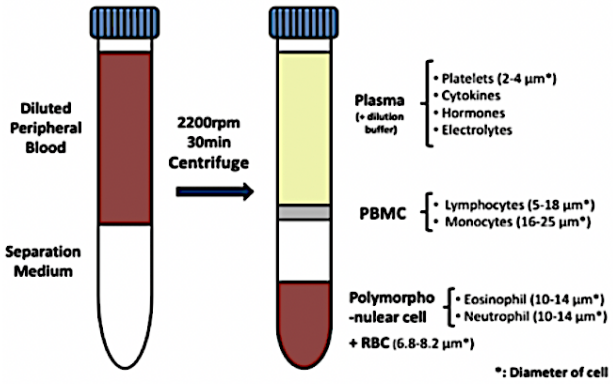

Figure 7 Isolation of blood by Density Centrifugation[90]

The middle cloudy layer of PBMCs is carefully transferred into a new tube and washed with PBS + 2%FBS at 300 G for 5 minutes. If the pellet still contains too many red blood cells, you can resuspend the pellet with red blood cell lysis buffer and let it stand for about 3 to 5 minutes, but not too long, because otherwise the important PBMCs will be also lysed. It is then centrifuged again under the same conditions and by estimating the pellet size it is resuspended again with the appropriate amount of PBS + 2%FBS. One ml should contain about 5 to 8 million cells and to ensure this, the cells are counted by mixing 10 µl of the cell suspension with 90 µl of Trypan Blue and from this mix 10 µl are done on the chamber and counted with the microscope. A hemocytometer (Neubauer Chamber) is always used for cell counting. The 4 squares at the corners are each divided into 16 smaller squares and are used for counting. At least 2 of the 4x4 squares are counted using the appropriate methodology (Eq 1) and formula. The dead cells are able to absorb the

trypan blue and therefore appear with the staining under the microscope. The PBMCs are roundish and glowing and provide a good contrast for counting. This procedure should always be done immediately after addition to the trypan blue because the dye has a toxic effect on the cells. If the desired number is not reached, one must make a dilution step and count the cells again. The cells are finally resuspended in 1 ml freezing medium consisting of 90% fetal calf serum (FCS) + 10% Dimethyl Sulfoxide (DMSO) and frozen at -80°C and later at -150°C. If you now have many more cells than the optimal amount, as much Freezing medium is produced in a single step to ensure the optimal amount of 5 to 8 million cells/ml. The pellet is then resuspended with the total freezing medium and 1 ml is distributed a corresponding number of cryotubes.

$$Quantity\ of\ cells\ per\ volume = \emptyset(per\ square) \times 10^4 \times 10\ (\mu l\ of\ cellsuspension\ added\ with\ Trypan\ Blue) \times 1(ml\ of\ resuspending\ medium)$$

Equation 1 Cell counting formula. Depending on the volume of medium, which is used, this must be adjusted in the formula

9.4.2 ISOLATION OF SPLENOCYTES AND THYMOCYTES

The tissue samples are collected in falcon tubes filled with PBS. In the laboratory the spleen and thymus tissue are excised into minor pieces in the hood under sterile conditions. The minor pieces are then passed through a 70 Mesh filter and gently pressed through the strainer, while continuously adding PBS. At this stage, enough care must be taken not to destroy the cells which cause a release of DNA, that gives the cells a sticky property. PBS is then added to the collected material in the tube until a final volume of 30 ml is reached before it is centrifuged for 5 minutes at 300G at 4°C. The resulting pellet is incubated as soon as possible with 5 ml ACK lysis buffer for 5 minutes to lyse the red blood cells. As with the PBMCs, one must pay close attention to the timing in order not to damage the desired isolated cells. After incubation, the tube is filled up to 40 ml with 1x PBS and centrifuged again for 7 minutes under the same conditions. The pellet is resuspended in 5 ml PBS and the cell suspension is passed through a new 70 mesh filter to remove dead cell lumps and other debris. The cell suspension is then left on ice for 10 minutes before a 1:10 dilution with Trypan Blue is made to count the cells. The desired concentration is

about 5 million cells/ml, as higher concentrations can affect the viability of the cells. The mononuclear cells are resuspended in 1 ml freezing medium (90% FCS + 10% DMSO) and frozen in cryotubes in an isopropanol container at -80°C. Later they are transferred to -150°C and are ready for the analysis methods.

9.4.3 ISOLATION OF LYMPH NODE STROMAL CELLS (LNSC)

With this protocol, an enzyme mix must first be prepared, which digests the lymph node. 1.3 mg collagenase P is dissolved in 6.5 ml DME/F-12 medium and then filtered sterile with a 22 µm filter. From the collagenase solution, the two halves are transferred each to a Dispase I vial (2mg) to obtain the final concentration of the solution (0.57 mg/ml Dispase and 0.2 mg/ml Collagenase P) before being mixed back into a tube. Finally, 120 µl DNase I from the stock (5mg/ml, -20°C) are added to reach the final concentration of the mix of 0.1 mg/ml. It should be noted that the volume of the enzyme mix is adjusted to the number of lymph nodes. If only one lymph node is present, 500 µl of the mix should be used per processing step. In addition, the culture medium has to be prepared, whereby 50 ml is first taken from the DME/F-12 bottle and stored at 4°C. The FBS is first inactivated by heat at 56°C in a water bath before 50 ml is added to the DME/F-12. Additionally, 5 ml each of L-glutamine and P/S are added to complete the medium.

In a preparatory step, the lymph nodes are first separated from the fatty tissue, chopped under sterile conditions in the hood and transferred to a digestion tube containing 2 ml of the produced enzyme mix. The collection tube is incubated in a 37°C water bath for 20 minutes to allow the enzymes to operate under the best possible conditions. If after the incubation time the lymph nodes are not digested enough, we can add another 0.5 ml of enzyme mix and leave it in the water bath for another 10 minutes. To facilitate the digestion of the lymph nodes, we have prepared the 1 ml pipette tip with scissors so that the lymph nodes can be pipetted up and down with the larger opening to release the cells. The supernatant is then transferred to a collection tube, 5 ml of culture medium is added, and the tube is centrifuged at 300 G for 5 minutes at room temperature. The supernatant is no longer needed because the cells are now in the pellet. An attempt is now made to collect the remains of the lymph node in the digestion tube by adding another 2 ml of the newly prepared enzyme mix and incubating it for another 10 minutes in the water bath with regular gentle mixing. The enzyme mix from the digestion tube is now

transferred to the collection tube using an 80 µm nylon mesh filter. You must always use this filter if you transfer something into the collection tube. If you still have remnants of lymph nodes in the digestion tube, you can repeat the mentioned step to get the highest possible harvest of cells. Once the entire contents have been transferred from the digestion tube to the collection tube, it is centrifuged again at 300 G for 5 minutes and depending on the size of the pellet, between 1 ml and 10 ml of culture medium can be used to resuspend the cells. Until you have some experience, use a little less in the beginning to resuspend the pellet, because you could still dilute the cell suspension. By adding a 1:10 dilution of the cell suspension with Trypan Blue to the chamber, the cells can be counted under the microscope using the well-known technique (Eq 8). The number of cells is used to calculate the amount of freezing medium (90% FCS + 10% DMSO) which is required for freezing. LNSCs are also stored in isopropanol-filled containers at -80°C before being transferred to -150°C.

9.4.4 EXTRACELLULAR STAINING OF PBMC FOR FACS ANALYZES

The PBMCs you want to analyze are frozen at -150°C at the end of the isolation phase and you have to defrost them first. Thaw the cells on ice for 30 minutes until only a small piece of ice is left or put the cells in a water bath (37°C) for 30-60 seconds. The cell culture medium must also be brought to room temperature. In a first step, the cells are placed in a 15 ml falcon tube containing 10 ml medium and washed at 400 G for 8 minutes at 4°C. The supernatant is removed, and the cells are centrifuged twice more with 5 ml medium under the same conditions. The cells are then resuspended with warm complete cell culture medium and placed in culture flasks in the incubator overnight at 37°C and 5% CO_2 so they can recover the epitopes. If the vials were overly reddish, the red blood cells can be mixed with 1.9 ml 1x Erythrocyte Lysis Buffer per tube and mixed or vortexed until the opalescence becomes clear. Additionally, they can be put in the fridge for 10 minutes until the color also becomes clear. This is an important step for extracellular staining, as it must be ensured that no red blood cells are present. Therefore, sometimes this step has to be performed several times. Finally, the cells containing the lysis buffer are centrifuged at 300 G for 5 minutes. Then they are washed once with PBS + 2%FBS at 300 G for 5 - 8 minutes. The viable cells are counted, resuspended in cell staining buffer to obtain 5 - 10 x 10^6 cells/ml and then 100 µl of the cell suspension is

distributed per FACS tube. To block the non-specific Fc-mediated reactions, 2.5 µg Fc Blocker per 10^6 PBMCs per tube are added and incubated for 10 minutes at room temperature. Subsequently, 1 µl of the fixable viability dye per tube is added and incubated light protected on ice for 15 minutes. Instead of the viability dye you can also use DAPI before putting the tubes into the machine. The cells are then washed with 1 ml Stain Buffer at 300 G for 5 - 8 minutes. Now you can prepare the master mix for extracellular staining, considering the number of tubes and the amount of antibodies which are used per tube. Once the antibodies are added, the tubes are incubated on ice for 45 minutes protected from light. An unstained tube should always be present as a control. The cells are then washed twice under the same conditions with Stain Buffer. The supernatants of the cell pellets are carefully aspirated, and the tubes are tapped to loosen the pellets. In this case the cells are washed two more times with Stain Buffer under the given conditions. The FACS tubes are transported in a polystyrene box. Once you are logged in and have determined the layout of your collection, you can add a few drops of the FACS flow into the Eppendorf tubes, mix well and transfer them to the FACS tubes.

9.5 DATA ANALYSIS

In FACS, antibody associated fluorescent markers are used to screen the subpopulations of lymphocytes in a successive procedure for analysis. With DAPI the dead cells can be stained and the living cells can be sorted out. The $CD4^+$ and $CD8^+$ cells are finally stained with the corresponding antibodies. After staining, the cell suspension is divided into individual units by a vibrating mechanism. These cell units receive a positive or negative charge depending on the reaction to the fluorescent markers, which are divided in the electric field according to the different cell types. The events are then analyzed with programs like Fiji or FlowJo. For the subdivision of the cell types, a threshold value must be determined which depends on the size and complexity of the cells. The graph shows on the y-axis the side scatter, which represents the complexity, and on the x-axis the forward scatter, which is associated with the size of the cells. Using applied markers, a step-by-step division of the desired cell types is now possible (Fig 8).

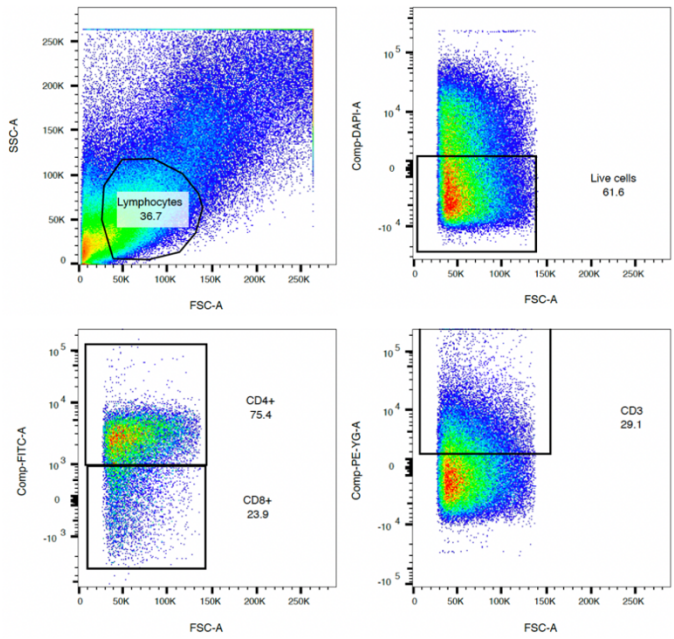

Figure 8 FACS outcomes with different threshold setting for successive separation of cell subpopulations with FlowJo (20200227 PBMC-DAPI-CD3-CD4) for Pig 6918 at POD7.

9.6 MATERIALS & REAGENTS REFERENCES

Table 4 Materials and reagents with the corresponding references used in the protocols.

Reagents	**Reference**
Antibodies: - CD4 (FITC Mouse Anti-Pig CD4) - CD3 (PE Mouse Anti-Pig GD3e)	 CD4: Clone 74-12-4 (Abcam. Ref: ab24989) CD3: Clone BB23-8E6-8C8 (BD. Ref: 561485)
Collagenase P	Roche Diagnostics, Ref: 11088882001
DAPI	Sigma-Aldrich, Ref: 32670-25MG-F
Density gradient (Lymphoprep)	Ficoll-Paque PLUS, Ref: 17144002
Dispase I	Roche Diagnostics, Ref: 04942086001
DNase I	Sigma-Aldrich, Ref: DN25-1G
DME/F-12	Sigma-Aldrich, Ref: D6421
DMSO (Dimethyl Sulfoxide)	Sigma-Aldrich, Ref: D2650
FBS (fetal bovine serum)	Sigma-Aldrich, Ref: F7524
Fc Blocking solution	Macs., Ref: 130-059-901
FCS (fetal calf serum)	Hyclone, Ref: SV30160.03
Fixable viability dye (eFluor 405)	Miltenyi Biotec, Ref: 130-109-816
L-Glutamine	Gibco, Ref: 21051024
Nylon Mesh filter	Clear Edge, Ref: PA75-1750
PBS (Phosphate-buffered saline)	Gibco, Ref: 10010023
Red blood cell lysis buffer	BioLegend, Ref: 420301
RPMI medium	Gibco, Ref: 31870-025
Stain Buffer	BioLegend, Ref: 420201
P/S (penicillin/streptomycin)	Gibco, Ref: 15140-122
Tacrolimus	LC Laboratories, Ref: F4900
TGMS	AK Scientific, Ref: 0570
Trypan Blue	Sigma-Aldrich, Ref: T8154 (0.04%)

10 RESULTS

10.1 SURVIVAL TIME OF TREATMENT GROUPS

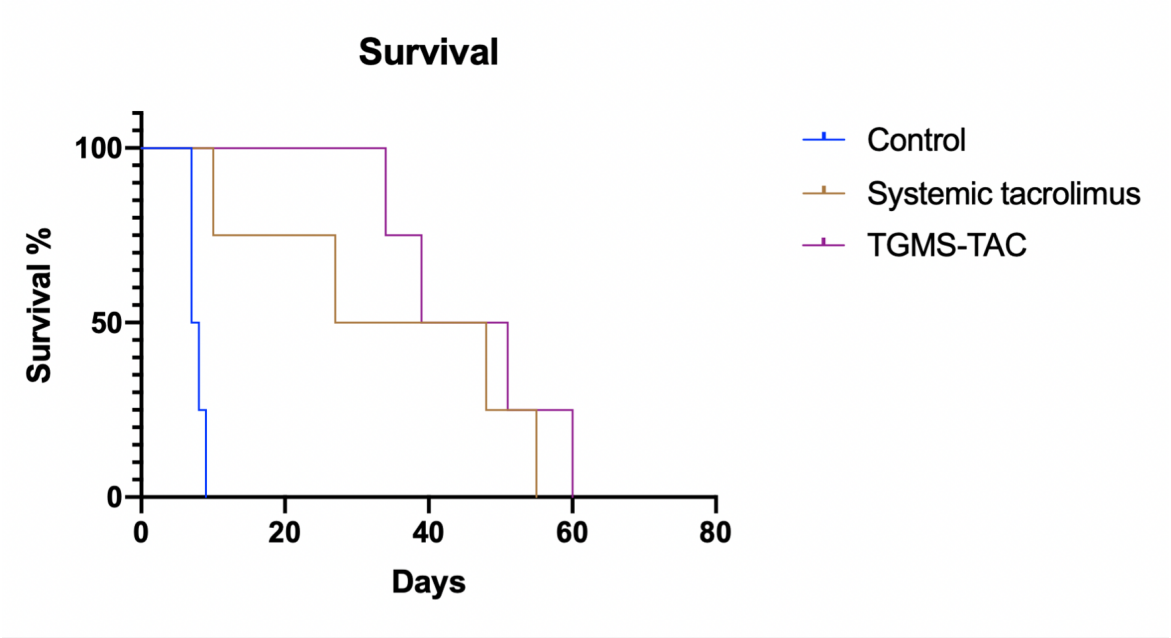

Figure 9 Survival staircase graph of the treatment groups.

One can see the course of deaths in the 3 different treatment groups during a period (Fig 9). Each death in the corresponding group is shown as e drop in survival. In this case, the survival of the pigs serves as the first of several parameters for the comparison between the systemic administration of the drug and the localized immunosuppressive therapy using the TGMS-TAC hydrogel. The control group shows a fast-successive drop of the curve with the last euthanasia on day 9. The systemic group also initially falls more in comparison to the TGMS-TAC group, although it recovers from day 40 and both curves are largely the identical. For the systemic group the last euthanasia is day 55 and for the TGMS-TAC day 60. The respective MST values are 7.75 days for the control group, 36 days for the systemic group and 46 days for the TGMS-TAC group. Of course, these data are not sufficient for a complete conclusion, but in the end, they should be put together with all dependent results to allow a scientific evaluation.

10.2 TACROLIMUS SKIN AND BLOOD LEVELS

The next parameter to add for the evaluation, which results and has been measured, are the Tacrolimus levels in skin and blood in the TGMS-TAC group (Fig 10 and 11). One of the specific aims of the project deals with the systemic exposure of

drugs during immunosuppressive therapy. In both presentations, the increase in the first week after the transplantation and the peak values of 80 ng/g in skin and 55 ng/ml in blood at POD7 is well recognizable. In addition, all pigs in the group show a similar course and a significant decrease in the values after POD7.

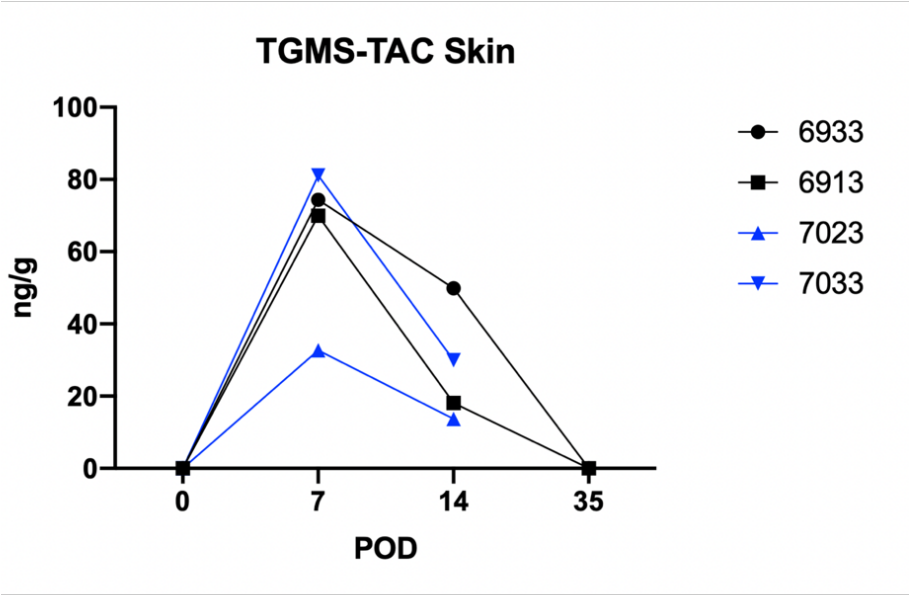

Figure 10 Tacrolimus skin levels (ng/g) of the TMGS-TAC group at different postoperative days (POD).

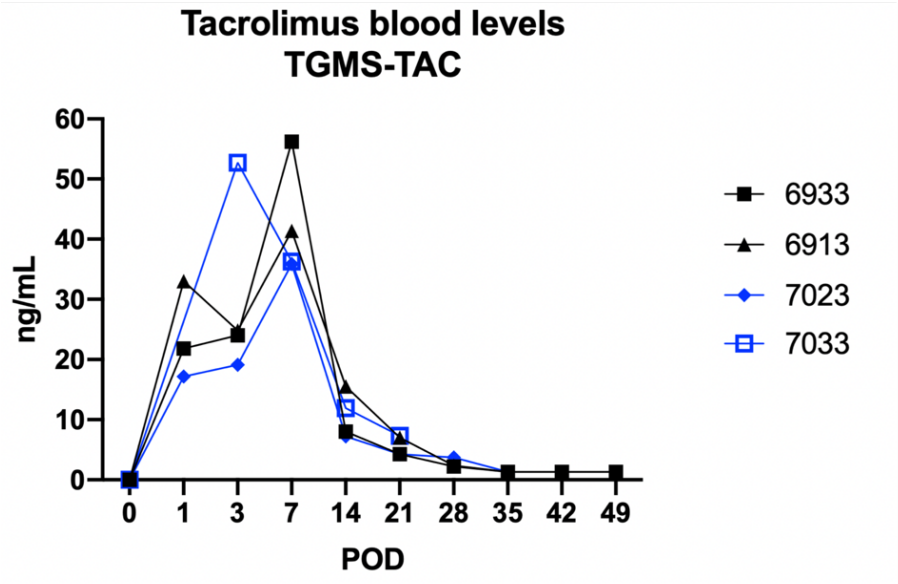

Figure 11 Tacrolimus blood levels (ng/ml) of the TGMS-TAC group at different postoperative days (POD).

Also related to the transplant, the Tacrolimus skin values of both groups for both sides are highest at POD7 (Fig 12). The reduction of local levels after POD7 remains the same as for blood and total skin concentrations. The Tacrolimus skin values of the graft shows a clear maximum value of 64.5% at POD7 during the

TGMS-TAC treatment compared to the contralateral side, which is 20%. The values in the systemic group are very similar for the graft and contralateral side with 13.5% and 10%.

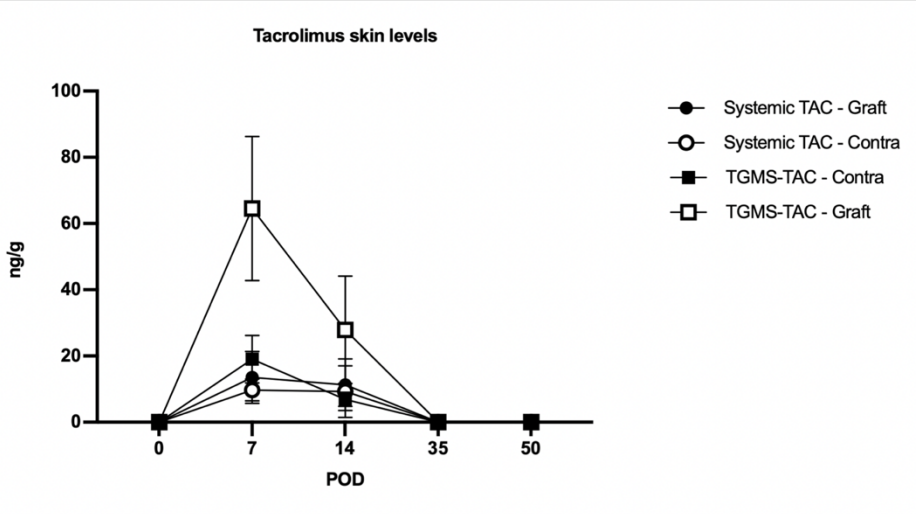

Figure 12 Tacrolimus skin levels of the systemic and TGMS-TAC groups of the graft and the contralateral side in comparison.

10.3 FACS OUTCOME – PBMC DYNAMICS

The last parameters represent the PBMC's occurrence and their dynamics during therapy. With the defined gating strategy, the cells were subordinated (Fig 8) and comparison of the immune response of the different treatment groups is possible. In FACS 250'000 cells per sample were used, except for the control group on POD0 where 2'500'000 cells were taken. Thus, this number always represents the total population and the percentages always refer to the total cell population that was examined.

In the control group, only a display up to POD7 was possible because, as already shown in the survival results, the pigs had to be euthanized between POD7 and POD9. These groups show a small increase in CD4+ cells and an equally small decrease in CD8+ cells compared to POD0 and POD7. This results in the total increase of T cells by 1.25% (Fig 13).

The systemic group first shows a decrease of 10% in T cells, whereby the stock then recovers from week to week and there is again an increase in T cells (Fig 14). The tendency of the CD4+ and CD8+ cells is the opposite of the control group. The $CD4^+$ cells experience a decrease of 9% while the $CD8^+$ cells increase by the same amount.

The TMGS-TAC group resembles the systemic group to a certain extent (Fig 15). The lymphocytes of both groups move in a bell-shaped curve. Again, there is a decrease in T cells after the first week and the relatively lighter recovery of the population up to POD28. In addition, there is approximately the same movement of CD4+ and CD8+ cells compared to the systemic group with a deviation of 1%.

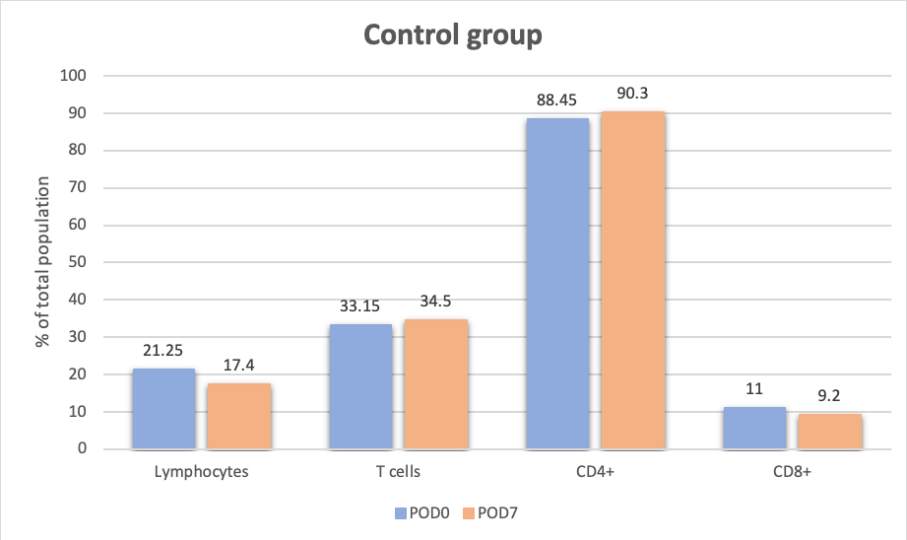

Figure 13 FACS outcome of different cell populations of control group represented by pig 6484 and pig 6587.

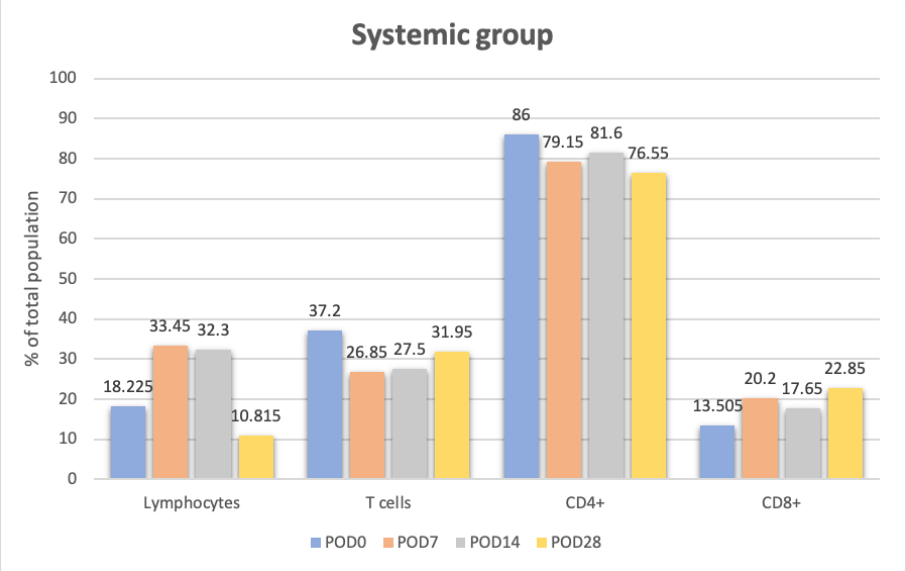

Figure 14 FACS outcomes of different cell populations of systemic group represented by pig 6916 and pig 6918.

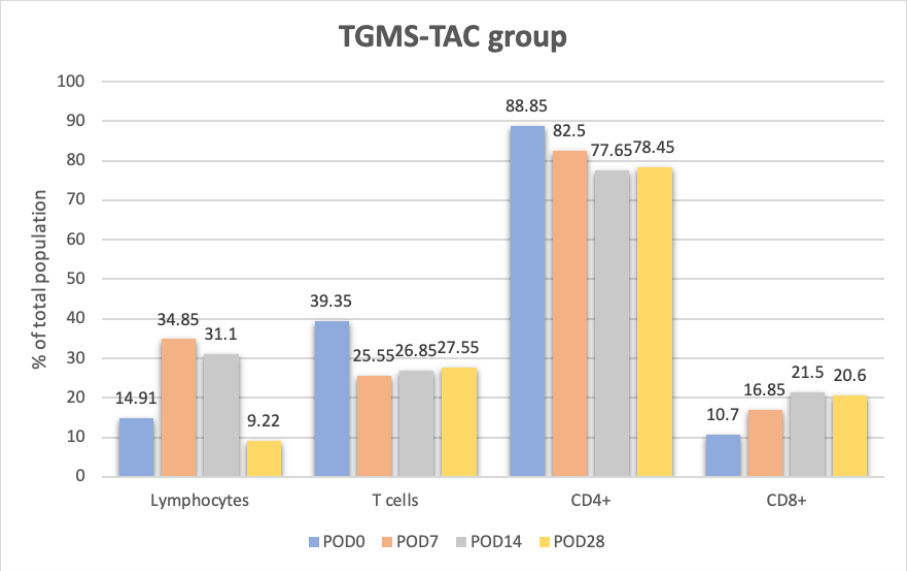

Figure 15 FACS outcomes of different cell populations of TGMS-TAC group represented by pig 6913 and pig 6933.

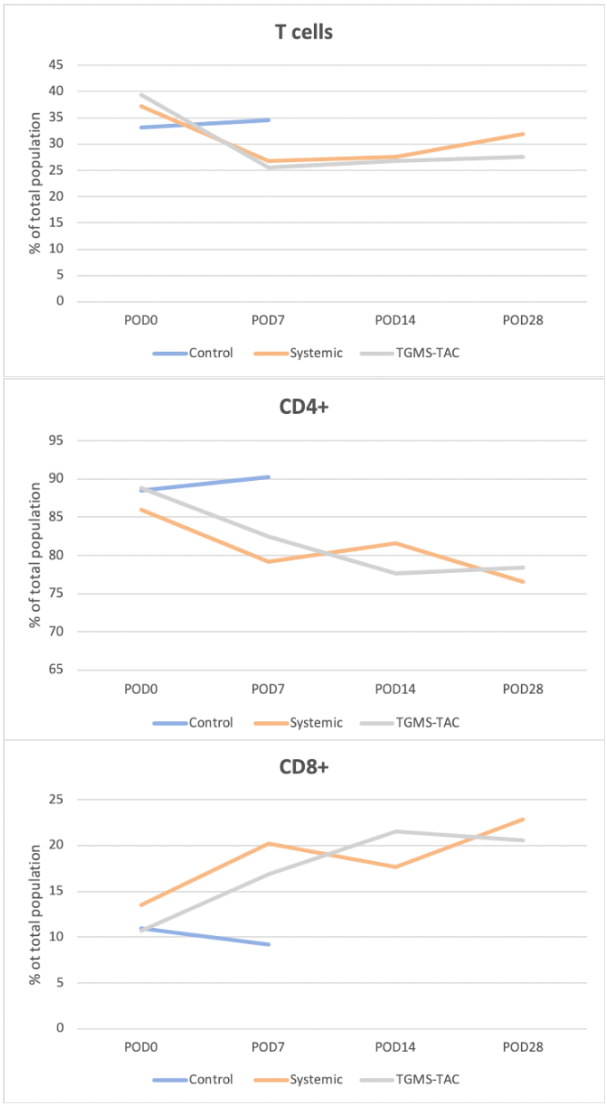

Figure 16 The dynamics of T cells and the subpopulations $CD4^+$ and $CD8^+$ in relation to the treatment group.

The tendency is additionally recognizable by the dynamics of the individual PBMC cell types (Fig 16). For example, the ratio of T cells in the TGMS-TAC group is 12% less, despite the increase from POD7. It can also be seen that the individual cell types between the systemic and TGMS-TAC group differ by ratio up to 4%. The dynamics of the individual cell populations correlate with the results of the amount of cell populations on the different days and also serve as a confirmation.

11 DISCUSSION

Currently, the adverse side effects of immunosuppression are a major concern of transplantation research and medicine. In VCA as a non-life-saving transplantation, the use of immunosuppressive drugs is the main factor preventing its widespread use. Therefore, there is a demand for an innovative strategy that reduces the side effects through decreased systemic exposure and at the same time prevents rejection reactions. In our project we want to show that an injection of TGMS-TAC into the transplant allows long-term survival of the VCA without inducing systemic toxicity. With a relevant porcine model, we also want to minimize the known side effects and improve patient compliance. Self-assembling hydrogels as drug delivery system are especially promising, because their properties allow the encapsulation of large amounts of drugs. Additionally, self-assembling hydrogels are biodegradable and provide sustained release and flexibility to develop an inflammation-responsive release by proteolytic enzymes.

Considering only the survival outcome (Fig 9), it can be argued that the TGMS-TAC injection group has an advantage over the systemic treatments, as this group has a higher MST value. As already indicated, however, it is not possible to draw a conclusion based on one parameter before the results are put together and analyzed as a whole. The Tacrolimus skin and blood levels (Fig 10 and 11) show a linear increase at first, which is possibly connected to the operation and the unavoidable immune response and thus a burst release of Tacrolimus can occur. The stress factors of transport, surgery and the unknown environment, to which the pigs are exposed, are not to be neglected, as they can contribute additionally to an inflammatory response. After one week the pigs recover in such a way that the concentrations of Tacrolimus in the skin and in the blood decrease significantly. This can be seen as a very positive result, as this is exactly what the TGMS-TAC Hydrogel was designed to achieve. It is a good sign for the minimization of the systemic strain and possible side effects. The mentioned results of TAC levels are additionally supported by the measurement of the Tacrolimus values in the graft and contralateral side of group II and III (Fig 12). The peak values of all groups in POD7 are related to previous results and the cause of the initial burst release of the drug. The subsequent reduction in concentrations also points to this explanation. It is clearly visible that the Tacrolimus concentration is relatively high only in the graft of pigs treated

with TGMS-TAC. Since the concentration of the contralateral side differs only by a small difference of 3.5% with the systmic group, it can be concluded that the TGMS-TAC hydrogels release Tacrolimus mainly at the graft. This corresponds to the expectations, because the DDS was intended to minimize the systemic burden and to achieve a high release locally where it is needed.

The control group showed an increase in $CD3^+$ and $CD4^+$ cells after the first week (Fig 13). Since this group did not receive any treatment, the rejection after one week is as expected in connection with the observed cell movement. One strongly assumes here a cell-mediated rejection, which is associated with an increase in $CD4^+$ cells. Whereas, the systemic Tacrolimus group first experiences a decrease of T cells which is connected with the administration of Tacrolimus (Fig 14). Therefore, it is understandable that the amount of $CD4^+$ cells decreases as well. However, it is unknown why the T cell population grows after the initial decrease originating from the treatment with Tacrolimus. This could be explained by a regular occurrence of rejection episodes, causing an increase in T cells. Yet, this explanation is controversial, as the administration of the immunosuppressive Tacrolimus should prevent exactly the increase of T cells. Theoretically, an infection could also boost the immune system and thus the body, despite immunosuppression, could be more on the side of ensuring immunity rather than suppression. In any case, these results show that it is impossible to predict how individual pigs will react to the treatments. This statement is confirmed by the survival results, because even if the treatment of the systemic group is discontinued after 14 days, one pig unexpectedly reached POD55 until the rejection reaction became overly strong and it had to be euthanized. One would expect that the animal would show strong signs of rejection a few days later. In the TGMS-TAC treated pigs, a reduction of T cells and the subpopulation $CD4^+$ is found (Fig 15). This is certainly due to the effect of the TGMS-TAC hydrogel. Just as before with the Tacrolimus treated porcine, there is no explanation why there is an increase in T cells and their subpopulations, except for the theoretical possibilities already mentioned for the systemic group. Nevertheless, the survival target of 90 days was clearly not reached, with the longest survival time being 60 days. One assumption is that the dosage of the hydrogel was not yet strong enough. As the dosage still ranges from 49 mg to 93 mg per 500g, it needs to be further investigated in further stages of the project. It is also significant that the pigs of both groups,

systemic and TGMS-TAC, have survived for a similar length of time with an advantage for those treated with TGMS-TAC. The difference in T cell dynamics of 1% could be an explanation for this result and thus could be related (Fig 16). With the combined results, it is not possible to show that TGMS-TAC prevents rejection reactions because the pig did not survive as long as expected. This is despite the fact that MST values as well as Tacrolimus concentrations in blood and skin provide an advantage over systemic treatment and stand for a promising functionality of TGMS-TAC. In rat models the strategy has already proven to be successful and has shown good results, thereby displaying the possibility that it can work. However, before moving on to clinical trials, it is necessary to reproduce similar results in larger animals such as porcine. The TGMS-TAC strategy is one of several options that still need to be improved in preclinical studies. Therefore, I expect to see combined strategies in the future that will improve the field of VCA and enable its widespread application.

12 REFERENCES

1. Shores JT, Malek V, Lee WPA, Brandacher G. Outcomes after hand and upper extremity transplantation. *J Mater Sci: Mater Med*. 2017;28(5):72. doi:10.1007/s10856-017-5880-0

2. Pomahac B, Gobble RM, Schneeberger S. Facial and Hand Allotransplantation. *Cold Spring Harbor Perspectives in Medicine*. 2014;4(3):a015651-a015651. doi:10.1101/cshperspect.a015651

3. Gordon CR, Nazzal J, Lozano-Calderan SA, et al. From experimental rat hindlimb to clinical face composite tissue allotransplantation: Historical background and current status. *Microsurgery*. 2006;26(8):566-572. doi:10.1002/micr.20296

4. Calne RY, Rolles K, Thiru S, et al. CYCLOSPORIN A INITIALLY AS THE ONLY IMMUNOSUPPRESSANT IN 34 RECIPIENTS OF CADAVERIC ORGANS: 32 KIDNEYS, 2 PANCREASES, AND 2 LIVERS. *The Lancet*. 1979;314(8151):1033-1036. doi:10.1016/S0140-6736(79)92440-1

5. Schneeberger S, Morelon E, Landin L. Vascularized Composite Allotransplantation: A Member of the Transplant Family? *Transplantation Journal*. 2012;93(11):1088-1091. doi:10.1097/TP.0b013e318254ad8a

6. Tuffaha SH, Cooney DS, Sopko NA, et al. Penile transplantation: an emerging option for genitourinary reconstruction. *Transpl Int*. 2017;30(5):441-450. doi:10.1111/tri.12928

7. Brännström M, Diaz-Garcia C, Johannesson L, Dahm-Kähler P, Bokström H. Livebirth after uterus transplantation – Authors' reply. *The Lancet*. 2015;385(9985):2352-2353. doi:10.1016/S0140-6736(15)61098-4

8. Thuong M, Petruzzo P, Landin L, et al. Vascularized composite allotransplantation - a Council of Europe position paper. *Transpl Int*. 2018;32(3):233-240. doi:10.1111/tri.13370

9. Shores JT, Brandacher G, Lee WPA. Hand and Upper Extremity Transplantation: An Update of Outcomes in the Worldwide Experience. *Plastic and Reconstructive Surgery*. 2015;135(2):351e-360e. doi:10.1097/PRS.0000000000000892

10. Bonastre J, Landin L, Diez J, Casado-Sanchez C, Casado-Perez C. Factors Influencing Acute Rejection of Human Hand Allografts: A Systematic Review. *Annals of Plastic Surgery*. 2012;68(6):624-629. doi:10.1097/SAP.0b013e318255a411

11. Carey BS, Poulton KV, Poles A. Factors affecting HLA expression: A review. *Int J Immunogenet*. 2019;46(5):307-320. doi:10.1111/iji.12443

12. Brandacher G. Vascularized composite allotransplantation: a field is maturing. Zachary AA, Leffell MS, eds. Published online October 2018. doi:10.1007/978-1-62703-493-7_5

13. Vajdová K, Graf R, Clavien P-A. ATP-supplies in the cold-preserved liver: A long-neglected factor of organ viability. *Hepatology*. 2002;36(6):1543-1552. doi:10.1002/hep.1840360633

14. Lanir A, Jenkins RL, Caldwell C, Lee RGL, Khettry U, Clouse ME. Hepatic transplantation survival: Correlation with adenine nucleotide level in donor liver. *Hepatology*. 1988;8(3):471-475. doi:10.1002/hep.1840080306

15. Burlage LC, Tessier SN, Etra JW, Uygun K, Brandacher G. Advances in machine perfusion, organ preservation, and cryobiology: potential impact on vascularized composite allotransplantation. *Current Opinion in Organ Transplantation*. Published online August 2018:1. doi:10.1097/MOT.0000000000000567

16. Etra JW, Raimondi G, Brandacher G. Mechanisms of rejection in vascular composite allotransplantation: *Current Opinion in Organ Transplantation*. 2018;23(1):28-33. doi:10.1097/MOT.0000000000000490

17. Diaz-Siso JR, Sosin M, Plana NM, Rodriguez ED. Face transplantation: Complications, implications, and an update for the oncologic surgeon: Face Transplantation. *J Surg Oncol*. 2016;113(8):971-975. doi:10.1002/jso.24211

18. Chandraker A, Arscott R, Murphy GF, et al. The Management of Antibody-Mediated Rejection in the First Presensitized Recipient of a Full-Face Allotransplant: AMR in Face Allotransplantation. *American Journal of Transplantation*. 2014;14(6):1446-1452. doi:10.1111/ajt.12715

19. Wilks DJ, Clark B, Kay SPJ. The histocompatibility and immunogenetics of hand transplantation. *Int J Immunogenet*. 2019;47(1):24-27. doi:10.1111/iji.12469

20. Landin L, Bonastre J, Casado-Sanchez C, et al. Outcomes with respect to disabilities of the upper limb after hand allograft transplantation: a systematic review. *Transplant International*. 2012;25(4):424-432. doi:10.1111/j.1432-2277.2012.01433.x

21. Gaynor JJ, Ciancio G, Guerra G, et al. Lower tacrolimus trough levels are associated with subsequently higher acute rejection risk during the first 12 months

after kidney transplantation. *Transpl Int*. 2016;29(2):216-226. doi:10.1111/tri.12699

22. O'Regan JA, Canney M, Connaughton DM, et al. Tacrolimus trough-level variability predicts long-term allograft survival following kidney transplantation. *J Nephrol*. 2016;29(2):269-276. doi:10.1007/s40620-015-0230-0

23. Wang S, Xiong Y, Wang Y, Chen J, Yang J, Sun B. Evaluation of PLGA microspheres with triple regimen on long-term survival of vascularized composite allograft – an experimental study. *Transpl Int*. 2020;33(4):450-461. doi:10.1111/tri.13574

24. Petruzzo P, Lanzetta M, Dubernard J-M, et al. The International Registry on Hand and Composite Tissue Transplantation: *Transplantation*. 2010;90(12):1590-1594. doi:10.1097/TP.0b013e3181ff1472

25. 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(8):1250-1252. doi:10.1097/TP.0000000000002207

26. Schneeberger S, Lucchina S, Lanzetta M, et al. Cytomegalovirus-Related Complications in Human Hand Transplantation: *Transplantation*. 2005;80(4):441-447. doi:10.1097/01.tp.0000168454.68139.0a

27. Solari MG, Washington KM, Sacks JM, et al. Daily Topical Tacrolimus Therapy Prevents Skin Rejection in a Rodent Hind Limb Allograft Model: *Plastic and Reconstructive Surgery*. 2009;123(Supplement):17S-25S. doi:10.1097/PRS.0b013e318191bcbd

28. Gharb BB, Rampazzo A, Altuntas SH, et al. Effectiveness of Topical Immunosuppressants in Prevention and Treatment of Rejection in Face Allotransplantation: *Transplantation Journal*. 2013;95(10):1197-1203. doi:10.1097/TP.0b013e31828bca61

29. Taddeo A, Tsai C, Vögelin E, Rieben R. Novel targeted drug delivery systems to minimize systemic immunosuppression in vascularized composite allotransplantation: *Current Opinion in Organ Transplantation*. Published online August 2018:1. doi:10.1097/MOT.0000000000000564

30. Howsare M, Jones CM, Ramirez AM. Immunosuppression maintenance in vascularized composite allotransplantation: what is just right? *Current Opinion in Organ Transplantation*. 2017;22(5):463-469. doi:10.1097/MOT.0000000000000456

31. Black KS, Nguyen DK, Proctor CM, Patel MP, Hewitt CW. Site-Specific

Suppression of Cell-Mediated Immunity By Cyclosporine. *Journal of Investigative Dermatology*. 1990;94(5):644-648. doi:10.1111/1523-1747.ep12876218

32. Unadkat JV, Schnider JT, Feturi FG, et al. Single Implantable FK506 Disk Prevents Rejection in Vascularized Composite Allotransplantation: *Plastic and Reconstructive Surgery*. 2017;139(2):403e-414e. doi:10.1097/PRS.0000000000002951

33. Feturi FG, Weinstock M, Zhao W, et al. Mycophenolic Acid for Topical Immunosuppression in Vascularized Composite Allotransplantation: Optimizing Formulation and Preliminary Evaluation of Bioavailability and Pharmacokinetics. *Front Surg*. 2018;5:20. doi:10.3389/fsurg.2018.00020

34. 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. *Science Translational Medicine*. 2014;6(249):249ra110-249ra110. doi:10.1126/scitranslmed.3008778

35. Dzhonova DV, Olariu R, Leckenby J, et al. Local Injections of Tacrolimus-loaded Hydrogel Reduce Systemic Immunosuppression-related Toxicity in Vascularized Composite Allotransplantation: *Transplantation*. 2018;102(10):1684-1694. doi:10.1097/TP.0000000000002283

36. Tchetverikov I. MMP profile in paired serum and synovial fluid samples of patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*. 2004;63(7):881-883. doi:10.1136/ard.2003.013243

37. Vemula PK, Cruikshank GA, Karp JM, John G. Self-assembled prodrugs: An enzymatically triggered drug-delivery platform. *Biomaterials*. 2009;30(3):383-393. doi:10.1016/j.biomaterials.2008.09.045

38. Hoyos IA, Yerly A, Sorvillo N, et al. Development of a surgical model for site-specific immunosuppression in vascularized composite allotransplantation. :1.

39. Maeda S, Sawai T, Uzuki M, et al. Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*. 1995;54(12):970-975. doi:10.1136/ard.54.12.970

40. Dzhonova D, Olariu R, Leckenby J, et al. Local release of tacrolimus from hydrogel-based drug delivery system is controlled by inflammatory enzymes in vivo and can be monitored non-invasively using in vivo imaging. Upendran A, ed. *PLoS ONE*. 2018;13(8):e0203409. doi:10.1371/journal.pone.0203409

41. Olariu R, Denoyelle J, Leclère FM, et al. Intra-graft injection of tacrolimus promotes survival of vascularized composite allotransplantation. *Journal of Surgical Research*. 2017;218:49-57. doi:10.1016/j.jss.2017.05.046

42. International Registry on Hand and Composite Tissue Transplantation. Published May 2020. https://www.handregistry.com

43. Barone AAL, Sun Z, Montgomery RA, Lee WA, Brandacher G. Impact of donor-specific antibodies in reconstructive transplantation. *Expert Review of Clinical Immunology*. 2013;9(9):835-844. doi:10.1586/1744666X.2013.824667

44. Heemann U, Lutz J. Pathophysiology and treatment options of chronic renal allograft damage. *Nephrology Dialysis Transplantation*. 2013;28(10):2438-2446. doi:10.1093/ndt/gft087

45. Win TS, Murakami N, Borges TJ, et al. Longitudinal immunological characterization of the first presensitized recipient of a face transplant. *JCI Insight*. 2017;2(13):e93894. doi:10.1172/jci.insight.93894

46. Clark RA, Chong B, Mirchandani N, et al. The Vast Majority of CLA $^{+}$ T Cells Are Resident in Normal Skin. *J Immunol*. 2006;176(7):4431-4439. doi:10.4049/jimmunol.176.7.4431

47. Fischer S, Lian CG, Kueckelhaus M, et al. Acute rejection in vascularized composite allotransplantation: *Current Opinion in Organ Transplantation*. 2014;19(6):531-544. doi:10.1097/MOT.0000000000000140

48. Cooney WP, Hentz VR. Hand transplantation—primum non nocere. *The Journal of Hand Surgery*. 2002;27(1):165-168. doi:10.1053/jhsu.2002.30367

49. Petruzzo P, Kanitakis J, Testelin S, et al. Clinicopathological Findings of Chronic Rejection in a Face Grafted Patient: *Transplantation*. 2015;99(12):2644-2650. doi:10.1097/TP.0000000000000765

50. Jablecki J, Kaczmarzyk L, Patrzałek D, Chelmoński A, Domanasiewicz A. Mild Rejection Episode Evoked by Modification of Immunosuppressive Protocol Observed in a Patient With Forearm Transplantation: Case Report. *Transplantation Proceedings*. 2009;41(2):557-559. doi:10.1016/j.transproceed.2009.01.007

51. Gasteiger G, Rudensky AY. Interactions between innate and adaptive lymphocytes. *Nat Rev Immunol*. 2014;14(9):631-639. doi:10.1038/nri3726

52. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *The Journal of Experimental Medicine*.

1983;157(1):202-218. doi:10.1084/jem.157.1.202

53. Zhou C-X, Zhou D-H, Liu G-X, Suo X, Zhu X-Q. Transcriptomic analysis of porcine PBMCs infected with Toxoplasma gondii RH strain. *Acta Tropica*. 2016;154:82-88. doi:10.1016/j.actatropica.2015.11.009

54. Wood KJ, Goto R. Mechanisms of Rejection: Current Perspectives: *Transplantation*. 2012;93(1):1-10. doi:10.1097/TP.0b013e31823cab44

55. Pober JS, Min W, Bradley JR. Mechanisms of Endothelial Dysfunction, Injury, and Death. *Annu Rev Pathol Mech Dis*. 2009;4(1):71-95. doi:10.1146/annurev.pathol.4.110807.092155

56. Black APB, Ardern-Jones MR, Kasprowicz V, et al. Human keratinocyte induction of rapid effector function in antigen-specific memory CD4+ and CD8+ T cells. *Eur J Immunol*. 2007;37(6):1485-1493. doi:10.1002/eji.200636915

57. Cavani CA andrea, Girolomoni G. Interferon-γ-Stimulated Human Keratinocytes Express the Genes Necessary for the Production of Peptide-Loaded MHC Class II Molecules. *Journal of Investigative Dermatology*. 1998;110(2):138-142. doi:10.1046/j.1523-1747.1998.00098.x

58. 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. doi:10.1111/ajt.14218

59. Matzinger P. The Danger Model: A Renewed Sense of Self. *Science*. 2002;296(5566):301-305. doi:10.1126/science.1071059

60. Larsen CP, Austyn JM, Morris PJ. The Role of Graft-derived Dendritic Leukocytes in the Rejection of Vascularized Organ Allografts: Recent Findings on the Migration and Function of Dendritic Leukocytes After Transplantation. *Annals of Surgery*. 1990;212(3):308-317. doi:10.1097/00000658-199009000-00009

61. Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. *The Journal of Experimental Medicine*. 1990;172(5):1483-1493. doi:10.1084/jem.172.5.1483

62. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *The Journal of Experimental Medicine*. 1990;171(1):307-314. doi:10.1084/jem.171.1.307

63. Oppenheim JJ. Cytokines: Past, Present, and Future. *Int J Hematol*. 2001;74(1):3-8. doi:10.1007/BF02982543

64. O'Sullivan RL, Lipper G, Lerner EA. The Neuro-Immuno-Cutaneous-Endocrine Network: Relationship of Mind and Skin. *Arch Dermatol*. 1998;134(11). doi:10.1001/archderm.134.11.1431

65. Eming SA, Krieg T, Davidson JM. Inflammation in Wound Repair: Molecular and Cellular Mechanisms. *Journal of Investigative Dermatology*. 2007;127(3):514-525. doi:10.1038/sj.jid.5700701

66. Friedman O, Carmel N, Sela M, et al. Immunological and inflammatory mapping of vascularized composite allograft rejection processes in a rat model. Palaniyandi S, ed. *PLoS ONE*. 2017;12(7):e0181507. doi:10.1371/journal.pone.0181507

67. Issa F. Vascularized composite allograft-specific characteristics of immune responses. *Transpl Int*. 2016;29(6):672-681. doi:10.1111/tri.12765

68. Pober JS, Orosz CG, Rose ML, Savage COS. CAN GRAFT ENDOTHELIAL CELLS INITIATE A HOST ANTI-GRAFT IMMUNE RESPONSE?1: *Transplantation*. 1996;61(3):343-349. doi:10.1097/00007890-199602150-00001

69. Hautz T, Zelger BG, Nasr IW, et al. Lymphoid neogenesis in skin of human hand, nonhuman primate, and rat vascularized composite allografts. *Transpl Int*. 2014;27(9):966-976. doi:10.1111/tri.12358

70. Shklovskaya E, O'Sullivan BJ, Ng LG, et al. Langerhans cells are precommitted to immune tolerance induction. *Proceedings of the National Academy of Sciences*. 2011;108(44):18049-18054. doi:10.1073/pnas.1110076108

71. 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(6):788-799. doi:10.1038/modpathol.2013.249

72. Clark RA. Skin-Resident T Cells: The Ups and Downs of On Site Immunity. *Journal of Investigative Dermatology*. 2010;130(2):362-370. doi:10.1038/jid.2009.247

73. Hettiaratchy S, Melendy E, Randolph MA, et al. Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine1: *Transplantation*. 2004;77(4):514-521. doi:10.1097/01.TP.0000113806.52063.42

74. Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The Majority of Human Peripheral Blood CD4 $^{+}$ CD25 ^{high} Foxp3 $^{+}$ Regulatory T Cells Bear Functional Skin-Homing Receptors. *J Immunol*. 2006;177(7):4488-4494. doi:10.4049/jimmunol.177.7.4488

75. Tacrolimus-2D-skeletal.

Published May 2020. https://upload.wikimedia.org/wikipedia/commons/0/04/Tacrolimus-2D-skeletal.png

76. Schreiber SL, Crabtree GR. The mechanismof action of cyclosporinA and FK506. Published online 1992:136-141.

77. Harding MW, Galat A, Uehling DE, Schreiber SL. A receptor for the immuno-suppressant FK506 is a cis–trans peptidyl-prolyl isomerase. *Nature*. 1989;341(6244):758-760. doi:10.1038/341758a0

78. Siekierka JJ, Hung SHY, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature*. 1989;341(6244):755-757. doi:10.1038/341755a0

79. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 1991;66(4):807-815. doi:10.1016/0092-8674(91)90124-H

80. Dumont F. FK506, An Immunosuppressant Targeting Calcineurin Function. *CMC*. 2000;7(7):731-748. doi:10.2174/0929867003374723

81. Bennett J, Cassidy H, Slattery C, Ryan M, McMorrow T. Tacrolimus Modulates TGF-β Signaling to Induce Epithelial-Mesenchymal Transition in Human Renal Proximal Tubule Epithelial Cells. *JCM*. 2016;5(5):50. doi:10.3390/jcm5050050

82. Flanagan WM, Corthésy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature*. 1991;352(6338):803-807. doi:10.1038/352803a0

83. Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SW15. *Cell*. 1991;66(4):743-758. doi:10.1016/0092-8674(91)90118-I

84. 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. Stepkowski S, ed. *PLoS ONE*. 2019;14(1):e0210914. doi:10.1371/journal.pone.0210914

85. Mezrich JD, Haller GW, Arn JS, Houser SL, Madsen JC, Sachs DH. Histocompatible miniature swine: an inbred large-animal model1: *Transplantation*. 2003;75(6):904-907. doi:10.1097/01.TP.0000054839.43852.BF

86. Ibrahim Z, Cooney DS, Shores JT, et al. A Modified Heterotopic Swine

Hind Limb Transplant Model for Translational Vascularized Composite Allotransplantation (VCA) Research. *JoVE*. 2013;(80):50475. doi:10.3791/50475

87. Ustüner ET, Zdichavsky M, Ren X, et al. LONG-TERM COMPOSITE TISSUE ALLOGRAFT SURVIVAL IN A PORCINE MODEL WITH CYCLOSPORINE/MYCOPHENOLATE MOFETIL THERAPY. *Transplantation*. 1998;66(12):1581-1587. doi:10.1097/00007890-199812270-00003

88. Chuang M, Orvieto M, Laven B, et al. Comparison of External Catheters With Subcutaneous Vascular Access Ports for Chronic Vascular Access in a Porcine Model. Published online March 2005:44(2):24-7.

89. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 Working Classification of Skin-Containing Composite Tissue Allograft Pathology: Banff CTA Allograft Pathology Classification. *American Journal of Transplantation*. 2008;8(7):1396-1400. doi:10.1111/j.1600-6143.2008.02243.x

90. Peripheral Blood Mononuclear Cells. Flow Contract Site Laboratory, Personalized Flow Cytometry Service. https://fcslaboratory.com/peripheral-blood-mononuclear-cells-pbmc/

13 DECLARATION OF CONSENT

Declaration of consent

on the basis of Article 30 of the RSL Phil.-nat. 18

Name/First Name:	Krasniqi Arber
Registration Number:	17-117-896
Study program:	Bachelor in Biology, Specialisation in Cell Biology
	Bachelor ☑ Master ☐ Dissertation ☐
Title of the thesis:	LOCAL IMMUNOSUPPRESSION FOR LONG-TERM MAINTENANCE OF VASCULARIZED COMPOSITE ALLOTRANSPLANTATION The evaluation of a drug delivery system in a clinically relevant large animal model
Supervisor:	Prof. Dr. Robert Rieben, Department of Biomedical Research Isabel Arenas Hoyos, PhD Student

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