University of Bern
Molecular Life Sciences

“Local immunosuppression using self-assembled hydrogel drug delivery system in vascularized composite allotransplantation and its effect on rejection in a swine model.”

Master Thesis submitted by

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Abstract

Background:

Vascularised composite allotransplantation (VCA) is a new field in transplantation aiming to restore severe tissue loss that are not amenable to conventional treatment. The major limits in a wider spread of clinical application of VCA are the need for a high-dose and lifelong immunosuppression treatment which is associated with numerous side effects, and graft rejection following allotransplantation. To avoid graft rejection by having a therapeutic sufficient local concentration of the drug while greatly reduce the systemic off-target toxicity, a drug delivery system (DDS) using a triglycerol monostearate [TGMS] gel loaded with tacrolimus (TAC) called TGMS-TAC, was developed. TGMS-TAC was tested both *in vitro* and *in vivo* using an experimental rodent model. It was shown that this DDS was very promising to promote graft survival with minimum off-target toxicity. **Aims:** In this study we aimed to 1) test the efficacy and 2) safety of the TGMS-TAC system in a clinically relevant large animal model, the swine; 3) elucidate differential immunological response leading to rejection following VCA transplantation in untreated vs tacrolimus treated patients. **Methods:** Outbred pigs underwent heterotopic hind limb transplantation and were randomly divided into three groups: control group, which were untreated, systemic group where the animals received daily oral administration of 1mg/kg of tacrolimus for 14 days, and TGMS-TAC group where they received a single subcutaneous injection of 140mg of tacrolimus per kg of graft at post-operative day (POD) 0. After transplantation, the pigs were monitored, and blood and tissue samples were collected at different timepoints throughout the follow-up. The endpoint of the experiment was set once the graft reached grade III rejection or survived up to POD90. Graft survival and off-target toxicity, cytokine secretion and related immune cell responses in the graft and in the periphery were evaluated. **Results:** Intra-graft injection of tacrolimus prolonged graft survival with a mean survival time (MST) of 46 days compared with the untreated animals which had an MST of 7,5 days (p=0,0062). With the TGMS-TAC treatment, the drug concentration was significantly higher in the graft when compared to its contralateral side (p=0,0001) and compared to the graft from the systemic group (p=0,0001). No off-target toxicity was observed in any experimental group. In the TGMS-TAC group, the proinflammatory cytokine IL-1β and Th2 related cytokine IL-4 were upregulated at endpoint, and there was increased number of B cells in the skin compared to healthy tissue. **Conclusion:** In the current study we validated the efficacy and safety of the TGMS-TAC system in a swine model of VCA which did improve graft survival while causing no off-target toxicity. The exact immunological mechanism leading to rejection in the untreated vs tacrolimus treated graft needs to be further studied.

1 Introduction

1.1 Vascularized composite allotransplantation

Vascularized composite allotransplantation (VCA) such as hand or face is a unique kind of transplantation. In contrast to the more common solid organ transplantation where the tissues of the graft are more or less homologous, VCA contains multiple tissues components such as skin, bones, muscles, nerves, vessels, and lymphatics system (Kaufman et al., 2016). VCA is a promising approach to restore extensive tissue loss of a non-vital body part which cannot be repaired with more conventional surgical techniques. The first successful hand allotransplantation was performed in 1998 in Lyon, France and the first partial face transplantation in 2005 in Amiens, France (Nassimizadeh et al., 2014; Uluer et al., 2016). It opened the door to a new area of reconstructive surgery and since then VCA has receive increasing interest. Up to date, more than 120 hand upper-extremity and 37 face transplantation were performed worldwide (Thuong et al., 2019). This unique surgical procedure promotes both functional and esthetic recovery of the lost body part, limb, and improve greatly the quality of life of the patients (Uluer et al., 2016).

However, VCA is a “life enhancing” and not “life-saving” procedure. Due to the allogenic nature of allotransplantation, the field has been challenged with rejection episodes. Lifelong systemic immunosuppression therapy commitment is unavoidable, and its side effects are one of the biggest challenges in the field. The side effects include opportunistic infections, malignancy, off-target toxicity such as renal failure, metabolic disorders and can even lead to death (Uluer et al., 2016). In VCA, the conventional immunosuppressive therapy consists first of induction agents such as monoclonal antibody that target different surface molecule of T cells to prevent acute rejection. Then a triple-drug combination is administered daily for the rest of the patient’s life as maintenance therapy. The intake of this immunosuppressive cocktail includes corticosteroids, antiproliferative agent, and calcineurin inhibitors (Sarhane et al., 2013). Furthermore, near all VCA patients who received immunotherapy, 87.8% for hand and 72.7% for face transplantation, experienced acute rejection within the first-year post-transplantation and 13.4% developed chronic rejection or graft vasculopathy.

The cost and benefits of systemic immunosuppression must be considered carefully. Specially, when the majority of patients who underwent VCA are otherwise young and healthy (Uluer et al., 2016). New solutions are thus urgently needed in order to avoid the life-threatening risks from the immunosuppression treatment, and this would promote wider use of this restorative surgical technique. A different drug administration alternative is being evaluated being a site-specific drug delivery system which would overcome the issue of off-target toxicity while at the same time increasing the survival of the graft.

The TGMS-TAC is a hydrogel made of triglycerol monostearate (TGMS) that is loaded with the therapeutic drug tacrolimus (TAC). The TGMS-TAC allows a local on-demand release of the drug. The hydrogel is degraded in an inflammation-responsive manner and thus release the tacrolimus in the environment. This new DDS has been tested *in vitro* and then *in vivo* in a rodent model of VCA and showed very promising results in promotion of the graft survival with high efficacy of the on-demand release of the drug (Dzhonova et al., 2018). In rodents, the graft remained rejection-free for over 200 days without using additional immunosuppressive therapy (Olariu et al., 2017). Moreover, the animals showed no sign of off-target toxicity (Olariu et al., 2017). Based on these results, our aim was to test the clinical use of TGMS-TAC in a clinically relevant large animal model, the swine. We hypothesized that with one single ingestion of TGMS-TAC in the transplanted graft, it would allow a high concentration of the drug locally and thus prevent the systemic off-target toxicity. The immune response leading to acute rejection after VCA transplantation was investigated to better understand its underlying process.

1.2 Immunological aspects of VCA: Innate and adaptive immune responses

The immune system is essential for survival. It protects the body from many kinds of pathogens. It is a complex system with numerus cellular components that can communicate, interact, and influence one another. In VCA, the immune system is the most important factor that will determine if the graft will be tolerated or rejected. The growing success of VCA transplantation were made possible by a better understanding of the basic immunology and transplantation immunology such as the importance of major histocompatibility complex (leukocyte antigen), antigen presentation and the two signals needed for T or B cell activation. Here are explained some of the most important mechanisms of the immune response that impact the outcome of allotransplantation.

The immune system is separated in two categories: the innate immune system and the adaptive immune system. The innate immune system is the first line of defense against non-self-antigen and pathogen such as virus, bacteria, fungi, and many other pathogens. Important cells of the innate immune system are macrophages, neutrophils, dendritic cells and natural killer cells. Those cells can rapidly migrate to the site of antigen invasion and fight against foreigners. With the help of pattern recognition receptors (PRR) on their membrane they can recognize and bind to pathogens-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). This type of response is fast and unspecific, since the cells do not need to be specialized or primed prior of activation. Macrophage and dendritic cells also play a major role in initiating the adaptive immune response. Indeed, they are called antigen presenting cells (APCs) as they are able to take in and digest antigen in order to present them on their major histocompatibility complex (MHC), also called leukocyte antigen LA), on their surface. The APCs can then travel to the spleen or lymph nodes and present the

alloantigen to T and B lymphocytes which are cells of the adaptive immune system (Kaufman et al., 2016; Siri et al., 2012; Zhang & Bevan, 2011).

On the other hand, the adaptive immune system is highly specific and can build a memory which means that upon a second contact with previously encountered antigen, the response will be faster and stronger. The most important cells of the adaptive immune system consist of the T and B cells. T cells make up the cell-mediate immune response whereas B cells induce the humoral immune response.

In the thymus, progenitors of T cells undergo a strict selection. The first selection is the positive selection, where the T cells which recognized self-MHC molecule are selected, then the second is the negative selection in which only the T cell that can recognized the MHC molecule with low affinity will survive and the ones that bind with high affinity to the MHC are eliminated to avoid autoimmunity (Zhang & Bevan, 2011, p. 8). The MHC system plays a critical role in immunity because all cells express MHC molecules on their surface, and it is essential to determine self to non-self. In a transplantation setting, the MHC molecule is therefore the most important determinant between graft acceptance or rejection. Similarity between donor and recipient MHC molecules promotes graft acceptance while disparity is associated with rejection response (Chinen & Buckley, 2010).

T cells are activated when they recognize and bind an alloantigen. T cell activation requires two signals (Figure 1). The first signal is the binding and recognition of the alloantigen-MHC complex on the APC by the T cell receptor (TCR) of the T lymphocyte. If the recognition occurs, a second activating signal is necessary to induce an immune response, it is called costimulation. Costimulation result in the binding of the B7 molecules on the surface of APC and CD28 molecule on the surface of T cells. After signal 1 and 2 are successfully completed, a cascade of cytoplasmatic event takes place that result in the transcription of and production of cytokines. The main cytokines are interleukin (IL-) 2, and 5 which

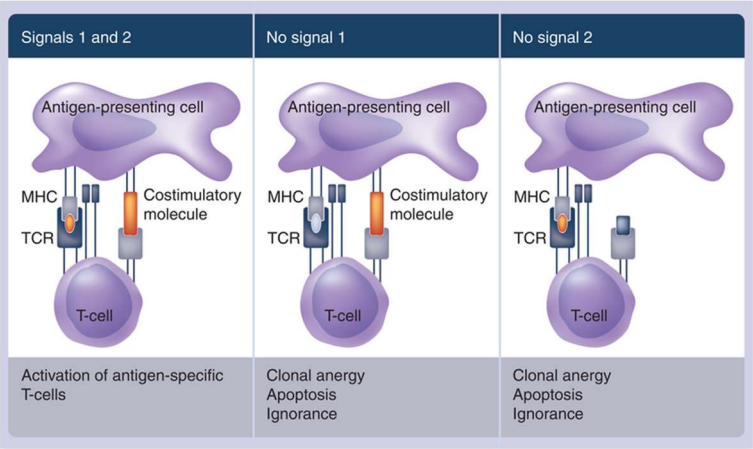


Figure 1: T cell activation through an antigen-presenting cell (APC). Signal 1 is the presentation and recognition by the TCR of an allopeptide loaded on the MHC of the APC. Then Signal 2 can occur which is costimulation. The costimulatory molecule, B7, on the APC bind to CD28 on the T cell. Both signals are needed for T cell activation. When signal 1 or 2 are missing, T cell go through apoptosis, anergy or ignorance. *(Arlen et al., 2009)*

induce the proliferation of the antigen-specific T-cell subset population as well as differentiating T cells into effector cells (Smith-Garvin et al., 2009; Wood et al., 2013).

There are two kinds of T cells: the $CD4^+$ T cells and the $CD8^+$ T cells. $CD4^+$ T cells recognize MHC class II molecules on APCs, they are also called T helper (T_H) cells as they help inducing an immune response from innate immune cells, B cells and T cells. $CD8^+$ T cells, called cytotoxic T cells, recognize MHC class I molecules which are present on all nucleated cell type and can directly eliminate the foreigner cells (Zhang & Bevan, 2011, p. 8).

B cells develop in the bone marrow where they go through positive and negative selection as for the T cells. Activation of the mature B lymphocyte occurs through T cell-dependent or T cell-independent activation mechanism (Bhattacharya, 2019). T cell-dependent activation takes place in secondary lymphoid organ such as lymph nodes and spleen or directly in tissues. When an antigen binds to a B cell receptor (BCR), the cell internalizes the antigen and process it into small pieces. A piece of the antigen is then loaded on a class II MHC molecule and exposed the cell's surface. Then a T_H cell can recognize and bind to the MHC peptide through its T cell receptor (TCR). Following the binding, the T_H cell express CD40L and release different cytokine such as IL-2, 4, 5. CD40L will bind to CD40 and the cytokines will bind to their respective receptors on the B cell. It is the costimulation. After costimulation, the B lymphocyte can either differentiate into plasma blast, a short-lived plasma cell secreting IgM with low affinity to the antigen, or they can migrate into the germinal center to undergo immunoglobulin class switch (Figure 2). T cell-independent activation occurs without the help of T_H cells. This activation is induced by foreign polysaccharides and unmethylated DNA (Bhattacharya, 2019).

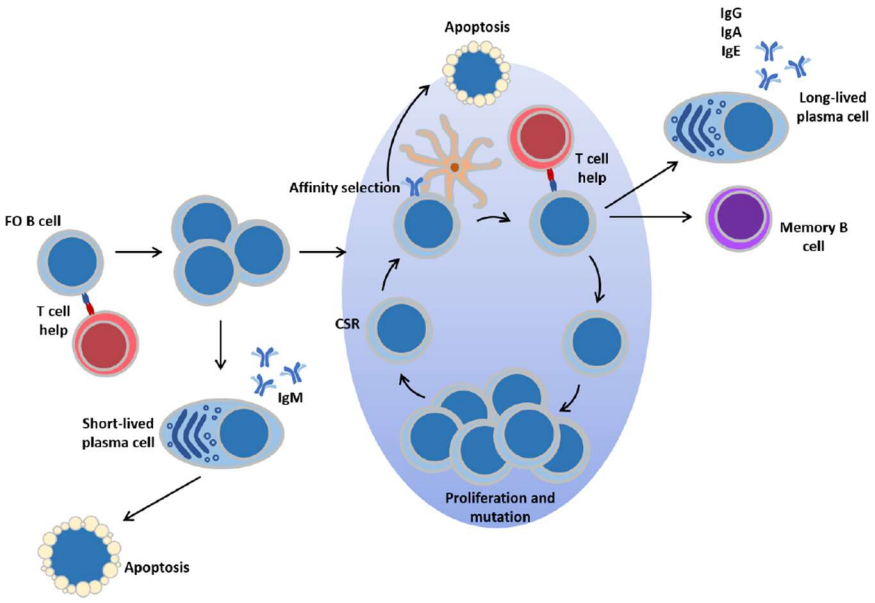


Figure 2: Schematic representation of T cell-dependent B cell activation. (Rebecca Newman)

1.3 Skin as an immune organ

Skin is the most immunogenic part of the body and an important part of the graft in VCA. It is also the primary site of acute rejection immune response of VCA graft (Salmon & Armstrong, 1994)(Quaresma, 2019).

In the last decades, it has been discovered that skin as more than just a physical barrier but could be consider as an immune organ itself where complex immune cells communication and processes take place. Indeed, skin has a unique immunogenicity. It is made of two layers, the epidermis, and the dermis. The epidermis lacks blood vessels but homes immune cells such as tissue resident dendritic epidermal T cells (DETCs) including langerhans cells, an APC. The dermis is highly vascularized and contains skin resident immune cells and lymphatics vessels (Leonard et al., 2013; Salmon & Armstrong, 1994). Under normal conditions, the dermis contains macrophages, mast cells, dendritic cells and twice as many T cells than in the periphery (Figure 3). Up to 10% of the T cells in the skin are regulatory T cells, a subpopulation of $CD4^+$ T cells which have the ability to inhibit the proliferation and activation of effector T cells and promote immune tolerance (Salmon & Armstrong, 1994; Siri et al., 2012; Kaufman et al., 2016; Quaresma, 2019).

In the context of VCA, the skin is where antigens from the allograft are being handled by APC and inflammation process starts, including expression of pro-inflammatory mediator, recruitment of immune cells (neutrophils, T cells ect…) and angiogenesis. Consequently, the skin is one of the main challenges in VCA transplantation by containing various immune cells that promote immune rejection. Indeed, about 80% of all patients experience an episode of acute rejection in the first-year post-transplantation most likely due to the skin contained in their VCA graft (Kaufman et al., 2016). On the

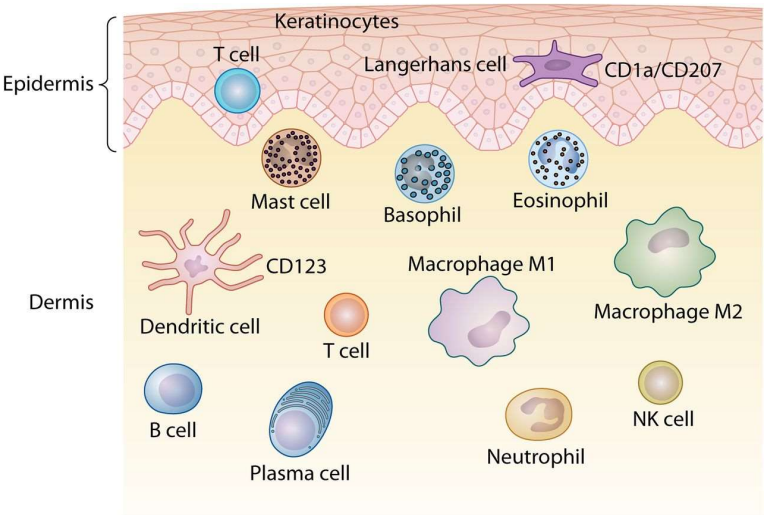


Figure 3. Schematic representation of the skin.

The epidermis is home to three main cell populations, hens' keratinocytes, melanocytes and Langerhans cells. The dermis can hosts various type of immune cells depending on parameters such as the host immune status or infection. The dermis can contain mast cell, basophils, eosinophils, different subpopulation of macrophages, neutrophils, NK cells, B and T cells, dendritic cells and plasma cells. These immune cells can be resident cell of the skin or they can migrate from the blood into the tissue when needed. (Quaresma, 2019)

other hand, due to its external location, the skin offers a unique opportunity for monitoring, prevention and treatment of VCA rejection. Its status, survival and signs of rejection can be visually observed.

1.4 Endothelial cells activation

As described above, the skin is the most immunogenic part of the VCA graft and can recruit immune cells leading to inflammation and rejection. Adhesion molecules expressed on activated endothelial cells play very important role to enable the adhesion and migration of immune cells from the circulating blood to the skin or any other tissues of the graft.

The endothelium is a monolayer of endothelial cells that cover that inner surface of vessels. Vascular endothelial cells make up the endothelium in the blood vessel and are in direct contact with blood, as for the lymphatic endothelial cells are in direct contact with the lymph and covers the lymphatic vessels. The endothelium forms a barrier between the circulatory system and the tissues. It controls the flow of molecules in and out and the infiltration of lymphocytes and other immune cells in the tissue. Endothelial cells are involved in many processes including vascular tonicity, permeability, coagulation, inflammation, secretion of protein such as Von Willebrand factors and cytokines (Barreiro & Sánchez-Madrid, 2009). In VCA, the donor endothelium plays an import role as it is the first to encounter the recipient blood. Indeed, in an allotransplantation setting, donor and recipient do not have the same MHC and blood component from the recipient blood will react against the foreign antigen and activate the endothelial cells. Endothelial cells become activated in response to different proinflammatory stimulus. This activation causes great changes in the endothelium such as expression of adhesion molecules, changing their phenotype from antithrombotic to prothrombotic, loss of vascular integrity, production of pro-inflammatory cytokines, and upregulation of type II leukocyte antigen molecules. The latest particularly is important in transplant rejection as the endothelium can act as an APC (Pober & Sessa, 2007).

The endothelial cell activation can be subdivided into two types: type I and type II. Type I activation is rapid and does not involve gene transcription and protein synthesis. It is mediated by the ligation of circulation blood components to the extracellular domain of G-protein-couple receptor (GPCR) on endothelial cells. The ligation activates the GPCR which lead to a cascade of intracellular pathway increasing cytoplasmic level of Ca^{2+}, releasing of von Willebrand factor and presenting P-selectin protein, that were stored in secretory vesicle called Weibel-Palade bodies, on the cell's surface. Type I activation last up to 20 minutes as the receptor become desensitized to prevent restimulation. Type II activation occurs when signs of inflammation persist, and a more enduring endothelial cell activation is needed. It typically needs more time to be active and involve gene transcription. The main mediators of the type II activation are tumor necrosis factors α (TNFα) and interleukin 1 (IL-1) that are secreted from activated leukocytes. In brief, they bind to their respective receptors present on the surface of endothelial cell which are TNF receptor 1 (TNFR1) and IL-1 receptor 1 (IL1-R1), and a series of intracellular signaling pathway are activated. Both lead to the activation of nuclear transcription factors, the nuclear factor kappa B (NF- κB) and activating protein 1 (AP1), which induce the transcription of proinflammatory proteins. These proteins include adhesion molecules such as E-selectin, intracellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1). These adhesion molecules are crucial for the adherence and invasion of leukocyte in the tissue (Pober & Sessa, 2007).

P-selecting and E-selecting are lectin-like adhesion glycoprotein and mediate the rolling of leukocyte on the endothelial cells via binding to L-selectin on leukocytes membrane (Barreiro & Sánchez-Madrid, 2009). ICAM1 and VCAM1 are immunoglobulin like adhesion molecules and are responsible for the

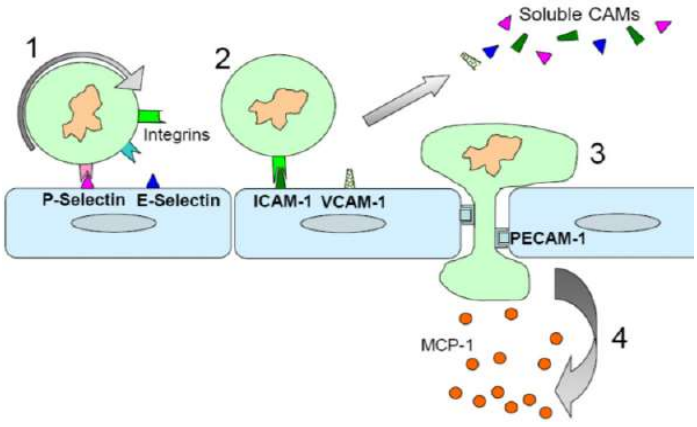


Figure 4. Leukocytes migration. 1) The immune cells present L-selectin on their surface, and they can bind on activated endothelial cell expressing P-selectin and E-selectin. The bond is weak and allow the cell to roll along the endothelium. 2) ICAM-1 and VCAM-1 are necessary for a stronger bond with integrin. 3) The leukocytes can then migrate through the endothelium by binding to PECAM-1 (CD31) present at the tight junction in a processed called diapedesis. 4) Migration inside the tissue is mediated via a chemokine gradient, here illustrated with MCP-1, that help the leukocyte to find the origin of the inflammatory stimulus. Adhesion molecules are later release as soluble CAMs. (Body and al., 2009)

firm adhesion via binding to integrin on the leukocytes surface (Barreiro & Sánchez-Madrid, 2009; Pober & Sessa, 2007). Once the leukocytes are firmly attached, they can transmigrate through the endothelial cells into the tissue. The transmigration occurs through binding of integrin situated on the leukocyte membrane and platelet-endothelial cell adhesion molecule 1 (PECAM, also known as CD31) on the endothelial cell surface. This process is called diapedesis (Figure 4) (Body et al., 2012).

1.5 The complement system in transplantation

The complement system activation is believed to contribute to allograft injury in transplantation by promoting ischemia reperfusion injury (IRI), antibody-mediated rejection as well as modulating the T and B cell response against alloantigen (Grafals & Thurman, 2019).

The complement system consists of various plasma protein that contribute to the innate immune response as well as the adaptive immune response. It allows the clearance of pathogen and dying cells via opsonization, induction of an inflammatory response and direct killing of pathogens. Under homeostasis, these proteins circulate in the body fluid as inactive precursors. Numerus of these plasma protein are protease which are themselves activated by proteolytic cleavage. When activated, the complement proteins are cleaved and in turn cleave other plasma protein resulting in an enzymatic cleavage cascade. The complement system has three functions. First it can bind to the surface of pathogens which result in their opsonization and allow their phagocytosis. Second, it can act as chemoattractant to recruit inflammatory cells. Third, it can form a membrane attack complex which make a pore in the membrane of bacteria and kill them (Riedemann & Ward, 2003; Zhu et al., 2017).

There are three pathways by which the complement system can be activated: the classical pathway, the alternative pathway, and the lectin pathway. The classical pathway is initiated by antigen-antibody interaction or the recognition of polymorphic leukocyte antigens present on the endothelial cells of the allograft which lead to activation of C1q. C1q cleave C2 and C4 proteins into C2a and C4b. these two fragments will unit and form C4b2a, also called C3 convertase. The lectin pathway occurs when mannose binding lectin (MBL) recognize mannose situated on the surface of bacteria. It also causes the cleavage of C4 and C2 as in the classical pathway. The alternative pathway is activated by lipopolysaccharide (LPS) and lead to spontaneous hydrolysis of C3. All pathways converge to the activation of the C3 convertase. The C3 convertase then cleave C3 into C3a and C3b which have different properties. C3a typically induce local inflammatory response as C3b bind to the surface of pathogen. C3b can also bind to the C4b2a complex, the C3 convertase, and form together the C5 convertase (Figure 5). The cleavage of C5 results in powerful pro-inflammatory complement product, hence C5a and C5b-9 (Asgari et al., 2010; Grafals & Thurman, 2019).

The complement system is known to be active in IRI which is inevitable in transplantation. IRI is the damage of the tissue caused after an episode of ischemia by the return of the blood supply in the vessels. Due to ischemia, cells and tissue experience changes that disturb different signaling pathways and the expression of surface molecules. Furthermore, the cells accumulate toxic products leading to their apoptosis or necrosis. As a result, to the lack of oxygen, neutrophils have increase adherence on endothelial cells due to the overexpression of their adhesion molecules. Growing evidence are also demonstrating the implication of the complement system in IRI with C5a and C5b-9 being the major contributors (Grafals & Thurman, 2019; Riedemann & Ward, 2003).

In VCA, little is known about the effect of ischemia/reperfusion injury on long term outcome on the graft (Riedemann & Ward, 2003). However, it was shown that the complement system triggers a pro-inflammatory response when activated in the allograft following IRI. Indeed, some complement proteins are also chemoattractant and activator of neutrophils and macrophages which in turn mediate the activation of T and B cell response (Grafals & Thurman, 2019). In a study on mice investigating the role of the complement in VCA, they show that blocking C3 convertase, thus all downstream cascades cannot happen, they can promote skin and muscle protection from IRI (Zhu et al., 2017). Indeed, they also showed that there was a significant decrease in neutrophils and leukocyte infiltration in the graft in the C3 deficient animals (Zhu et al., 2017).

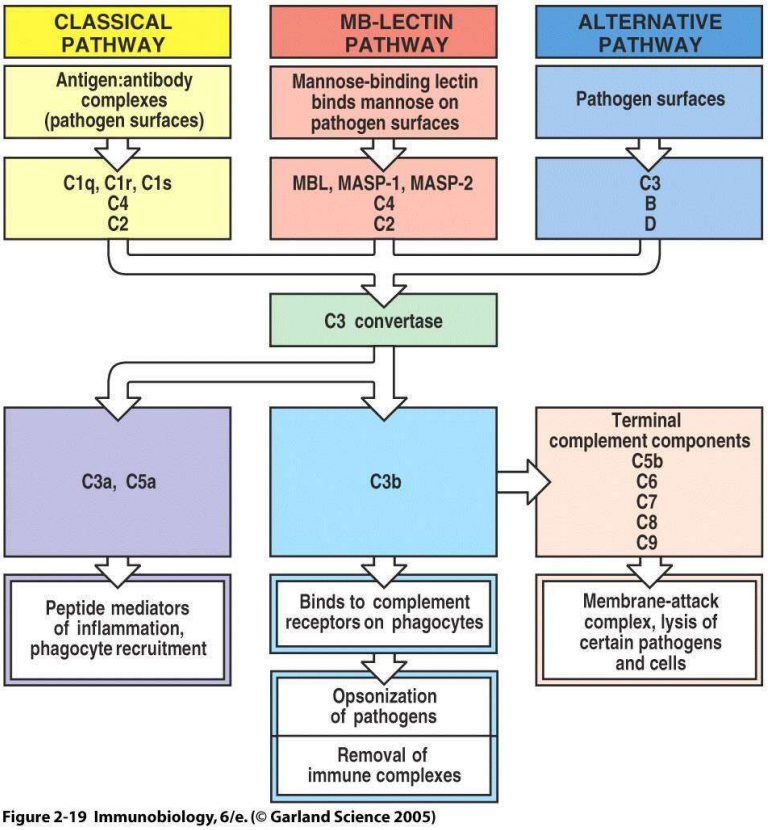


Figure 2-19 Immunobiology, 6/e. (© Garland Science 2005)

Figure 5: The complement system summarized. There are three different pathways by which the complement system can be triggered. The classical pathway which is initiated by antigen-antibody complex and activate C1q as well as C1r, C4, C2. The MB-Lectin pathway also simply called the lectin pathway, which recognize mannose bound on the surface of pathogen via their mannose binding lectin (MBL). In turn it activates different protein, MASP-1 and MASP-2, and cleave C4 and C2. Last, the alternative pathway recognizes lipopolysaccharides (LPS) on pathogen surface and thus activate C3 convertase. The C3 convertase in turn will cleave C3 into C3a and C3b. Both have distinct properties. C3a promotes inflammation and C3b opsonize the pathogen. C3b can also form a complex with other complement protein and result in a C5 convertase (not shown here). The C5 convertase then continue the complement cascade and produce C5b, C6, C7, C8 and C9. Together, they form a membrane attack complex (MAC).

1.6 Graft rejection in VCA

Graft rejection is one of the major challenges in VCA. The immune system as seen earlier protects the body from alloantigens, without it the body would be expose to many dangers. It is based on the recognition of self-versus non-self and works to eliminate all that is considered as foreign. VCA has an allogenic nature which means that the donor and the recipient are genetically different. The immune system will then attack the newly transplanted graft and caused rejection. Rejection is cause by various mechanisms involving the innate and adaptive immune system as well as the complement system. Here are describe the two mechanisms of rejection: Cell mediated rejection and antibody mediated rejection.

1.6.1 Cell mediated rejection

Cell mediated rejection (CMR) is induce by both the innate and the adaptive immune system with T cells being the most important player in this process.

In transplantation, tissue injury is inevitable. Indeed, transplantation include the removal, transplantation, and reperfusion of a new tissue in the recipient. The procedure leads to tissue injury and stress response that influence the immune response. Tissue injury result in the release of DAMPs. DAMPs are danger signals release from damaged or dying cells such as reactive oxygen species, heat shock protein or heparan sulfate. DAMPs activate the innate immune system by binding to their surface PRR. Inflammation is then initiated by activation of the inflammasome, upregulating gene transcription and release of proinflammatory mediator that will activate in turn the adaptive immune system. It is important to remember that the innate immune system is the first cellular line of defense of the body. Hence, the early post-transplantation reaction is due to tissue injury and not due to genetic differences. However, since in VCA, donor and recipient face in most of the case MHC mismatch, APC that are part of the innate immune response will take up the alloantigen from the graft and migrate to secondary lymphoid organ to present it to the adaptive immune cells (Lin & Gill, 2017).

In the secondary lymphoid organ, APC will present the alloantigen to T cells and initiate the adaptive immune response. Allorecognition can happen in three different ways: direct, indirect, or semi-indirect allorecognition. Direct allorecognition occur via direct binding of recipient TCR to allogenic MCH-peptide complex from a donor derived APC. Indirect allorecognition is the interaction of recipient TCR and MHC-peptide complex presented by a recipient APC in which a donor antigen is processed and presented on the MHC. Semi-indirect allorecognition pathway occurs when an intact donor MHC molecule is taken up and presented on the surface of recipient APC by a membrane fusion mechanism and then interact with recipient TCR. Two class of rejection mechanisms can be differentiated based on the MHC alloreactivity of the T cell (Lin & Gill, 2017; Wood et al., 2013). Cognate alloreactivity

describes T cells that recognize and bind donor MHC molecule, thus the direct pathway. As for noncognate T cells are those that can only react to donor peptide presented on recipient MHC molecule, thus the indirect pathway (Figure 6) (Lin & Gill, 2017). After alloantigen recognition via one of the three pathways, the T cells can be activated. Activation is initiated by the two signals that make up the costimulation as describe above.

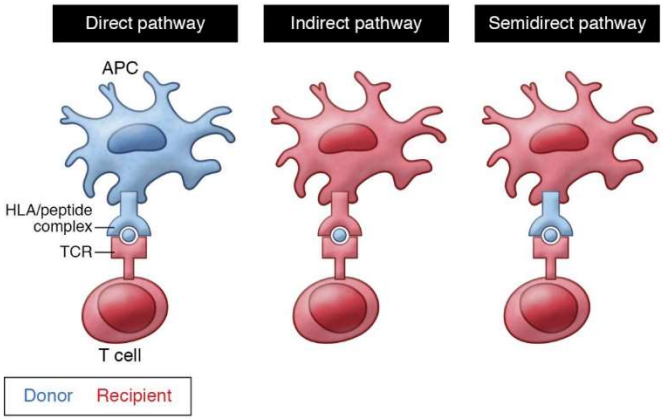


Figure 6. Allorecognition of donor peptide by recipient T cell. The allorecognition can occur via the direct pathway where donor APC present its MHC to a recipient T cell. It can happen via an indirect pathway in which the donor peptide is presented by a recipient APC to a recipient T cell. Lastly, it can occur via a semidirect pathway. The semidirect pathway is when a recipient APC has integrated a donor MHC via membrane fusion and present it unprocessed to a recipient T cell. *(DeWolf & Sykes, 2017)*

Following activation ad costimulation, T cells will differentiate into different subsets. Differentiation depends on a multitude of parameters such as the immune status of the recipient, degrees of genetic mismatch, degrees of ischemia-reperfusion injury, number of alloantigen present and the immunosuppressive drug cocktail prescribed. All of those influence the chemokines and cytokines being secreted from the transplanted tissue, the migration of APC, the recruitment of immune cells and differentiation of leukocytes (Wood et al., 2013).

1.6.2 Antibody mediated rejection

Antibody mediated rejection (AMR) or humoral rejection occurs when plasma cells produce donor-specific antibodies (DSA) (Garces et al., 2017).

These antibodies are directed against the leukocyte antigen molecules, blood antigen, ABO, and endothelial cell antigen. The main mechanism of action of antibody mediated rejection is the activation of classical complement system by the binding of DSA on endothelial cells (Puttarajappa et al., 2012). The DSA bind to the donor endothelium and form an antigen-antibody complex. This complex activates the complement cascade causing the formation of a membrane attack complex which leads to microcirculatory changes and tissue injury. In addition, endothelial cell damage promotes platelets

activation, coagulation, and thrombotic event. The complement system activation also leads to endothelium cell activation which induce the recruitment and infiltration of lymphocytes, neutrophils, natural killer cells and monocytes in the site of activation and causes cell mediated rejection. C4d deposition, a degradation product of the complement pathway that binds to the endothelium, has been discovered recurrent in VCA. Thus, C4d seems to be a good marker for antibody mediated injury (Garces et al., 2017). AMR can be classified according to their time of action: Hyperacute, accelerated and acute and chronic AMR. Hyperacute AMR occurs within second to minutes after transplantation due to the presence of preformed DSA. Accelerate AMR happens without the first 24h-48h after the operation. Acute AMR can take place days, weeks or even month after the graft transplantation by either preformed or *de novo* DSA. At last, chronic AMR can happens years after the graft transplantation (Garces et al., 2017; Puttarajappa et al., 2012).

1.7 Transplant rejections: subtypes

Transplant rejection happens when the immune system of the recipient attacks the donor graft either by CMR or AMR. It can be divided into three subtypes depending on their mechanisms of actions and their tempo.

1.7.1 Hyperacute rejection

Hyperacute rejection occurs when preformed recipient antibodies recognize and bind to donor MHC, ABO or other tissue antigen. The recipient can have performed anti-donor antibody if he was already exposed to donor antigen in blood transfusion for example or in previously pregnant women where paternal antigen was exposed with the fetus (Moreau et al., 2013).

Following transplantation, hyperacute rejection happen within minutes to hours post-operation. In this case, anti-donor antibodies bind to donor antigen and activate the complement system as well as recruit recipient macrophage and neutrophil to the graft. Follows, thrombotic event due to coagulation cascade. The graft cannot be saved and must be removed. Fortunately, hyperacute rejection occurs rarely as antigen matching test is performed prior to the transplantation.

1.7.2 Acute rejection

Acute rejection occurs days to weeks after the transplantation. It is the result of the adaptive immune system from the recipient directed against the donor cells. It can be caused by either cell-mediated rejection or humoral rejection or even both. It induces a severe inflammation and damage of the donor graft. The extend and pattern of inflammation may vary depending on which rejection process is the cause, cell-mediated or humoral (Moreau et al., 2013).

Acute rejection can be treated with immunosuppressive therapy to slow down or prevent this process (Moreau et al., 2013).

1.7.3 Chronic rejection

Chronic rejection occurs months to years after the transplantation. It can be cause by either humoral and cell mediated immunity linked to memory, plasma cells and antibody. Repetitive inflammation process over time lead to gradual loss of function and damage of the graft via fibrosis. Tertiary lymphoid organs are also characteristic of this type of rejection. It results to slowly loss of function of the graft till inevitable graft loss. Nowadays, it seems to be the major cause of graft failure (Moreau et al., 2013).

1.8 Immunosuppression in VCA

After VCA transplantation, donor MHC antigens from the graft are presented by APC to the recipient T cells which recognized them as foreign and lead to T cell activation. The immune response from the recipient can lead to damage and rejection of the graft. Consequently, immunosuppression therapy aims to avoid this adverse response and hence graft rejection.

There are two types of immunosuppressive drug therapy: induction and maintenance therapy. Induction therapy are monoclonal or polyclonal antibodies that binds to immune cell receptors to avoid acute rejection. Such induction agents are for example Basiliximab or Alemtuzumab, two monoclonal antibodies which block respectively IL-2 receptors on T cells and CD52 receptor on B cells. Maintenance therapy is usually a triple-drug treatment. It includes corticosteroids that act as an unspecific anti-inflammatory agent, an anti-proliferative agent like mycophenolate mofetil (MMF) or azathioprine that inhibit the proliferation of immune cells through diverse intracellular pathways, and a calcineurin inhibitors such as cyclosporine or tacrolimus which inhibit the production of IL-2 amount other cytokines in T cells (Enderby & Keller, 2015; Gutierrez-Dalmau & Campistol, 2007; Sarhane et al., 2013).

As the immunosuppressive therapy promotes graft survival and acceptance from the body, it has also diverse and severe side effects. Indeed, the immune system as well as cell proliferation is downregulated and therefore the patients have higher risk of infections, malignancy, nephrotoxicity, myelosuppression and so on.

Here we focus on the calcineurin inhibitor tacrolimus as it is loaded in the novel and promising drug delivery system that is tested in this study.

1.8.1 Tacrolimus

Tacrolimus is a macrolide discovered in 1984 from a fermentation both of *Streptomyces tsukubaensis*. It is now used as an immunosuppressive agent. Tacrolimus is a calcineurin inhibitor. Calcineurin is an intracellular protein phosphatase found in the cytosol of cells. When an APC present and bind an antigen to the T cell receptor on a T cell, it leads to an increase of cytoplasmic calcium level. This increase of calcium activates the calcineurin protein and it dephosphorylates the nuclear factor of activated T cells (NF-AT), a transcription factor that is found when inactive in the cytoplasm. Once dephosphorylated, NF-AT translocate in the nucleus where it starts the transcription of different cytokines such as IL-2, 3, 4, 5, TNF-alpha, IFN-gamma and GMCSF. IL-2 is an important cytokine responsible for the activation of T cells, as for the others they contribute also to T cell activation as well as proliferation and differentiation (Chinen & Buckley, 2010; Rusnak & Mertz, 2000). Tacrolimus inhibits the action of calcineurin phosphatase by binding to the intracellular protein FKBP-12. The complex, tacrolimus-FKBP-12, bind the calcineurin and prevent the dephosphorylation of NF-AT and thus the transcription of the cytokines (Figure 7) (Mejia et al., 2014).

Tacrolimus is an efficient immunosuppressor, but it has nonetheless adverse effects. The side effects following an extended administration of Tacrolimus include nephrotoxicity, metabolic disturbances, hyperglycemia, and others (Mejia et al., 2014).

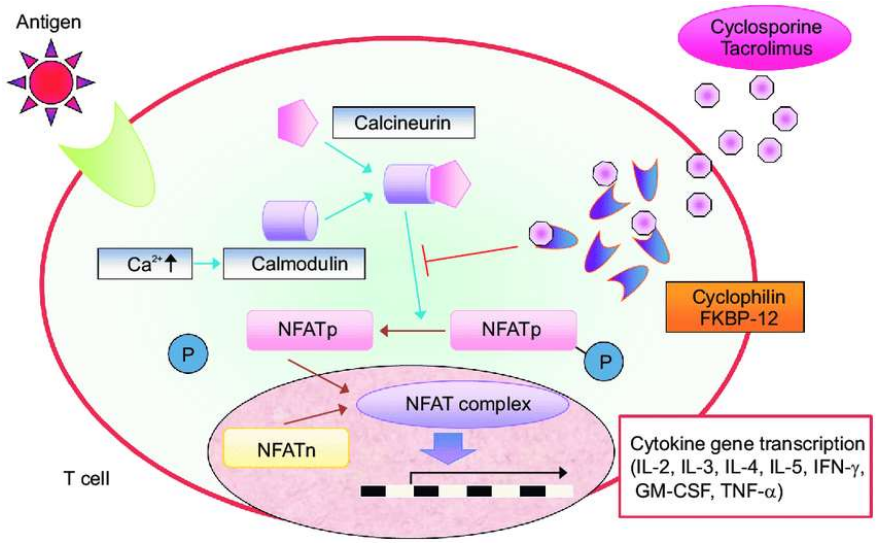


Figure 7. Mechanism of action of tacrolimus. When an antigen binds to a TCR, cytoplasmatic level of Ca^{2+} increases. Ca^{2+} activates calmodulin that will form a complex with calcineurin. Calcineurin is now active. Calcineurin inhibitors such as cyclosporine and tacrolimus bind to cyclophilin and FKBP-12 respectively. The complex that is formed prevent the dephosphorylate of the cytoplasmatic NF-AT (NFATp). If dephosphorylated, NFATp migrates to the nucleus where is forms a complex with the nuclear NFAT (NFATn). The complex is a transcription factor that initiate different cytokine gene transcription. Consequently, tacrolimus inhibits the transcription and release of specific cytokine from T cells. (Nakase, 2019)

1.8.2 Drug delivery systems

Drug delivery system (DDS) is a useful way to deliver a therapeutic drug in a specific, targeted site of action. Thus, it avoids a systemic effect of the drug and its related side effects on off targeted cells, organs, and tissues. VCA offers a unique opportunity to use a DDS. Indeed, unlike in solid organ transplantation where grafts are hidden in the body, in VCA grafts are easily accessible to administer the DDS. Moreover, the graft can be visually monitored for signs of inflammation or rejection so the treatment can be adapted quickly if for any reason the one administered is not sufficient.

1.8.2.1 TGMS-TAC

TGMS-TAC is an enzyme responsive tacrolimus-encapsulated hydrogel. Tacrolimus (TAC) is loaded in a triglycerol monostearate gel (TGMS), an agent generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA). TGMS can self-assemble after being administer in the patient into a hydrogel that encapsulate the drug tacrolimus. The TGMS is partially degraded in an enzyme dependent manner in the present of proteolytic enzyme such as metalloproteinases (MMP), esterase and other enzymes that are upregulated during an inflammatory response. As the TGMS is degraded, tacrolimus is therefore release in the tissue (Fries et al., 2019). This enzyme-responsive tacrolimus-encapsulated hydrogel was made in collaboration with the group of Praveen Vemula at the InStem Research Institute in Bangalore, India and the group of Jeffrey M. Karp at Harvard-MIT Division of Heath Sciences and Technology, Boston (MA), USA.

2 Aims

In this study we aimed to 1) validate the efficacy and 2) safety of the TGMS-TAC system in a clinical large animal model, the swine 3) and elucidate differential immune response leading to rejection following VCA transplantation in untreated vs tacrolimus treated patients.

By comparing different groups, we aimed to assess graft survival as well as off-target toxicity. Based on the results given from the rodent model, we hypothesized that local drug delivery via TGMS-TAC promotes long term graft survival by maintaining high intra-graft drug level while reducing off-target toxicity that is caused by a systemic exposure of the immunosuppressive drug in a swine model of VCA. As for the immune response, it plays a critical role in determining graft survival or graft rejection and was never fully characterized in previous research on the TGMS-TAC system. Consequently, we also characterize of the immune responses locally in the tissue but also in the periphery.

3 Materials and Methods

3.1 Ethical statement

Animal experiments were planned and carried out in agreement with current 3R principle and was approved according to Swiss animal protection laws by the Veterinary Authorities of the Canton Bern, Switzerland (license no. BE48/19). State-of-the-art anesthesia and pain management were used to reduce the exposure of the animals to stress and pain during the experiments.

3.2 Study design

In this study, we aimed to evaluate site-specific immunosuppression achieved with a DDS loaded with Tacrolimus, hence TGMS-TAC, on a clinically relevant large animal model. To this end the heterotopic swine hind limb transplantation model was used. Outbred pigs were divided into donors (N=8) and recipients (N=16) and were paired based on their swine leukocyte antigen (SLA). The pigs were randomly divided into three different groups: control (N=4), systemic tacrolimus (N=3), and TGMS-TAC (N=4) group. Both males and females were included in all groups.

3.2.1 Groups and treatments

A total number of 24 outbred pigs were used in the study, including 8 donors and 16 recipients. The pigs originated from a local farm certified for laboratory animal breeding. The swine leukocyte antigens (SLA) were determined by SLA-typing done with polymerase chain reaction (PCR) in collaboration with Dr. Sabine Hammer at the Institute of Immunology, University of Veterinary Medicine, Vienna, Austria. Then based on their SLA-typing, groups of males and females were paired for transplantation. This current study aimed to achieve at least two complete mismatches between donor and recipient to be as clinically relevant as possible.

To assess the efficacy and effect of local drug delivery through TGMS-TAC on graft survival and immune response, the pigs were allocated into three different groups:

Group 1 (N=4) is the control group where the pigs did not receive any treatment. Therefore, graft survival without any immunosuppression therapy could be assess. Group 2 (N=3) received systemic tacrolimus orally daily for the first 14 days to achieve a target level of 10-15ng/mL of systemic tacrolimus, then the treatment was stop. Group 3 (N=4) was given the TGMS-TAC system. Directly after the operation, a single dose of 140mg of TGMS-TAC per kg of graft was injected subcutaneously in the graft though multiple injections. The animal number and group allocation are described in table 1.

Out of the 16 recipients, three pigs from the control and one pig from the systemic group had to be excluded from the experiment due vascular complications and thus were not included in the data. Additionally, one animal served as a model for the ameliorated surgical procedure.

Table 1: Group allocation

Groups	Pig number
CONTROL	Pig 1
	Pig 2
	Pig 3
	Pig 4
SYSTEMIC	Pig 5
	Pig 6
	Pig 7
TGMS-TAC	Pig 8
	Pig 9
	Pig 10
	Pig 11

3.2.2 Heterotopic swine hind limb transplant model

Outbred swiss pigs between 18 to 30 kg were selected according to their SLA typing and matched to have one donor (N=8) for two recipients (N=16). Males and females were both used so that if the donor were a female, the recipients would be males and vise-versa. The pigs were sedated (Propofol 1-4 mg/kg, ketamine 1mg/kg and inhaled isoflurane) and then put under general anesthesia. Additional muscle relaxant was administered, if the anesthesia allowed it, to facilitate the procedure. An osteo-myocutaneous limb allograft containing lymph nodes was harvest from the donor pig and transplanted on one of the recipients. The limbs were always placed on the contralateral side of the recipient meaning that the donor left limb was transplanted on the right side of the recipient and vise-versa. The harvested graft was placed in a subcutaneous pocket made from the groin to the dorsolateral abdominal wall. The donor vessel from the graft were ligated. Donor and recipient were then linked by an arterial end to end anastomosis. On the recipients, the skin paddle from the donor graft was exteriorized on the dorsolateral side of the pig. The skin paddle and the position of the graft on the dorsolateral side enabled an easy visual monitoring of the graft post-transplantation. A port-a cath was also inserted in the external jugular vein so that the port would be placed subcutaneously in the posterior neck area. This method allowed post-operative venous blood sample collection.

Then the second graft was harvest and transplanted on the second recipient in the same manner. The donor pig was euthanized immediately after the second limb was harvest.

New from prior heterotopic limb transplantation is the inclusion of draining lymph node in the graft as well as a muscle sparing technique which allows a faster recovery of the animals.

All the surgeries were performed in the Experimental Surgery Facility (ESF) of the Department for Biomedical Research, University of Bern and in collaboration with the Department of Plastic and Hand Surgery, University hospital of Bern, Switzerland.

3.2.3 Follow-up and grading of the grafts

After transplantation, the pigs recovered first for 24h in a recovery cage at the ESF and then were maintained for 10-15 days for a full recovery at the Clinic for Ruminants and Pigs, Faculty of Veterinary Medicine, University of Bern. Based on their health status after this period, they were transferred in a local farm until endpoint.

Pigs were monitored regularly, and the graft rejection status was graded based on the following macroscopic criterium of skin evaluation:

- Grade 0: normal skin appearance
- Grade I: edema and erythema
- Grade II: limited epidermolysis and exudation
- Grade III: extensive epidermolysis with signs of desquamation and necrosis
- Grade IV: necrosis and mummification

Endpoint was settled when grade III rejection or graft survival till POD90 was reached.

3.3 Sample collection and euthanasia

Samples were collected at different time points after transplantation (See Table 2). Blood samples were taken at baseline (POD0), which is the day of the surgery and then at POD 1, 3, 7, 14 and afterwards weekly until endpoint. Skin punch biopsies from the graft and the contralateral side were taken at POD 7, 14, 35 and at endpoint. At endpoint, pigs were euthanized in a surgery room and samples were collected under sterile conditions. Blood and skin were collected as mentioned as well as muscle samples. A piece of all tissues was individually stored in cassette placed in formaldehyde (for histopathology analysis) and cryopreserved in cryomold (for tissue immunofluorescence analysis) covered with Tissue-Tek O.C.T. (Sakura Finetek, 4583).

Table 2: List of samples collection and their timepoint

	Group name	Donors	Control				Systemic Tacrolimus											TGMS-TAC										
	Animal number	8	4				4											4										
	POD	0	0	1	3	Endpoint POD6-9	0	1	3	7	14	21	28	35	49	daily	weekly	Endpoint POD27-55	0	1	3	7	14	21	28	35	49	weekly	Endpoint POD39-60
Surgery																													
Hindlimb transplantation		X	X				X												X										
Samples																													
Blood		X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Skin (graft)		X				X				X	X			X	X			X				X	X			X	X		X
Skin (contraleteral)		X				X				X	X			X	X			X				X	X			X	X		X
Muscle (graft)		X				X												X											X
Treatments																													
Tacrolimus							X	X	X	X	X					X													
TGMS-TAC																			X										

Note: Post-Operative Day (POD)

3.4 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from whole blood samples. Blood from the patients were collected at defined timepoint through a port-a-cath and placed immediately in EDTA tubes. The blood was first centrifuged 15 minutes at 1000G at RT. The cellular part of the blood was re-suspended in 1x phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) (Sigma, F7524 579), 1:1 dilution. After adding the solution on top of the density gradient medium Ficoll-Paque TM PLUS (GE Healthcare, 17-1440-02), it was centrifuged for 20 minutes at 400G at RT. The PBMCs were collected from the gradient and re-suspended in 90% FBS-10% Dimethyl Sulfoxide (DMSO) (Sigma, D4540) for storage at -150°C.

3.5 Plasma and serum collection

Blood taken via the pot-a-cath from different timepoint were collected in EDTA and serum tubes. The tubes were centrifuge centrifuged 15 minutes at 1000G at RT. The plasma from the EDTA tubes and serum from the serum collection tubes were collected and aliquoted in Eppendorf tubes before storage at -150°C.

3.6 Immunofluorescence

Immunofluorescence staining was used to in order to visualized specific cells and structures in the tissues. Skin and muscles were collected at baseline or endpoint and preserved in a tissue mold with Tissue-Tek O.C.T. (Sakura Finetek, 4583). The molds were frozen at -20°C and later stored at -80°C. The frozen samples were cut into 5µm thick tissue samples with the Cryostat (Zeiss, Hyrax C60) at -20°C and placed immediately on glass slides and stored at -20°C until used.

The sections were air dried prior to the staining for 15 minutes at RT. Then, the samples were fixed in acetone and methanol of a ratio 1:1 for 10 minutes. After fixation, the slides were rehydrated with 1x tris-buffered saline (TBS) for 5 minutes at room temperature on a shaker. The slides were slightly dried around the tissue and marked with a hydrophobic Dako pen (Dako, cat. s-2002) and then blocked in TBS-3% bovine serum albumin (BSA) for 1h at RT. The unlabeled primary antibodies (Table 3) were diluted in 1x TBS-1% BSA-0.05% Tween and applied overnight at 4°C on the sample. After washing 3x 20 minutes in TBS 1x on a shaker, the secondary antibodies (labelled, table 3) were diluted in 1x TBS-1% BSA-0.05% Tween and applied on the tissue for 90 minutes at RT. After being washed 3x 20 minutes with 1x TBS, the slides were mounted with ProLong™ Gold Antifade Mountant (P36934, Thermofisher). Images were obtained with confocal microscope Zeiss LSM 710 with 10X or 20X objectives and analyzed with Fiji, ImageJ software (Schindelin et al., 2012).

Immunofluorescence signal quantification was obtained with Fiji by evaluating the raw integrated density (referred as Int/Den) and data are presented as mean ± SD.

Table 3: List of antibodies used for tissue immunostaining.

Name	Species	Clone	M/P	Dilution	Reference N°	Company
PRIMARY						
CD31	IgG1 Rat anti-pig	/	M	1:100	MAB33871	R&D System
E-selectin	IgG1 Rat anti-pig	1.2B6	M	1:100	Sc-18852	Santa Cruz
MPO	Rabbit anti-human	/	P	1:500	A0398	DAKO
CD3	Mouse anti-pig Cy5	/	M	1:100	4510-13	Biotech
CD4	Mouse anti-pig PE	/	M	1:100	4515-09	Biotech
CD79a	IgG1 Mouse anti-human	HM47/A9	M	1:100	MA5-13212	Thermofisher
CD163	Mouse anti-pig	/	M	1:100	MCA23116A	Biorad
Macrophage	IgG2b Mouse anti-pig macrophage	BA4D5	M	1:100	MCA2317GA	Biorad
C1q	Rabbit anti-human	/	P	1:100	A0136	DAKO
C3c	Rabbit anti-human FITC	/	P	1:100	F0201	DAKO
C5b-9	IgG2 Mouse anti-C5b-9	aE11	M	1:200	DIA 011-01	Antibodyshop
IgM	Goat anti-human FITC	/	P	1:100	F-5384	Sigma
IgG	Goat anti-human FITC	/	P	1:100	62-8411	Invitrogen
Phalloidin	Acti-stain 555 Phalloidin	/	/	1:500	PHDH1	Cytoskeleton
Dystrophin	Rabbit anti-human	/	P	1:100	Ab15277	Abcam
SECONDARY						
DAPI	4 ' ,6-Diamidine-2 ' -phenylindole dihydrochloride	/	/	1:1000	10236276001	Sigma
	Donkey IgG anti-rat, Alexa Fluor®488	/	P	1:500	A21208	Invitrogen
	Donkey anti-mouse, Alexa Fluor®568	/	P	1:500	A10037	Invitrogen
	Goat anti-rabbit, Alexa Fluor®568	/	P	1:500	A11036	Invitrogen
	Donkey anti-goat, Alexa Fluor®488	/	P	1:500	A11055	Life Technology

Note: Monoclonal antibody (M), Polyclonal antibody (P).

3.7 Flow cytometry

After the PBMCs were thawed, erythrocytes were lysed if needed with lysis buffer. The cells were resuspended in complete cell culture medium (RPMI (Gibco, 31870-025), Penicilin/Stretomycin, L-glutamine and FBS), at a concentration of 10^6 cells/ml at 37°C with 5% CO_2 overnight. After spinning down at 300G 4°C for 5 minutes, the cells were resuspended in FACS buffer containing 1x PBS and 2% FBS (Sigma, F7524 579), at a concentration of 10^6 cells/ml. 5 to $10x10^5$ cells were distributed in FACS tubes. The cells were blocked for non-specific Fc-mediated interaction for 10 minutes at 4°C. The blocking solution is a mix between FcR Blocking Reagent (Macs, 130-059-901) and FACS buffer in a ratio of 1:8 respectively. Cells were washed with stain buffer and centrifuged at 300G for 5 minutes, and stained in the LIVE/DEAD Fixable Yellow Dead Cell Stain (Thermofisher, L34959) for 30 minutes at RT in the dark. After the cells were washed with stain buffer, they were stained in the master mix (antibody Table 4) mixture for extracellular staining by considering the amount of antibody per number of cells recommended on the manufacturer's instruction for 45 minutes on ice in the dark. The cells were washed twice with stain buffer. After washing, the cells were resuspended in FACS buffer and DAPI (1:1000) was added prior of acquisition. The data were acquired with the FACS machine LSRII/SORP (BD Bioscience), and analysed with the FlowJo software. Gating strategy is shown in supplementary figure 2.

Table 4: Panel for flow cytometry

Panels for flow cytometry				
Panel #	Target	Color	Firm	Clone
Panel 1	Dead/Live		Thermofisher	L34960
	CD3ε	PerCp-Cy5.5	Bioscience	BB23-8E6-8C8
	CD4	PE	Thermosfisher	74-12-4

3.8 Cytokine level

Plasma levels of 15 different cytokines were measure by using Bio-Plex and Milliplex multiplex immunoassays. The 7/15 of these cytokines including IL-6, IL-10, C5a, C5b-9, bFGF, VEGF and MCP-1 were analyzed with Bio-Plex. To this end, plasma sample were diluted 1:3 and the standards were prepared according to the instruction manual. Magnetic beads were added to the plates and then washed 3x with washing buffer on a shaker. 50µl of standards and samples were added to the wells and incubated for 60 minutes at RT on a shaker set at 300 rotation per minutes (rpm). After the plates were washed 3x with washing buffer, 25µl of biotinylated detection antibodies were added and incubated on a shaker at 300rpm for 30 minutes. After 3x washing, PE-streptavidin was added in all wells and incubated for 10 minutes on a shaker. The beads were washed and resuspended in wash buffer, measured by FLEXMAP 3D® system (Luminex) following the set up in the user guide.

The other 8/15 cytokines including IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-8, IL-12 and IL-18 were analyzed with MILLIPLEX® MAP porcine cytokine/chemokine magnetic bead panel kit (cat#PYCTMAG-23K). The standards were prepared according to the user guide. 200µl of assay buffer was added in each well and the plate was placed on a shaker for 10 minutes at 300 rpm. Then either 25µl of standards or controls were added into the wells. Then the samples were mixed with 25µl of assay buffer while the background, standards and controls were mixed with 25µl of the respective matrix solution. At the end, the beads were added to each well and incubated overnight at 4°C. Washed 3x with wash buffer, then the detection antibodies were added in all wells and incubated for 2h on a shaker at RT. Streptavidin-Phycoerythrin was added and incubated shaking for 30 minutes at RT. At last, the 100µl of Sheath fluid was added to all wells and after resuspension of the beads, the plate was run according to the instruction guideline on the FLEXMAP 3D® system (Luminex).

Results are presented in picograms per milliliter(pg/ml) for the Bio-plex multiplex assay and in nanogram per milliliter (ng/ml) for the Milliplex.

3.9 Blood and tissue levels of Tacrolimus

The level of tacrolimus in the blood and skin were evaluated at defined timepoint by either blood collection via the port-a-Cath or skin punch biopsies. The blood was collected at POD0, POD1, POD3, POD7 and then weekly until end point. The skin biopsies were taken at POD7, POD14, POD35 and POD49. The punch biopsies were taken from the skin of the graft and the skin of the recipient on the contralateral side of the graft. Tacrolimus levels were determined by using liquid chromatography mass spectrometry at the Center of Laboratory Medicine at the University Hospital of Bern.

3.10 Histopathology

Skin and muscle from the grafts were collected at endpoint. The tissues were stored in cassette were sent to the institute for pathology of the university of Bern where they were fixed in 4% formaldehyde prior of being imbedded in paraffin and then stained with matoxylin and eosin for microscopic evaluation. Skin samples were scored based on the modified Banff classification of different stages of swine VCA skin rejection (Figure 8)(Etra et al., 2019). The muscle tissues were evaluated based on the degrees of necrosis and atrophy as well as the amount of leukocyte infiltration: 0: non; 1: minimal; 2: moderate; 3: extensive.

Swine VCA skin rejection classification (modified Banff criteria)

Grade	Dermal inflammation	Epidermal involvement
0	None to minimal	None
1	Mild	None
2A	Moderate	None
2B	Mild to moderate (may be paucicellular)	Infiltrating inflammatory cells (may be few) without keratinocyte necrosis
3A	Moderate or severe	Multifocal single cell epidermal necrosis, variable infiltrating inflammatory cells
3B	Mild to severe (may be paucicellular)	Multifocal epidermal necrosis (may be full thickness, not diffuse), infiltrating inflammatory cells
4	Mild to severe (may be paucicellular)	Diffuse full thickness necrosis (entire epidermis is necrotic and/or sloughed off)

Figure 8: The Banff 2007 working classification of skin-containing composite tissue allograft pathology.

3.11 Statistical analysis

The data were analyzed by using Prism software version 9 (GraphPad software). The data are represented with mean ± SD except for the cytokines graphs which are presented with median ± SD. Statistical analysis between the different groups was acquired with ordinary one-way ANOVA with Tukey's multiple comparisons tests. Statistical analysis comparing multiple parameters within and between groups were acquired with a two-way ANOVA with Šídák's multiple comparisons test. Unpaired t-test was used to analysis the statistical significance in the total amount of tacrolimus given to each treated group. P value < 0.05 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4 Results

4.1 Macroscopic evaluation of the grafts

The transplanted limbs were macroscopically evaluated via the skin paddle for signs of rejection such as erythema, oedema, epidermolysis, exudation, desquamation, necrosis, and mummification. The grafts were also given grade of rejection based on specific criteria (see above in Groups and Treatment). The pigs from all groups showed edema and erythema in the graft as grade I rejection at POD4, suggesting that the surgery triggers an inflammatory response, and the body needs some time to recover from the trauma of the surgery. In the control group, the grafts showed signs of inflammation quickly after the surgery and reached grade III rejection around POD8 with clear signs of necrosis and epidermolysis. While for the same POD8 in the systemic tacrolimus and TGMS-TAC groups, the grafts showed almost no signs of inflammation nor rejection. Furthermore, the inflammation in the graft following tissue injury from the surgery may be prohibited faster in the TGMS-TAC group due to local tacrolimus levels. At POD14, the skin of the grafts in the treated groups resemble the healthy skin of the recipient pigs, they only reach grade III rejection between 2-6 weeks later. (Figure 9)

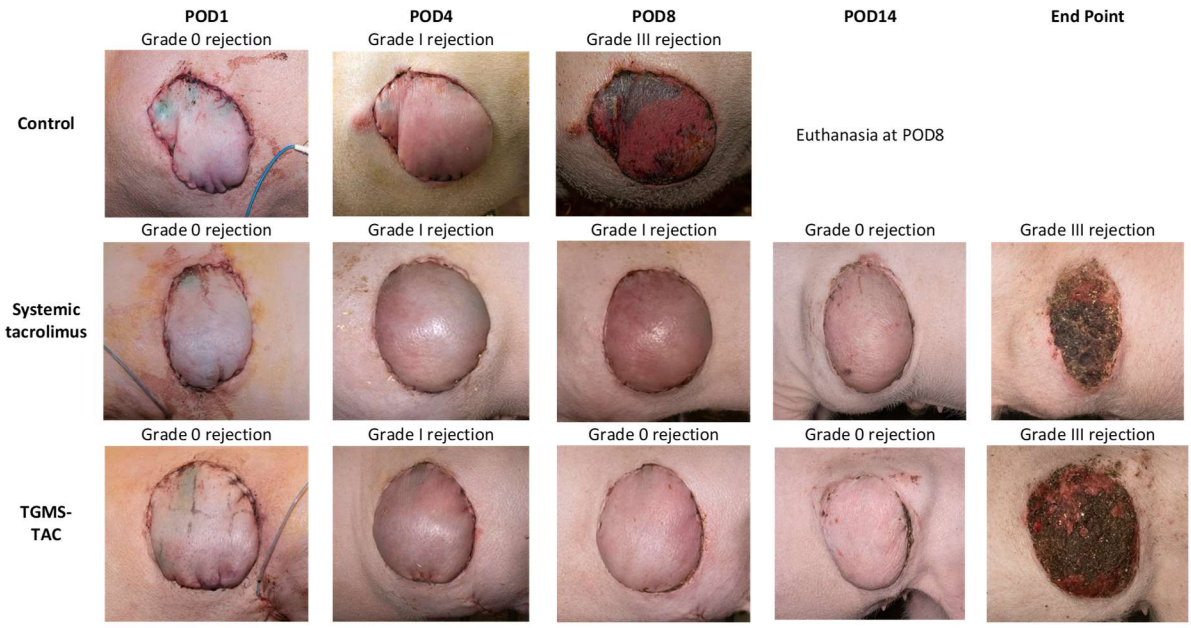


Figure 9: **Macroscopic evaluation of the transplanted graft from POD0 until grade III rejection**. The pigs were monitored daily for the first 7 to 8 days and then weekly. Pictures were taken at each visit and shown in the figures are the most representative skin paddles for each group at a specific timepoint. Grades of rejection were given for each pig at each visit. When grade III rejection was reach, the pigs were euthanized.

4.2 Intra-graft administration of Tacrolimus prolonged graft survival.

In the control group, the graft survived for a maximum of 9 days. Two pigs rejected at POD7 while one at POD8 and one at POD9. The mean survival time is of 7.75 days.

In the systemic groups, the pigs reach grade III rejection at an average of 43 days. The TGMS-TAC group showed the highest graft survival time with a mean of 46 days.

Our results showed that without immunosuppression therapy, the graft could not survive more than a week. When treated with the TGMS-TAC, the survival time of the graft increased significantly compared to the untreated group (p= 0.0062). Moreover, a single administration of local tacrolimus via the TGMS-TAC is as efficient as the daily oral administration of the drug. (Figure 10)

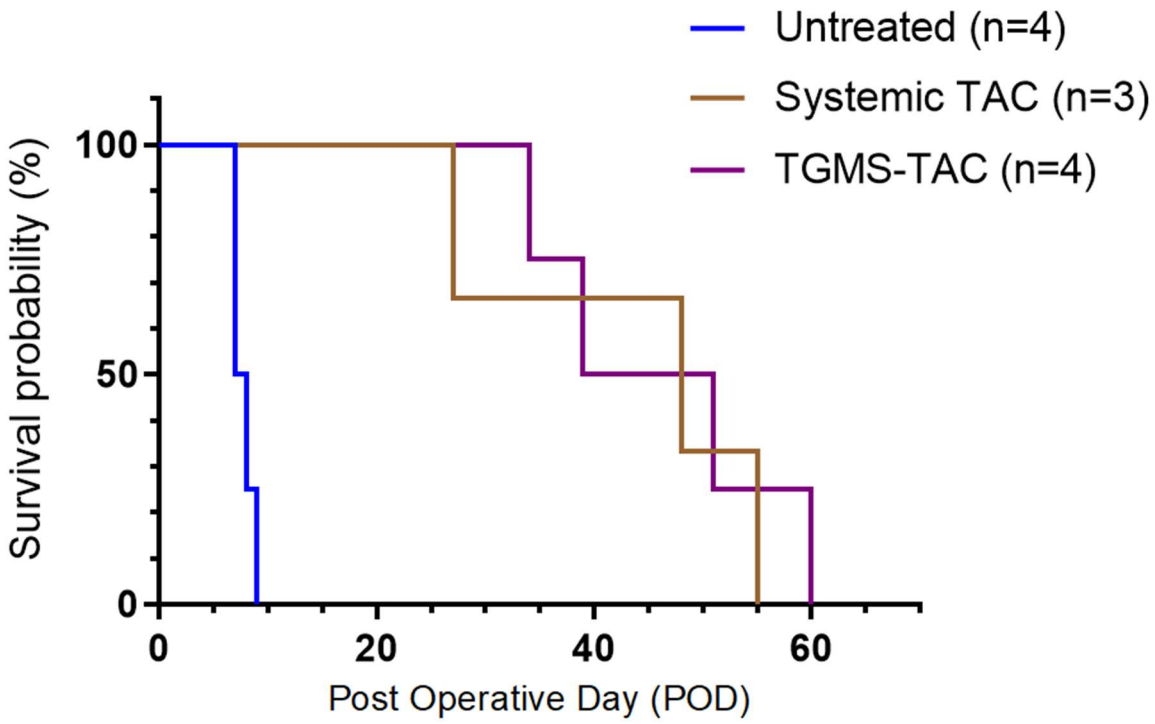


Figure 10: **Graphic representing the survival of the grafts in the different groups**. The grafts in the control group (n=4) reached grade III rejection around POD8. The grafts in the systemic (n=3) and TGMS-TAC (n=4) groups survived up to POD55 and POD60 respectively. The TGMS-TAC system increased graft survival compared to the control (p=0,0062).

4.3 Total Tacrolimus

In the systemic groups, tacrolimus was given daily for 14 days to achieve a target level of 10-15ng/mL of systemic tacrolimus. To this end, 1.0 to 1.4 mg/kg of tacrolimus was administered oraly to the pigs depending on their weight. At the end of the treatment a mean of 400 ± 42,46 mg of tacrolimus per animal was given to the systemic group. In the TGMS-TAC, 140 mg of tacrolimus per kg of graft was injected subcutaneously at POD0. With a mean graft weight of 439 ± 31,89g, the mean tacrolimus levels injected was 61 ± 4,359mg per pig. With our data, we can see that the TGMS-TAC system decreases greatly the amount of tacrolimus needed to avoid graft rejection (p=0,0002) (Fig.11).

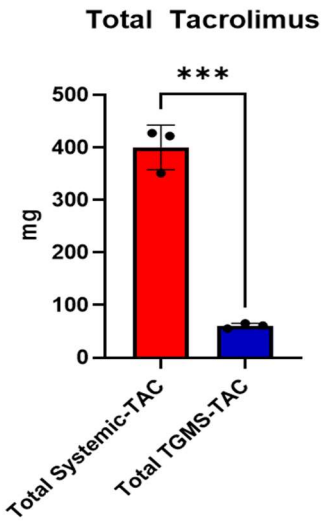


Figure 11: **Total amount of Tacrolimus.** The overall amount of tacrolimus given to both treated groups was calculated based on the dose needed for each treatment to avoid rejection. For systemic tacrolimus, the patients required 10-15ng of tacrolimus per ml of blood and it was achieved with daily administration of 1-1,4mg of tacrolimus per kg. In the TGMS-TAC group, tacrolimus was injected to reach 140mg of the drug per kg of graft. The total amount of tacrolimus given to each group was compared. Statistical analysis was done using the unpaired t-test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.4 Tacrolimus levels

In the systemic group, the tacrolimus levels in the blood were stable from the beginning of the oral administration, POD1, till the end of the treatment, POD14 (POD3: 8,333 ± 4,165; POD7: 11.067 ± 1,877; POD14: 10,433 ± 2,146 ng/ml). From POD21, the blood level of tacrolimus was not any more detectable. There was no difference in the skin levels of tacrolimus in the graft (POD7: 13,687 ± 9,603 ng/ml; POD14: 11,327 ± 7,760 ng/g) compared to the contralateral side (POD7: 9,617 ± 4,040; POD14: 9,270 ± 7,807 ng/g) at POD7 (p=0,9553) and POD14 (p=0,9938), suggesting the drug is distributed uniformly in the whole body. One week after the cessation of the treatment, the drug level both in the blood and the skin has dropped to 0.

In the TGMS-TAC group, we observed an initial burst of tacrolimus in the blood (42 ± 9,522 ng/ml) and in the skin (64,635 ± 21,726 ng/g) at POD7. The level of tacrolimus dropped in the blood at POD14 (10,650 ± 3,830 ng/ml) and kept decreasing until POD35 where the levels were under detection limit. The skin levels of tacrolimus were significantly higher in the graft (POD7: 64,535 ± 21,726; POD14: 27,915 ng/g) compared to the contralateral side (POD7: 19,045 ± 7,202; POD14: 6,905 ± 2,142 ng/g) both at POD7 (p=0,0001) and POD14 (p=0,0227). From POD35 on, the tacrolimus levels were not detectable anymore (Figure 12).

When comparing the systemic administration of the drug and the TGMS-TAC system, we can notice that the TGMS-TAC has locally significantly higher concentration of the drug in the skin of the graft at POD7 compared to the graft from the systemic group (p=0,0001). On the other hand, the TGMS-TAC group also has a higher concentration of the tacrolimus on the blood compared to the systemic group at POD1, 3 and 7 (all p values p=0,0001) and overall, the tacrolimus was present longer in the blood of the TGMS-TAC groups (Figure 12).

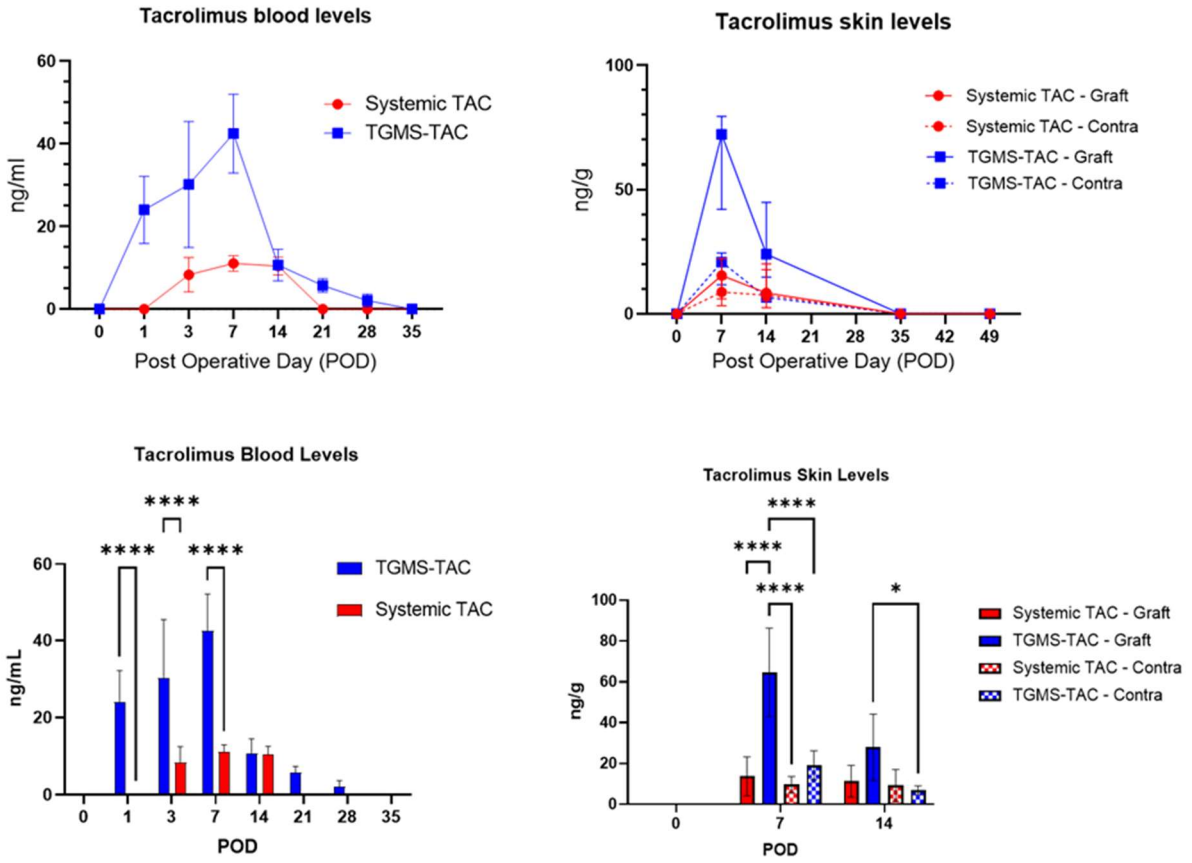


Figure 12: **Tacrolimus levels in blood and in skin**. The drug level was measured in the plasma at defined timepoint to compare the systemic distribution of tacrolimus in the body (ng/ml). To assess the local concentration of tacrolimus, skin punch biopsies were taken from the grafts and from the contralateral side (ng/g skin). For each timepoints, data was presented as mean ±SD (systemic, N=3 and TGMS-TAC, N=4). Statistical analysis was done by two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* p ≤ 0.05, ** p ≤0.01, *** p ≤ 0.001, **** p ≤ 0.0001).

4.5 Off Target Toxicity

The adverse effects of calcineurin inhibitors, i.e tacrolimus, include acute renal failure, cardiovascular disease caused by dyslipidemia and liver dysfunction.

For the off-target toxicity of tacrolimus and its nephrotoxicity, the plasma creatine levels were measured at defined timepoint throughout the study. Triglycerides and cholesterol levels were also assessed to evaluate the effect of the drug on the lipid homeostasis. Alanine transaminase (ALAT) and aspartate transaminase (ASAT) were used to measure the liver function and its potential damages. All the markers for kidney and liver function were measured and analyzed at the Center of Laboratory Medicine at the University Hospital of Bern using liquid chromatography mass spectrometry.

The treated groups follow the same curve pattern and the amount of cholesterol, triglycerides, ALAT, ASAT and creatinine were not high enough to cause off-target toxicity. There was no significant difference in the levels of the biochemicals parameters measured between the systemic tacrolimus group and the TGMS-TAC, suggesting that systemic tacrolimus did not cause off-target toxicity for the duration of the experiment. (Figure 13)

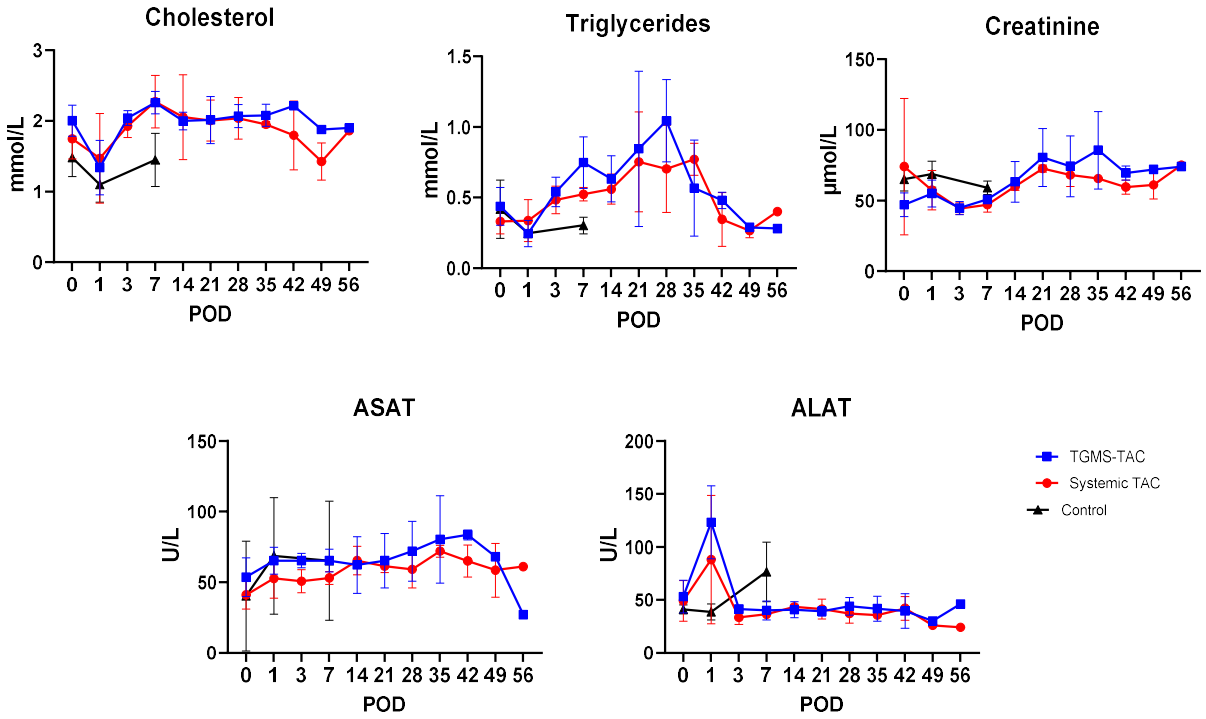


Figure 13: **Biochemical markers used to evaluate the off-target toxicity.** To evaluate the potential off-target toxicity of tacrolimus, plasma levels of cholesterol, triglyceride, ALAT, creatinine and ASAT, were measured at defined timepoint by using liquid chromatography mass spectrometry. The biochemicals were measured in the recipients from the control (N=4), systemic (n=3) and TGMS-TAC (n=4) group in the plasma via liquid chromatography mass spectrometry. Data are presented as mean ± SD.

4.6 Cytokine levels

To explore the rejection mechanisms, the cytokines were measured at defined timepoints in the different groups. 15 cytokines were selected as follows: pro-inflammatory cytokines IL-1α, IL-1β, IL-6; the receptor antagonist IL-ra which inhibit the action of IL-1α and IL-1β; T cell regulatory cytokines such as IL-2, IL-4, IL-12, IL-18; Innate immunity regulatory cytokine like IL-8 and MCP-1; the growth factors bFGF and VEGF; two complement system proteins, C5a and sC5b-9 and anti-inflammatory cytokine IL-10.

It was shown that the pro-inflammatory cytokine IL-1β significantly increased at endpoint both in the systemic (p= 0,0124) and TGMS-TAC (p= 0,0341) group (See supplementary figure 1). IL-1β levels were the highest at POD3 in the untreated group (0,065 ± 0,078 ng/ml) which may correlate with the start of the graft rejection because the pigs rejected 4-5 days later (Baseline vs POD3 p=0,0477) (Figure 14). In the TGMS-TAC group, IL-1β level remained stable till POD21 (0,093 ± 0,111 ng/ml), then dropped until POD49 (0,049 ± 0 ng/ml). From this timepoint, the TGMS-TAC pig 11 started rejecting and its cytokine level increased until endpoint. IL-1β level was increased and reached a peak at POD7 (0,06

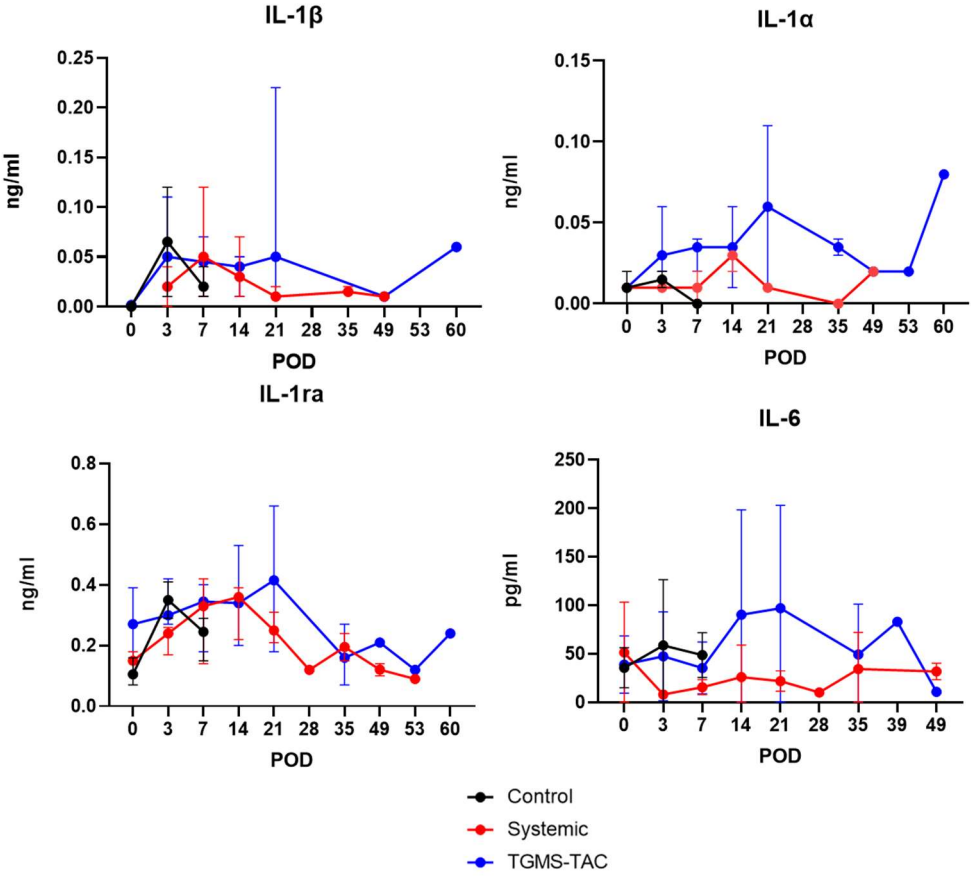


Figure 14: **Pro-inflammatory cytokines levels**. The plasma levels of selected cytokines were measured by suing Milliplex (IL-1α, IL-1β and IL-1ra) and Bio-plex (IL-6) and analysed with Luminex multiplex assay.-Data are presented as median ± SD (N=4 for control, N=3 for systemic, N=4 for TGMS-TAC group).

± 0,056 ng/ml) in the systemic group, and then rapidly decreased overtime. This peak of IL-1β level at POD7 however did not corelate with any acute rejection episode (Figure 14).

IL-1α is an equally compelling pro-inflammatory cytokine as IL-1β. It is produced by monocytes as well as macrophages, endothelial cells and lymphocytes upon for example traumatic injury (Di Paolo & Shayakhmetov, 2016). In this current study, it was shown that IL-1α level was higher in the TGMS-TAC groups during the whole follow-up when compared to the systemic group (p=0,0258). In the TGMS-TAC group, there was an increase in IL-1α level at POD21 (0,06± 0,048 ng/ml), but might not be due to the rejection because pigs rejected between 2-5 weeks later. The second peak IL-1α level at POD60 (0,08± 0 ng/ml) may possibly corelated with rejection of the last surviving pig of this group, pig 11 (pig 11 baseline vs POD60 p=0,0269). In the systemic group, the small increase of IL-1α at POD49 (0,02± ng/ml) may be due to the rejection however, it was not significantly higher compared with baseline (p=0,8553) (Figure 14).

IL-1 receptor antagonist (IL-1ra) is secreted in the acute phase of inflammation in order to modulate the inflammatory response by prohibiting the action of IL-1α and IL-1β(Mehta et al., 2019). IL-1ra secretion varies throughout the follow-up. IL-1ra levels were increased over the time and reached their highest levels at POD14 (0,323 ± 0,091 ng/ml) in the systemic group (compare with baseline p=0,1371) and POD21 (0,418 ± 0,233 ng/ml) in the TGMS-TAC group (compared with baseline p=0,989). These variations may correlate with the increase of IL-1α and IL-1β at these different timepoints. Then, the levels drop in both groups with some small variation towards the endpoints (Figure 14).

IL-6 is produced at the site of tissue injury and promotes acute inflammation reaction. There is a minimum increase in IL-6 levels in the control and TGMS-TAC group at POD3 (control: 58,595 ± 67,691; TGMS-TAC: 47,336 ± 45,848 pg/ml) however it did not reach statistical significance when comparing with baseline. The systemic group show steady levels thought the different timepoints. In the TGMS-TAC group, two peaks were observed, the first one around POD21 (97,075 ± 105,855 pg/ml) and the second at POD39 (83.09 ± 51,88 pg/ml). The first peak does not relate to an episode of acute rejection, Pig 11 which shows the highest amount of interleukin 6 at POD 21 only rejected at POD60. The second peaks, on the other hand, could match with the acute rejection episode where the one pig having the highest level of IL-6, pig 8, rejected at POD39 (83,09 pg/ml) (Pig 8: baseline vs POD39, p= 0,2720) (Figure 14).

The innate immune system plays an important role as the first line of defense against pathogens. In transplantation, they are the first to react to tissue injury following the surgery and recognize foreign antigen and thus initiate the cell-mediated rejection process via antigen presentation (Ref). Interleukin 8 (IL-8) is a potent chemoattractant and activator for neutrophils (Borst et al., 2015, p. 8). It was shown that IL-8 level was increased at POD3 in the untreated group (0,04 ± 0,014 ng/ml) which correlated with acute rejection in the pigs from this group. However, it did not reach statistical significance compare to the baseline (control: baseline vs POD3, p= 0,0714) In the treated group, levels of IL-8 stayed unchanged except at POD14 (0,04 +0,14 ng/ml) in the TGMS-TAC and POD21 (0,04 ± 0 ng/ml) in the systemic group. There was no correlation in those cases with episode of acute rejection, which was observed only 2-5 weeks later. There was a high level of IL-8 in pig 11 from the TGMS-TAC group at POD14 (0,05 ng/ml) but this pig rejected at POD60, and in pig 9 from the systemic group with a peak at POD21 (0,04 ng/ml) but this pig rejected at POD55 (Figure 15).

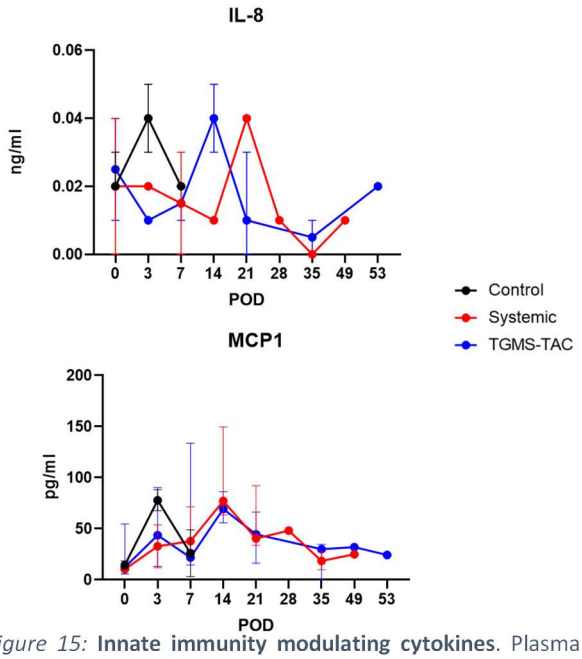


Figure 15: **Innate immunity modulating cytokines**. Plasma levels of Interleukin 8 (IL-8) and monocyte chemokine protein 1 (MCP-1) were measured by using Milliplex and Bio-plex respectively. Data are presented in nanogram (ng) and picogram (pg) per milliliter as median ± SD (N=4 for control, N=3 for systemic, and N=4 for TGMS-TAC group).

Monocyte chemokine protein 1 (MCP-1) regulates the migration and infiltration of monocytes and macrophages (Deshmane et al., 2009). We showed that MCP-1 was increased and reached the peak in all groups at POD3 (control: 77,930 ± 14,651 pg/ml; systemic: 32,855 ± 29,748 pg/ml; TGMS-TAC: 47,718 ± 35,530 pg/ml), suggesting an upregulation of this chemokine in the inflammatory stage after transplantation (control: baseline vs POD3, p= 0,0293; systemic: baseline vs POD3, p=0,2230; TGMS-TAC: baseline vs POD3, p=0,2535). In the control group it may even suggest an upregulation of MCP-1 in the acute phase of rejection with a significant increase prior of reaching grade III rejection (p=0,0293). There was a significant increase in MCP-1 levels in both treated groups at POD14 (systemic: 96,787 ± 46,256; TGMS-TAC: 70,218 ± 12,774 pg/ml) (systemic: baseline vs POD14, p=0,0301; TGMS-TAC: baseline vs POD14, p=0,0,0097), without any acute rejection in any of the pigs in the following days (Figure 15). Overall in the systemic group, there was also an increase in MCP-1 at endpoint compared with baseline (p=0,0037) (see complementary Figure 1).

To further study the role of tacrolimus on T cell mediated immune response, two cytokines, IL-2 and IL-4, that are directly prohibited by tacrolimus, were measured by using Multiplex. IL-2 is crucial for T

cells in their differentiation, activation and maintenance. IL-2 kept low level in the systemic tacrolimus group, except at POD14 (0,06 ± 0,04 ng/ml). The TGMS-TAC group show a non-significant increase of IL-2 expression at POD3 (0,145 ± 0,123 ng/ml)(TGMS-TAC: baseline vs POD3, p=0,1901), then the amount of the cytokine decreases till POD53 (0,01 ± 0 ng/ml). Only in one pig of the TGMS-TAC group, pig 11, there was a high level of IL-2 expression at the time of rejection, POD60 (0,11 ± 0 ng/ml). The p value comparing the baseline and POD60 of pig 11 is significant (p=0,0099) (Figure 16). On the other hand, that is the only pig where IL-2 is significantly increased at rejection time.

IL-4 maintained at lower level in the systemic TAC group even after the cessation of the oral administration of the drug compared to the TGMS-TAC group. There was an increase in IL-4 levels at POD21 (0,483 ± 0,433 ng/ml), however it did not reach significance (TGMS-TAC: baseline vs POD21, p=0,1031). This could be due to the fact that in one group tacrolimus worked systematically on the whole body while in the TGMS-TAC group, tacrolimus only acted locally in the grafts with little effect on the rest of the immune system (Figure 16). Overall, in the TGMS-TAC group, IL-4 levels were significantly increased at endpoint compared to baseline (p=0,0379) (see complementary Figure 1).

Important also for the T cells immune response are IL-12 and IL-18. IL-12 is secreted by activated macrophage as well as dendritic cells and neutrophils. In turn, IL-12 and IL-18 activate T cell mediated immunity by promoting the differentiation of Th1 cell and their production of IFN-γ(Tominaga et al.,

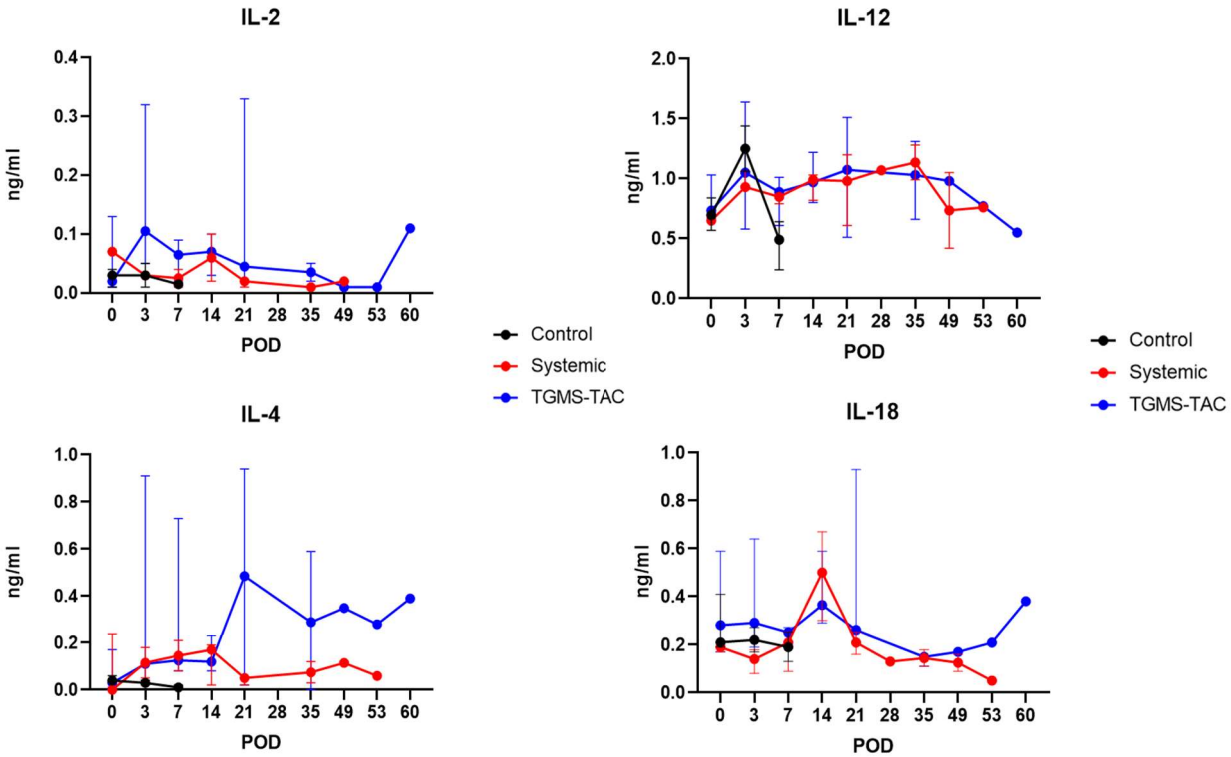


Figure 16: **Cytokines inhibited by Tacrolimus and T cell related cytokines.** Il-2 and IL-4 are cytokines affected by the drug tacrolimus which prohibits their production and secretion in T cells. IL-12 and IL-18 are cytokines that are important for T cell differentiation. The plasma levels of these cytokines were measured from all groups by using Multiplex. Data are presented in nanogram (ng) per milliliter (ml) as median ± SD (N=4 for control, N=3 for systemic, and N=4 for TGMS-TAC group).

2000, p. 12). There was a significant increase level of IL-12 in the untreated group at POD3 (1,25 ± 0,269 ng/ml), when acute inflammation reaction happened in the graft (control: baseline vs POD3, p=0,0259). In the treated group, IL-12 level also significantly increased at POD3 (systemic: 0,963 ± 0,085 ng/ml; TGMS-TAC: 1,08 ± 0,461 ng/ml) (systemic: baseline vs POD3, p=0,0046; TGMS-TAC: baseline vs POD3, p=0,2840). Then maintained unchanged till POD35 (systemic: 1,135 ± 0,205 ng/ml; TGMS-TAC: 1 ± 0,326 ng/ml) and decreased till the endpoint. As for IL-18, there was an increase in both treated groups at POD14 (systemic: 0,49 ± 0,185 ng/ml; TGMS-TAC: 0,403 ± 0,13 ng/ml) (Systemic: baseline vs POD14, p=0,0,458; TGMS-TAC: baseline vs POD14, p=0,5439). The levels then decreased from POD21 on (systemic: 0,207 ± 0,045 ng/ml; TGMS-TAC: 0,415 ± 0,345 ng/ml) (Figure 16).

In transplantation, growth factors have a broad range of action from wound healing after tissue injury, cell proliferation and survival as well as their migration. Moreover, basic Fibroblast Growth Factor

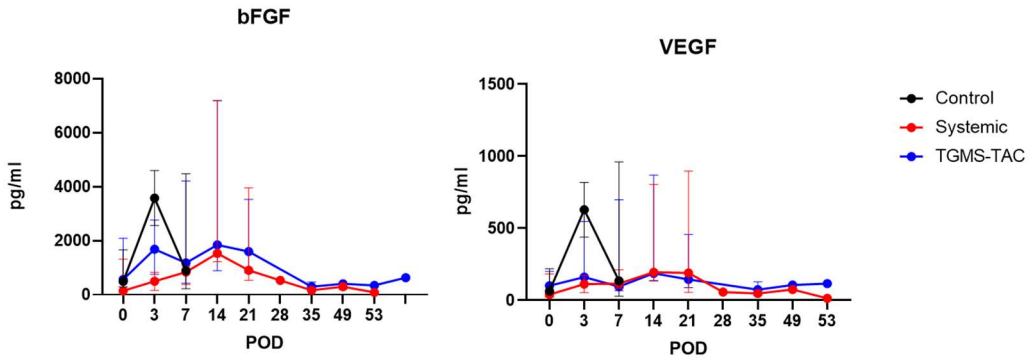


Figure 17: **Growth factors**. The growth factors, Basic Fibroblast Growth Factor (bFGF) and Vascular endothelial growth factor (VEGF), were acquired in plasma samples from the control (N=4), systemic (N=3) and the TGMS-TAC (N=4) groups and analysed with Bio-plex. Data are presented in picogram (pg) per milliliter (ml) and showing median value ± SD.

(bFGF) and Vascular endothelial growth factor (VEGF) may promote immunoinflammatory response and angiogenesis following transplantation (Zhang et al., 2014).

bFGF in the control and the TGMS-TAC group shows an increase at POD3 (Control: 3579,485 ± 1438,206; TGMS-TAC: 1720,678 ± 921,079 pg/ml) (control: baseline vs POD3, p=0,0219; TGMS-TAC: baseline vs POD3, p=0,2195). The levels of bFGF have a non-significant peak at POD14 in both treated groups (systemic: 3315,280 ± 3361,466; TGMS-TAC: 2940,340 ± 2928,923 pg/ml) (Systemic: baseline vs POD14, p=0,2020; TGMS-TAC: baseline vs POD14, p=0,3703) and then decreased till the endpoint (Figure 17).

Concerning VEGF, in the untreated group, there was a significant increase in the level of VEGF at POD3 (628,86 ± 267,343 pg/ml) (control: baseline vs POD3, p=0,0154). In both treated groups, VEGF kept unchanged throughout the follow-up (Figure 17).

The complement system has a wide role both in the innate and adaptive immune system. Studies have shown that complement activation contributes to allograft injury in several clinical settings, including ischemia/reperfusion injury, antibody mediated rejection and modulates T and B cells response (Grafals & Thurman, 2019). It is also potent chemoattractant for inflammatory cells such as neutrophils and monocyte as well as T cells and thus contribute to acute and chronic inflammation(Asgari et al., 2010). Here, we aimed to explore whether these complement components were involved in acute rejection. It was shown that sC5a levels remained unchanged in the systemic group while in the TGMS-TAC group, there was a peak at POD14 (801,248 ± 610,341 pg/ml) (TGMS-TAC: baseline vs POD14, p=0,4229) which then decreased till POD35 (439,86 ± 512,199pg/ml) and then maintained at the same level until the endpoint. In the other hand, sC5b-9 levels are more tumultuous. It significantly increased in the TGMS-TAC group at POD3(10,795 ± 4,922pg/ml) (TGMS-TAC: baseline vs POD 3, p=0,0093) and POD49 (15,250 ± 0pg/ml) (TGMS-TAC: baseline vs POD49, p=0,0564). In the systemic group, the peaks are at POD14 (11,053 ± 3,941pg/ml) (systemic: baseline vs POD14, p=0,1040) and POD49 (7,825 ± 5,24pg/ml) (systemic: baseline vs POD49, p=0,5244). In both treated groups, pigs showing the higher level of sC5b-9 at POD49 reached grade III rejection between 2-5 days later (Figure 18).

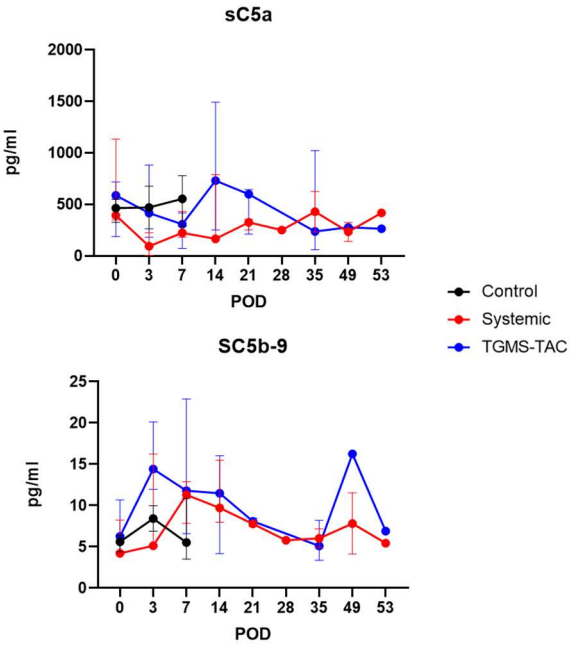


Figure 18: **Complement system proteins.** sC5a and sC5b-9 protein levels were measure in plasma samples from the control (N=4), systemic (N=3) and the TGMS-TAC (N=4) groups and analysed with Bio-plex. Data are presented in picogram (pg) per milliliter (ml) and showing median value ± SD.

IL-10 is an anti-inflammatory cytokine that down-regulates Th1 cells as well as NK cells and macrophages (Ref). In this current study, IL-10 maintained at low level and unchanged during the whole follow-up of the pigs in both untreated and treated groups (Figure 19).

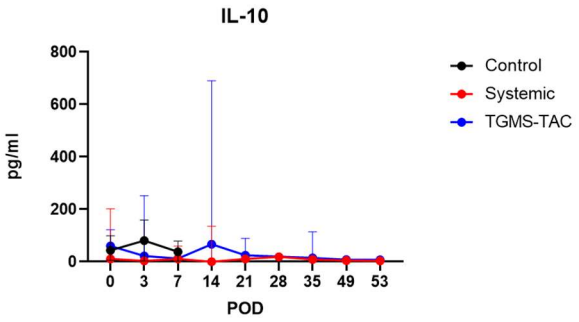


Figure 19: **Anti-inflammatory cytokine.** Plasma levels of IL-10 were measured by using Bio-plex. Data are presented in picogram (pg) per milliliter (ml) as median ± SD (N=4 for control, N=3 for systemic, and N=4 for TGMS-TAC group).

4.7 Flow cytometry

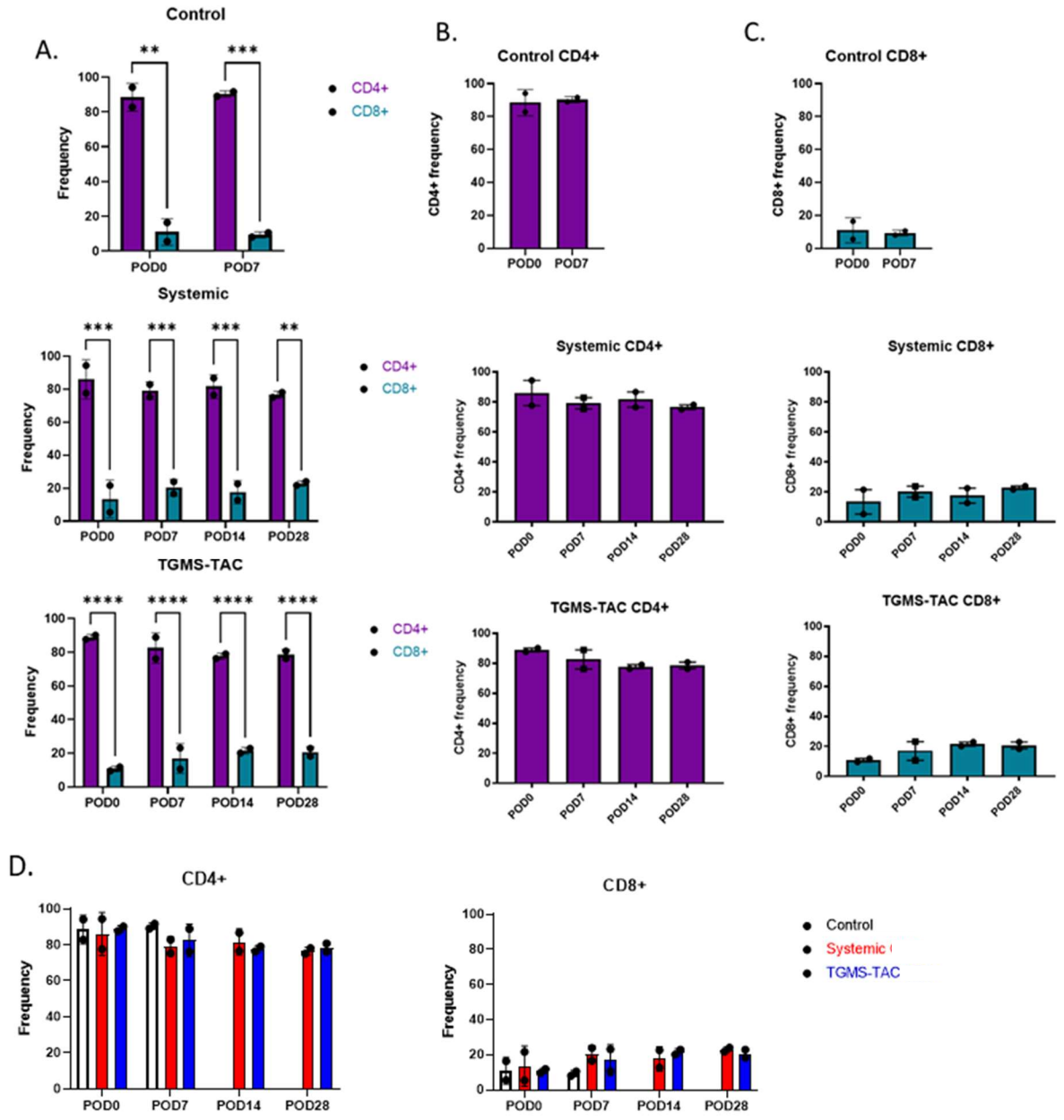


Figure 30: **Peripheral CD4⁺ and CD8⁺ T cells.** The amount of CD4⁺ and CD8⁺ T cells was measure in the blood at different time throughout the study. A. Comparison of CD4⁺ vs CD8⁺ frequency. B. Comparison of CD4⁺ frequency at different timepoint. C: Comparison of CD8⁺ frequency at different timepoint. D. comparison of CD4⁺ and CD8⁺ T cells between all groups. Statistical analysis were done with a two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented showing the mean values ± SD. N=2 for control, N=2 for systemic and N=2 for TGMS-TAC.

The peripheric number of CD4⁺ T cells were significantly higher compared to CD8⁺ T cells in the control, systemic and TGMS-TAC group at all timepoints (figure 20, A). There were no differences in the amount of CD4⁺ or CD8⁺ T cells between the various timepoints (Figure 20, B and C). When comparing the different treatments, they all have similar levels of both CD4⁺ and CD8⁺ cells from POD0 to POD7 and then from POD14 to POD28 between the systemic and TGMS-TAC group (Figure 20, D).

4.8 Histology

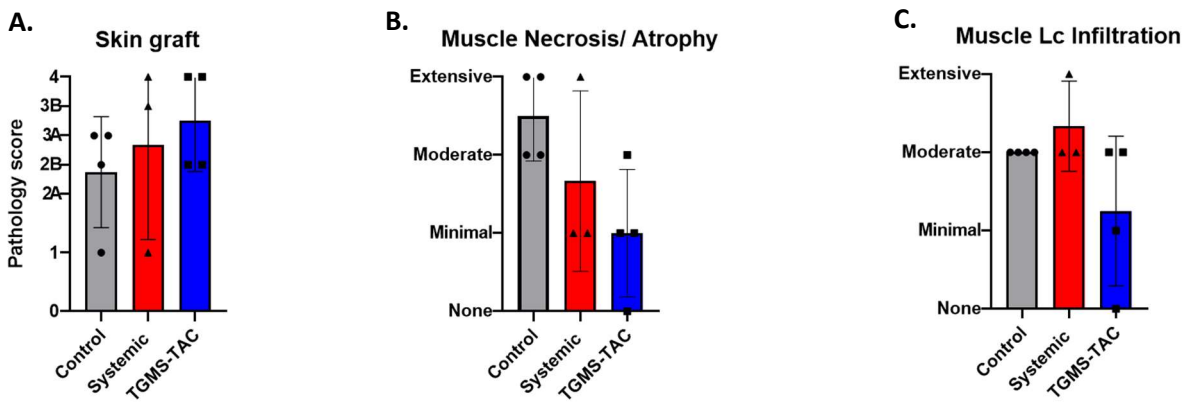


Figure 21: **Histological evaluation**. A. Skin from the graft was graded based on the Swine VCA skin rejection classification (modified Banff criteria). Levels of muscle changes from the graft area was evaluated based on the extend of B. necrosis and atrophy and C. Leukocytes infiltration. Statistical analysis was done using one-way ANOVA with Turkey multiple comparison test. p value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). N=4 for control, N=3 for systemic, N=4 for TGMS-TAC.

To further investigate the mechanism of rejection, rejected skin and muscle were histologically evaluated. For each pig, a score was given by a pathologist from the institute for pathology of the university of Bern. In the untreated group, 75% of the subject had mild to moderate dermal inflammation with multifocal epidermal necrosis and inflammatory cells infiltration. As for their muscle samples, half of the pigs showed signs of edema and perivascular inflammation while the other half had severe muscle damage including extensive necrosis, atrophy, and inflammation. All of the untreated pigs also had moderate leukocytes (Lc) infiltration in the muscle. In the systemic treatment, two of the animals had severe dermal inflammation with signs of extensive necrosis in the epidermis. While one of these two pigs also had extensive muscle necrosis and Lc infiltration, the other pig only had minimal muscle necrosis and atrophy with moderate Lc infiltration. The third pig of the systemic group on the other hand only showed signs of mild skin inflammation with minimal muscle necrosis and atrophy, and moderate leukocyte infiltration. In the TGMS-TAC group, half of the pigs presented signs of moderate dermal inflammation without any epidermal involvement, in addition to minimal muscle necrosis/atrophy, and minimal to moderate cells infiltration. The other half had severe dermal inflammation with extensive epidermal necrosis. Their muscles, however, showed none to moderate muscle necrosis with no to moderate Lc infiltration (Figure 21).

Overall, there were no significant differences in the skin pathological score between the TGMS-TAC group and the control (p=0,5398) or with the systemic group (p=0,8801), and no significant difference in the muscle Lc infiltration between the TGMS-TAC and the control (p=0,2912) or the systemic group (p=0,1367). Nevertheless, there is favorable trend showing the decrease in the severity of muscle necrosis/atrophy in the TGMS-TAC group compared to the control (p=0,0821) (Figure 21).

4.9 Immunofluorescence

Immunofluorescence staining was used to characterize the local immune responses in the graft. Skin and muscle from the donors and from the graft of the transplanted animals were stained with different markers and the groups were compared.

4.9.1 Cell infiltration and endothelial cell activation

Cell infiltration was observed in the treated group compared to the untreated one, suggesting immune cell recruitment in the graft. In the next step, we evaluated whether the endothelial cells were actively recruiting immune cells in the graft and thus were expressing adhesion molecules. Therefore e-selectin was used as a marker for endothelial cell (EC) activation. The treated group shows substantial EC activation compared to the donor and control group (Figure 22).

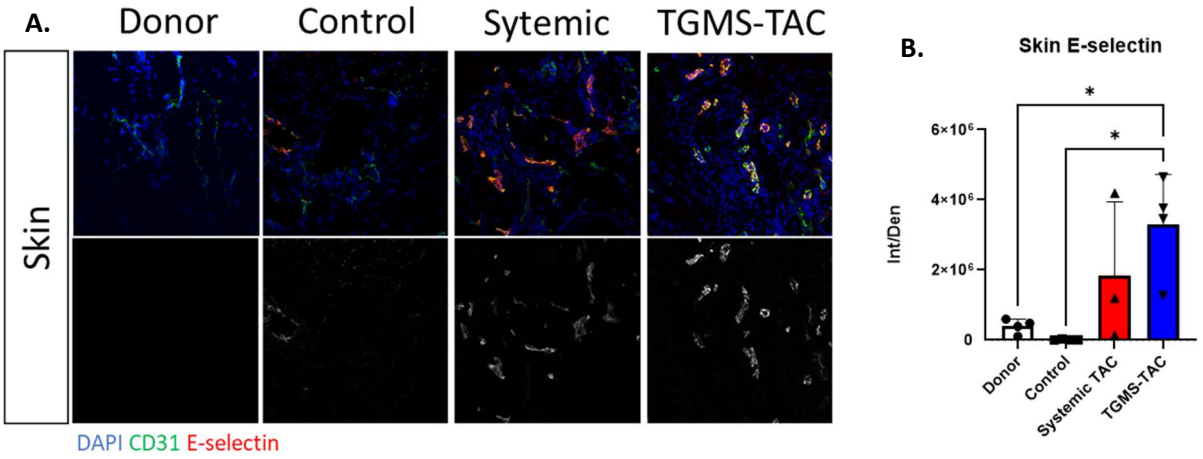


Figure 22: **Immuno-staining of E-selectin in the skin**. Skin from the donors and from the grafts of the transplanted animals were stained for e-selectin to visualize the level of endothelial cell (EC) activation. A, Representative pictures of immuno-staining of E-selectin. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group). One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. p value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.9.2 Innate immune system investigation of the graft

For the innate immune responses, myeloperoxidase (MPO) was evaluated as a marker for neutrophils and CD163 for macrophages.

In the skin, we showed a significantly higher number of neutrophils in the TGMS-TAC group when compared to the donors and un-treated group. There was also neutrophil infiltration in the skin in the systemic group but the difference with the other groups was not significant. In the un-treated group, there were significantly higher number of neutrophils in the muscle compared to those in the skin. For

the donors and treated group, there were no significant difference in the numbers of neutrophils between the skin and the muscle (Figure 23).

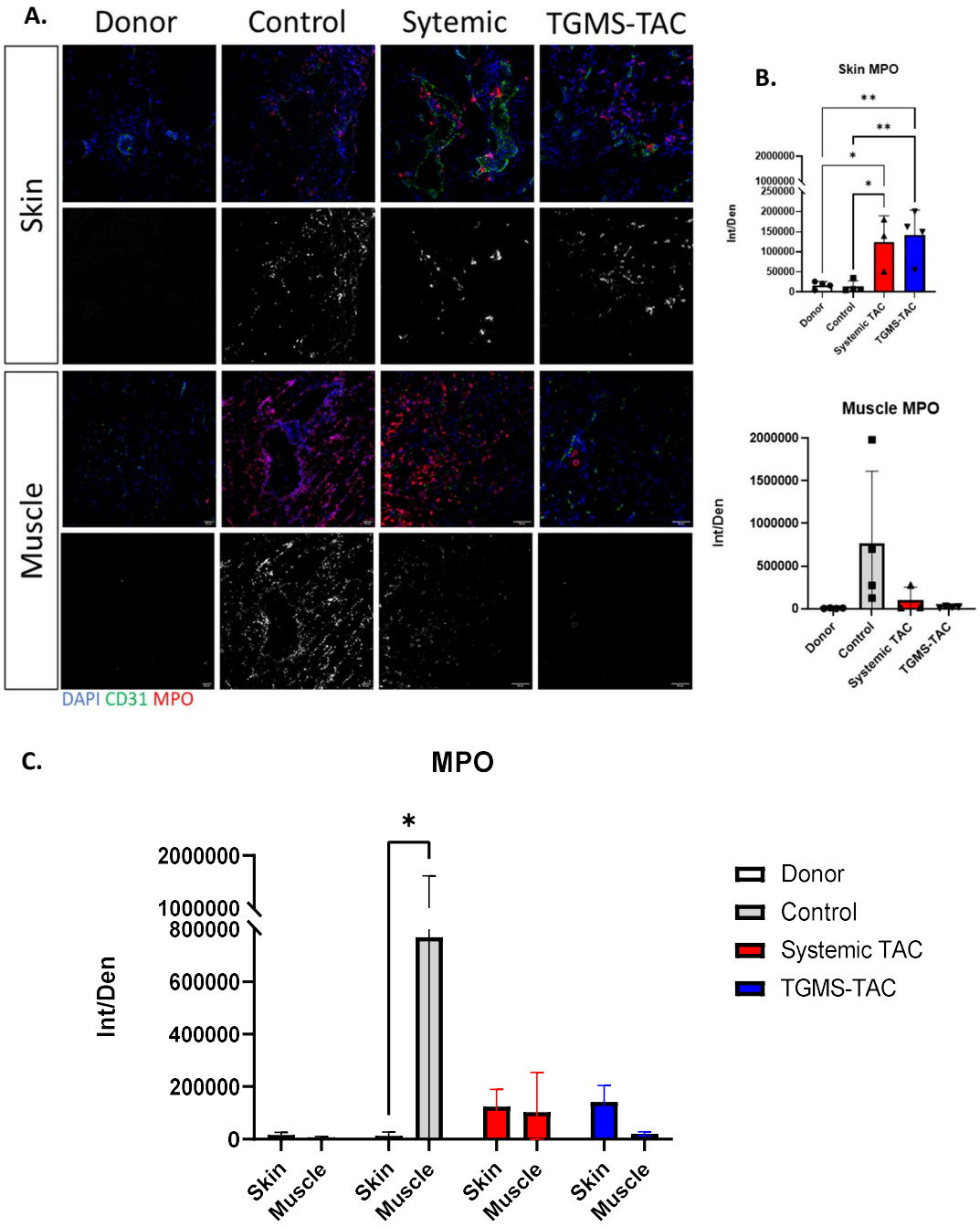


Figure 23: **Immunofluorescence of neutrophils with MPO**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with MPO to assess the presence of neutrophils. A, Representative pictures of immuno-staining of MPO. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of MPO in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

There were higher numbers of macrophages in the un-treated and treated groups when compared to the donors. However, there were no significant difference between the groups in the skin. In the muscle, there were significantly more macrophages from the pigs which receive the systemic tacrolimus when compared to the donors and the TGMS-TAC group. Comparing the neutrophils and macrophages between the skin and muscle from each group, only the TGMS-TAC group showed a significantly higher number of neutrophils in their skin than in their muscle (Figure 24).

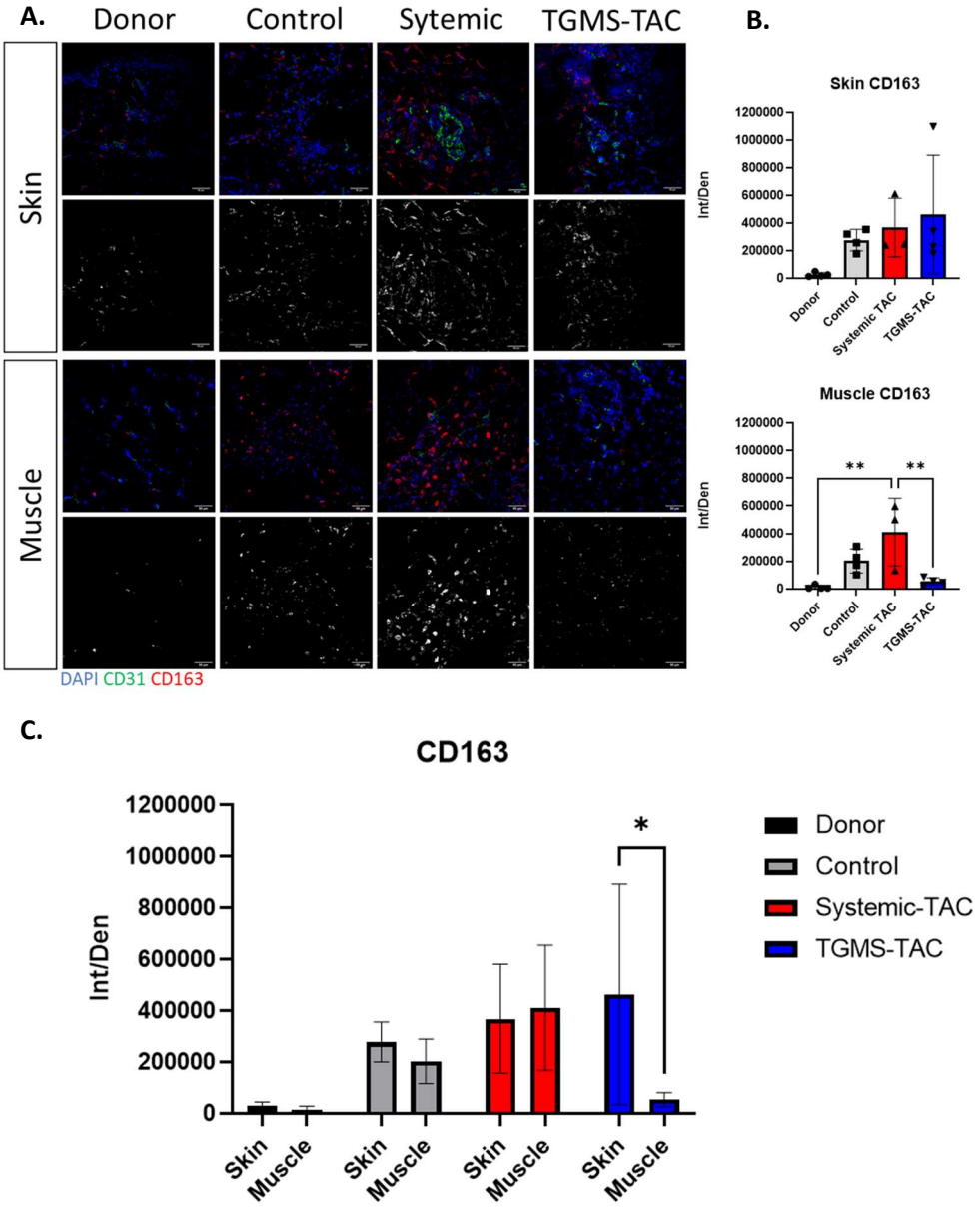


Figure 24: **Immunofluorescence of macrophage**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with CD163 to assess the presence of macrophages. A, Representative pictures of immunostaining of CD163. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of CD163 in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

4.9.3 The local adaptive immune system

The involvement of the adaptive immune system in rejection was assessed by the presence of T and B cells. CD3 was used as a general marker for T cells, and CD4 as a marker for $CD4^+$ T cells and CD79a for B cells.

In the skin and muscle, there was no significant difference in T cell numbers between the donors, untreated and treated groups. However, $CD4^+$ T cells in the skin from the systemic group were significantly higher compared to the donor group. In the muscle, there were more $CD4^+$ T cells in the systemic group compared to other groups. $CD4^+$ T cells equally distributed in the skin and the muscle from each pig (Figure 25, 26).

In the skin, there were higher numbers of B cells in all the experimental groups compared to the donors. Though, only the TGMS-TAC pigs had a significantly higher number of B cells compared to the donors. In the muscle, there were no significant difference in B cell numbers between the groups. However, when comparing skin and muscle within each group, there were significantly more B cells the TGMS-TAC group in in their skin than in their muscle (Figure 27).

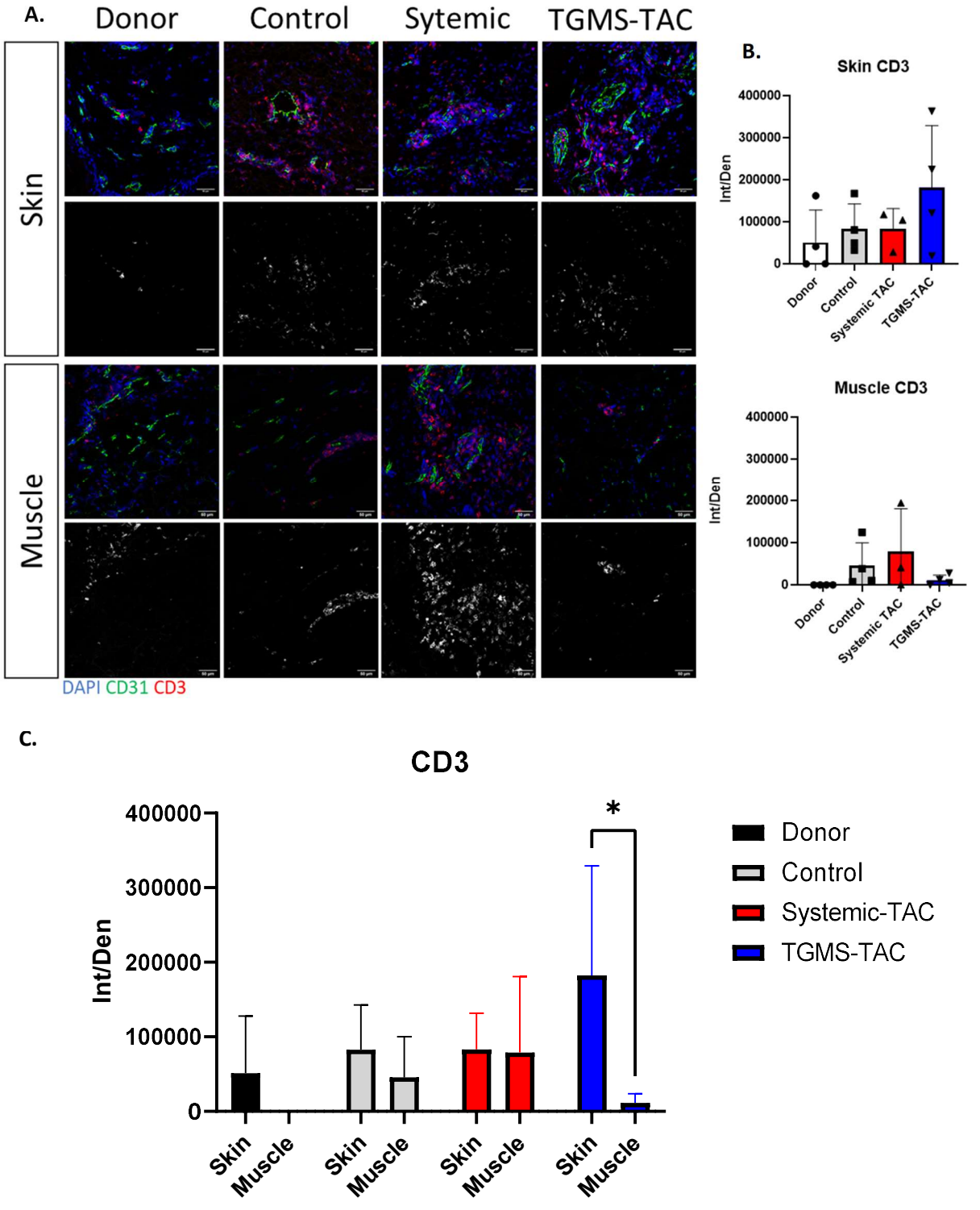


Figure 25: **Immunofluorescence of T cells with CD3**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with CD3 to assess the total number of T cell present. A, Representative pictures of immuno-staining of CD3. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of CD3 in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

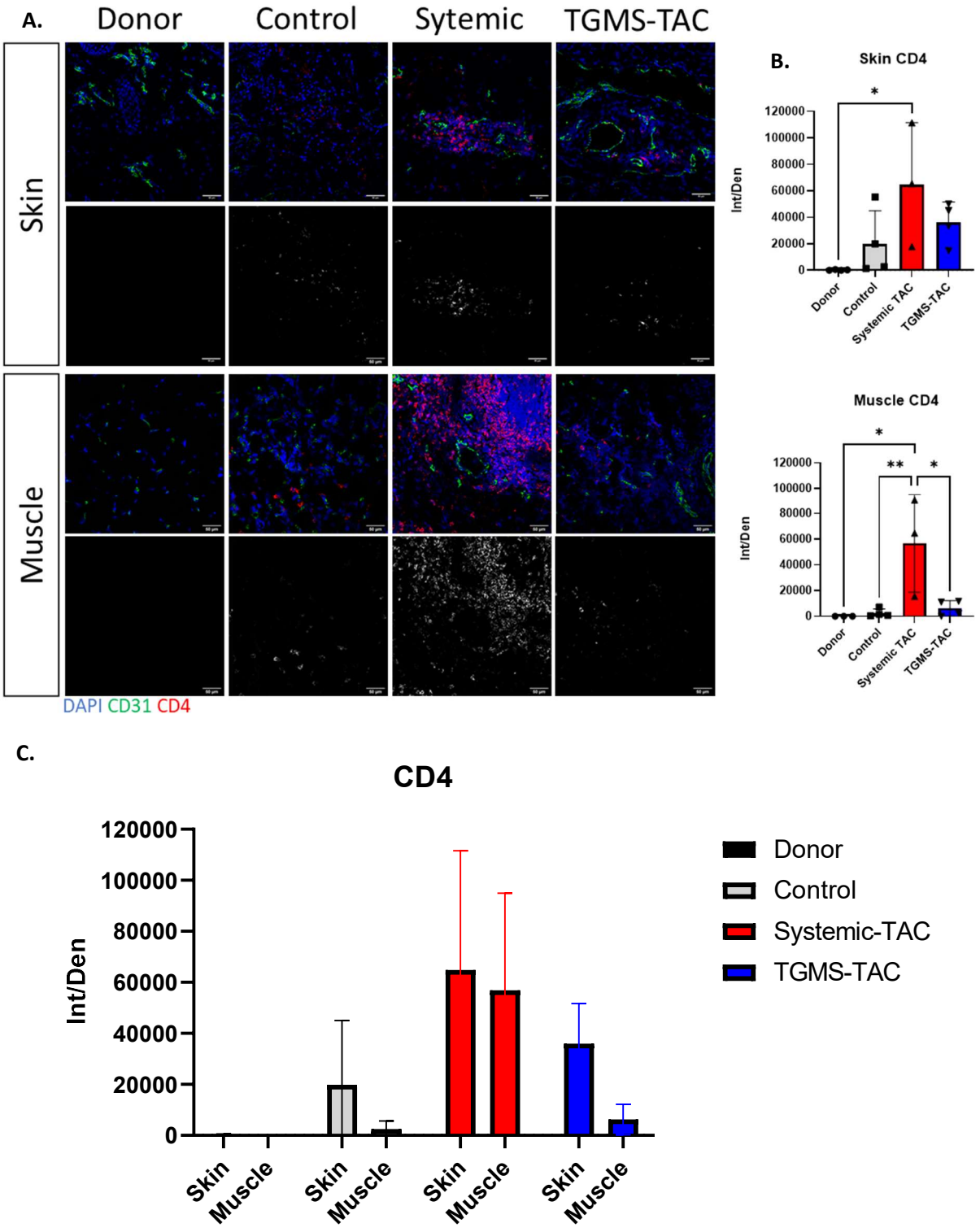


Figure 26: **Immunofluorescence of T helper cells with CD4**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with CD4 to assess the presence of Th4 cells. A, Representative pictures of immuno-staining of CD4. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of CD4 in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

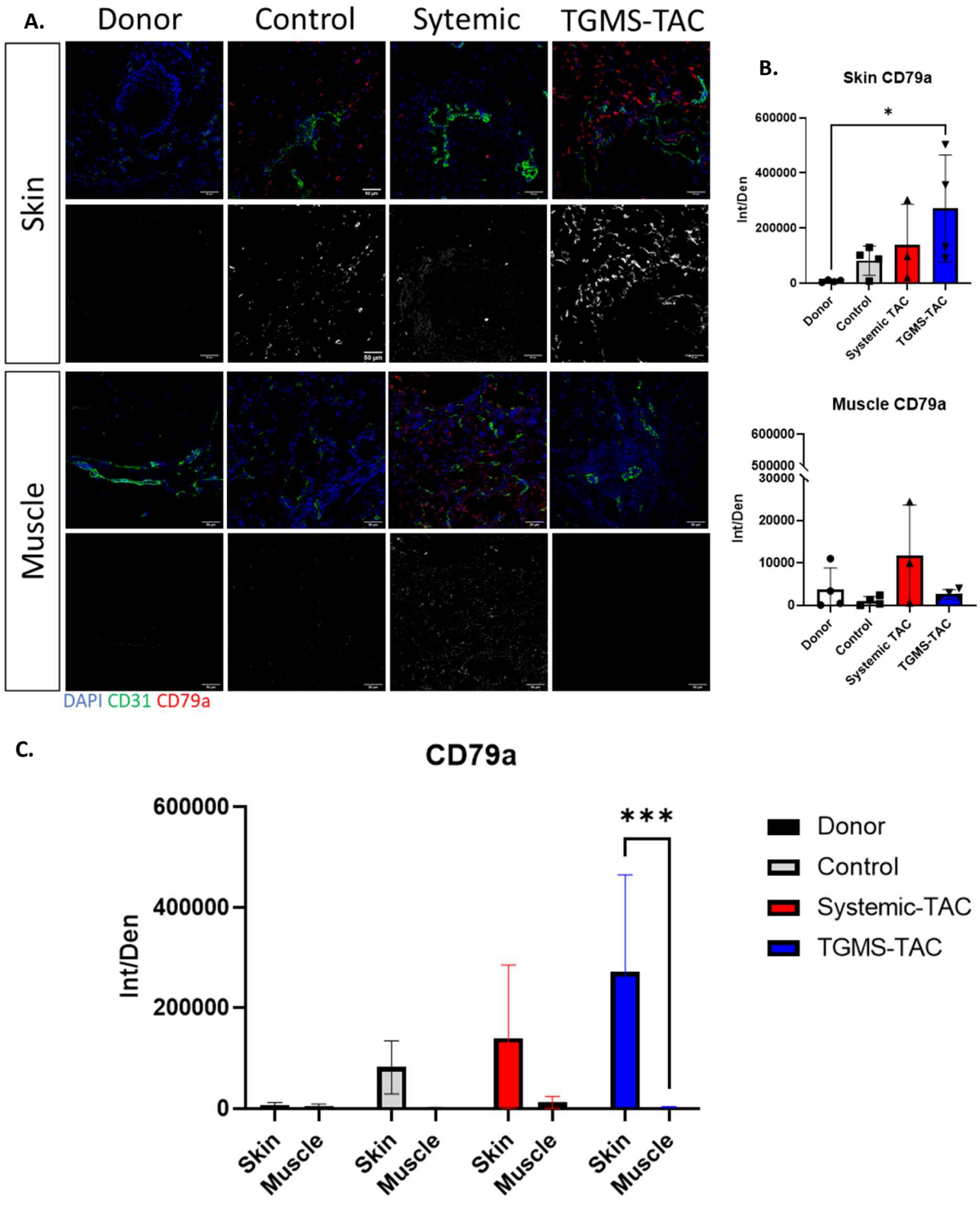


Figure 27: **Immunofluorescence of B cells with CD79a**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with CD79a to assess the presence of B cells. A, Representative pictures of immuno-staining of CD79a. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of CD79a in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

4.9.4 The complement system activation in the graft

Complement system deposition was investigated using the markers C1q, C3c and sC5b-9.

It was shown that there was no significant difference in C1q, C3c deposition either in the skin or in the muscle from the different groups. (Figures 29, 30). However, C5b-9 levels were significantly higher in the skin from un-treated group compared to any other groups (Figure 28).

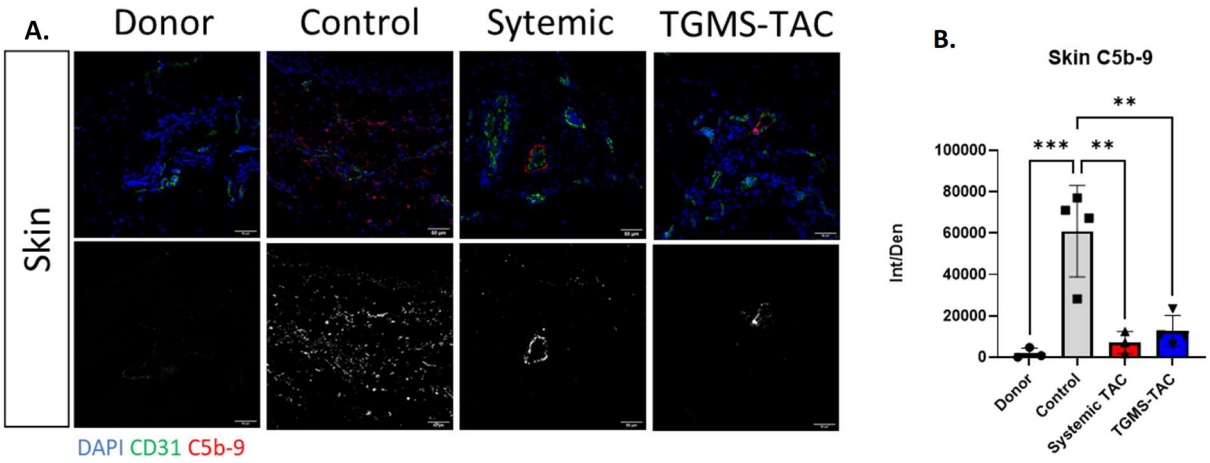


Figure 28: **Skin immunofluorescence analysis of C5b-9**. Skin from the donor and from the graft of the transplanted animals were stained using the complement marker C5b-9. A. For each group, a representative picture shows the amount of activation of EC. B. Immunofluorescence quantification was obtain using ImageJ software by measuring the integrated density. Each dots represent the mean integrated density from 5 pictures taken for each pig from the control (N=4), systemic (N=3) and the TGMS-TAC (N=4) groups. Data are presented showing the mean values ± SD. Statistical analysis was done by one-way ANOVA with Turkey multiple comparison test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

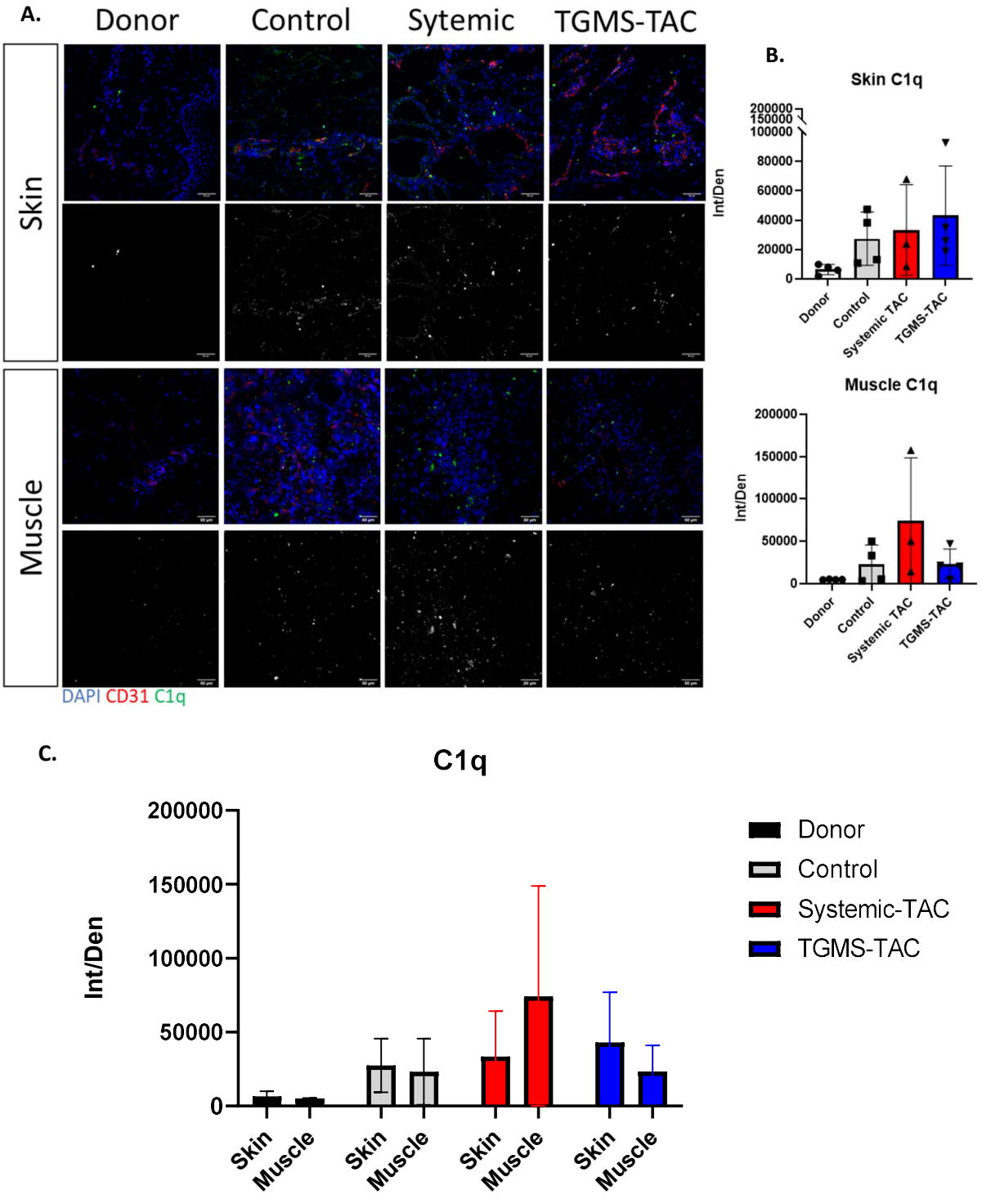


Figure 29: **Immunofluorescence of the complement system with C1q**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with C1q to investigate the presence of the complement system and it's role in rejection. A, Representative pictures of immuno-staining of C1q. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of C1q in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

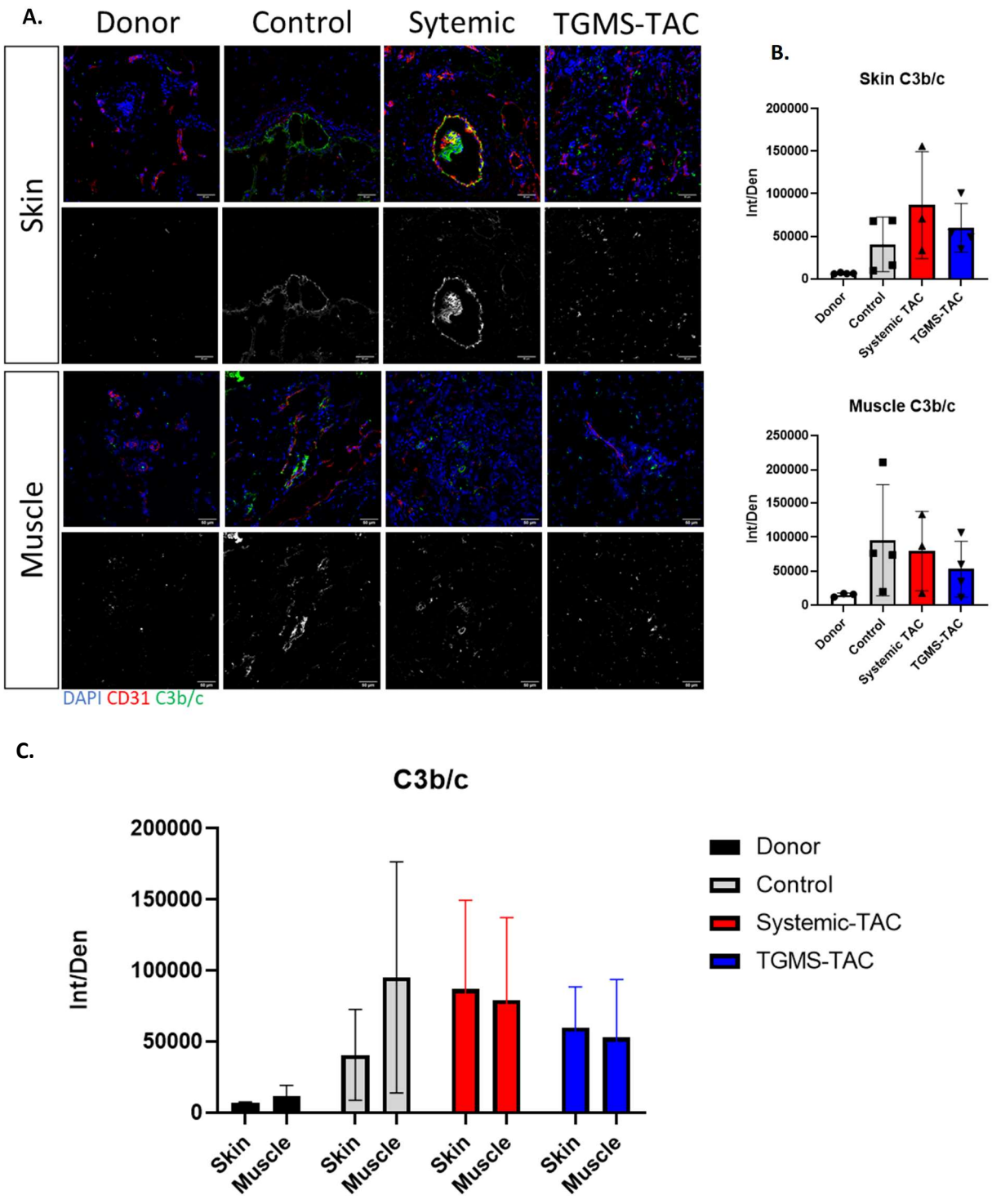


Figure 30: **Immunofluorescence of the complement system with C3c**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with C3c to assess the presence of the complement system. A, Representative pictures of immuno-staining of C3b/c. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of C3b/c in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

4.9.5 Antibody deposition in the graft

The presence of immunoglobulins in the graft was investigated using IgM and IgG antibodies. There was some antibody deposition of both IgG and IgM in the skin and muscle in all groups. However, the difference was not significant (Figure 31).

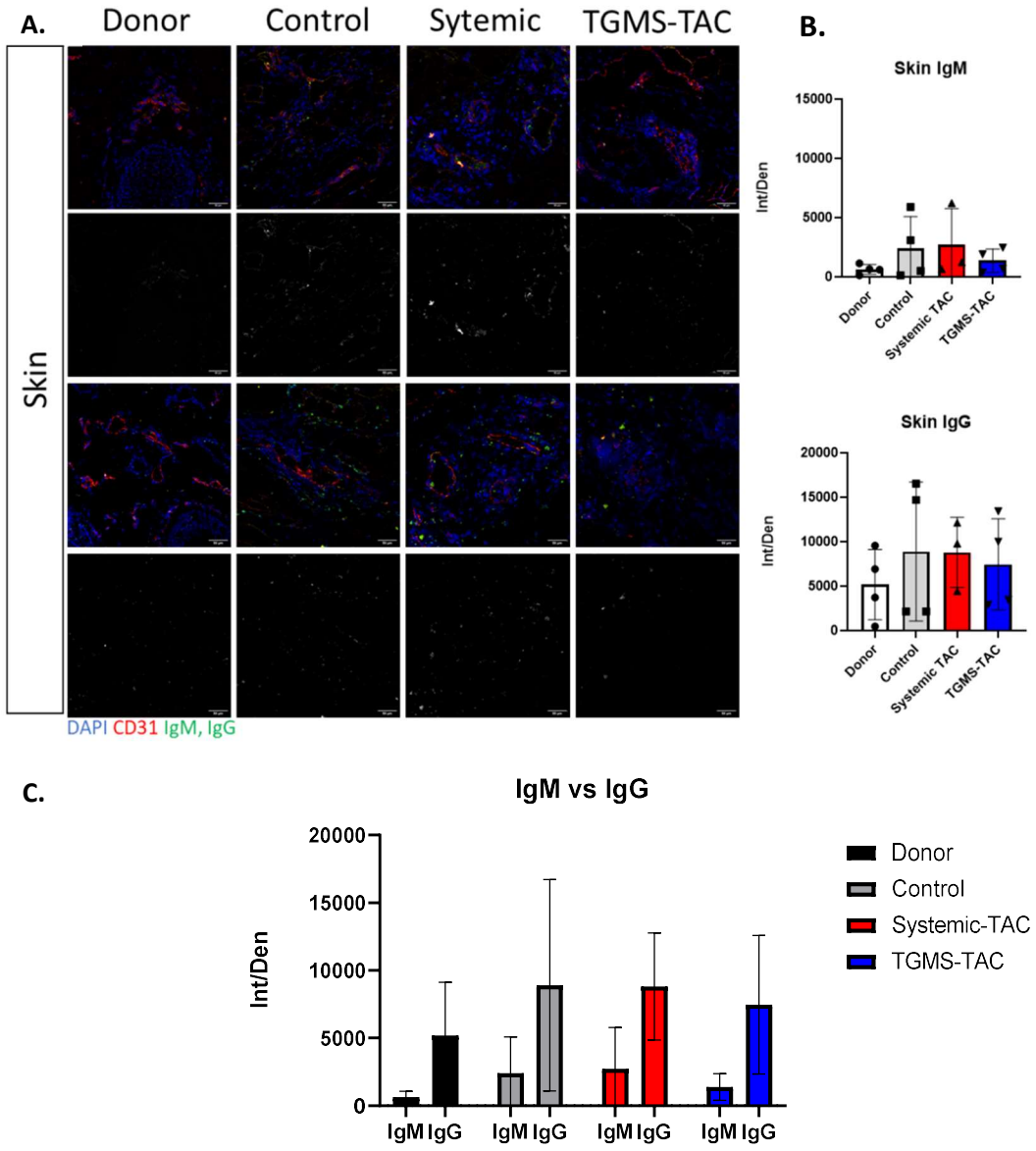


Figure 31: **Immunofluorescence of immunoglobulin with IgM and IgG**. Skin and muscle from the donors as well as from the grafts of the transplanted pigs were stained with IgM and IgG to investigate the presence of antibody deposition. A, Representative pictures of immuno-staining of IgM and IgG. B, Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each dot represents the mean integrated density from 5 pictures randomly taken from each pig. One-way ANOVA with Turkey multiple comparison test was used for statistical analysis. C, statistical analysis was done to evaluate the presence of both immunoglobulin in the skin compared to the muscle within the same group by using the two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as mean ± SD (N=4 for control, N=3 for systemic and N=4 for TGMS-TAC group).

5 Discussion

Vascularized composite allotransplantation (VCA) is facing multiple issues that prevent a wider use of this unique restorative surgical technic. Indeed, the high incidence of acute rejection episodes within the first-year post-transplantation and the strong life-long immunosuppressive treatment administered causing life-threatening side effects are the mayor withdrawal to VCA procedures. Therefore, a site-specific immunosuppressive treatment is desirable where it enables increase graft survival while decreasing the off-target toxicity. Furthermore, a better understanding of the immune response would help underlying the cause of the rejection mechanism.

In this study, we were able to evaluate the efficacy and safety of the TGMS-TAC DDS in a porcine model of VCA. As we can see in Figure 9 and 10, the TGMS-TAC system does increase the survival of the grafts. Pigs that received the TGMS-TAC had a mean survival time (MST)of 46 days (N=4) compared to 7,5 days (N=4) for the untreated animals (p=0,0062). Surprisingly, the short systemic administration of tacrolimus for 14 days reached similar MST, 43 days, compared with the TGMS-TAC. In a similar study from Fries et al. where they tested the TGMS-TAC DDS on an orthotopic porcine limb model of VCA, they reported a graft survival ranging from 56 to 93 days (Fries et al., 2019). However, they used a mini swine with only one SLA mismatch whiles here we achieved two complete SLA mismatches. This increase graft survival may then be explained by this higher compatibility between donors and recipients. In another study using TGMS-TAC on rats, the grafts treated with the TGMS-TAC remained rejection free for 200 days. However, divergent results are expected when using a larger and more complex model.

Depending on the therapeutic dose of tacrolimus needed to avoid rejection, a mean of 61mg of tacrolimus was injected in the graft from the TGMS-TAC group while in the systemic group they received in 14 days a mean total dose of 400mg of TAC (Figure 11). The TGMS-TAC system then decreases over six time the amount of drug administered to the patients. It is important to note that in the oral administration of tacrolimus, the bioavailability is greatly reduced due to the extensive metabolism from the liver as well as from enzymes from the gastrointestinal track, bacterial enzymes, hepatic enzymes and possible renal clearance (Fries et al., 2019). Finally, only a small percentage of the actual amount of drug administered orally reaches the blood stream and the targeted graft tissues.

It was also observed that the TGMS-TAC also may enables a faster reduction of the surgically triggered inflammatory response in the graft, shown in figure 9, by having locally high drug levels. Indeed, in Fig.12, Tacrolimus level were significantly higher in the graft's skin compared to the contralateral side in the TGMS-TAC groups both at POD7 (p=0,0001) and POD14 (p=0,0227). In the systemic groups, the drug levels were comparable whether it was measured in the graft or in the contralateral side.

Moreover, with the help of the drug delivery system, we achieved higher local tacrolimus concentration, thus in the graft, compared to the systemic administration at POD7 (p=0,0001).

In the blood, on the other hand, the tacrolimus levels were extremely high in the first days after the surgery in the TGMS-TAC group. The TGMS-TAC pigs had at POD3 and POD7 a mean of 30,15± 15,243 and 42,46± 9,522ng/ml respectively of tacrolimus in the blood compared to 8,333± 4,165 and 11,067± 1,877ng/ml in the systemic group (p=0,0001 in both cases) (Figure 12). The phenomenon is explained by the release system of the drug from the hydrogel. The TGMS-TAC hydrogel disassembles in an enzyme-responsive manner to release the drug in the presence of proteolytic enzymes that are expressed during inflammatory conditions. As the surgery itself causes trauma and triggers inflammation, the hydrogel is actively releasing tacrolimus within the graft which can then be distributed systemically though the blood. Our results are compatible with other studies on the release of the drug from the hydrogel both in rodent and swine models of VCA where they also observed this initial burst between POD1-7 (Fries et al., 2019; Olariu et al., 2017). In our study, this initial burst also causes a premature drug exhaustion in the gel and therefore a lower level of tacrolimus is available in the graft in the long term. Since a large amount of the drug is already release in the first two weeks, from POD35 on the level of tacrolimus are below the detection limit. As a possible correlation to this absence of tacrolimus drug in the graft, pigs treated with TGMS-TAC started rejecting from POD34.

Based on our preliminary data, the treatment with TGMS-TAC cause no to minimum off-target toxicity (Figure 13). When comparing the untreated pigs with the treated ones, we could not assess significant differences in the biochemical parameters suggesting that in our project neither the systemic tacrolimus nor the TGMS-TAC treatment is affecting liver or kidney function in the time lapse that lasted the experiment. However, it is important to point out that tacrolimus does cause off-target toxicity when administer over a long period of time as it was systemically observed in treated patients, both in rat models and in the clinic (Dzhonova et al., 2018; Lloberas et al., n.d.). In our study, the systemic administration of tacrolimus lasted only 14 days, when we would expect to have significant effect on the toxicity after a period of six months of treatment.

We also studied different important cytokines that can promotes inflammation as well as stimulate the innate immune system or T cell mediated rejection. As expected, the pro-inflammatory cytokine IL-1β levels is higher in the first days after the surgery in the control group as they rejected within a week after transplantation. Il-1β was also shown to be significantly increased at endpoint in the systemic (p=0,0124) and TGMS-TAC (p=0,0341) groups compared to their baseline. However, in the treated groups there was not always a correlation between high levels of inflammatory cytokines such as Il-1α and IL-6 with rejection episode (see complementary figure 1). In the study from Friedman and

al. on a rat model of VCA, they could show a significant increase of the proinflammatory cytokines, IL-1α, IL-1β, IL-6, IL-18 and IL-10, in the acute rejection phase of the graft. These findings were based on samples taken five days after transplantation where the grafts shown signs of acute rejection. However, no grading system was used to evaluate the degree of rejection at this timepoint. In our study, since the animals are euthanized when the graft reach grade III rejection, the cytokines profile may differ.

Concerning the innate immune cell modulating cytokines, we selected IL-8 and MCP-1 as neutrophils are believed to play an important role in rejection and monocytes such as dendritic cell and macrophages are essential for antigen presentation to induce cell-mediated rejection. Only in the systemic group, we could observe a clear augmentation in MCP-1 levels at endpoint compared to baseline (p=0,0037) (complementary figure 1). Interleukin 8 shows great fluctuation in its circulating levels in the treated groups without being specially a correlation with rejection episodes as seen in Figure 15. Still, pigs in the control group that reach grade III rejection had an increase level of IL-8 when rejecting and we can visually observe numerous neutrophils within the muscle of the graft (Figure 23). As for the treated groups, IL-8 levels vary slightly (Figure 15) throughout the follow-up and the small increase of the cytokine did not show any correlation with acute rejection. However, when investigating the presence of neutrophils in the grafts at rejection, we can see that we actually have the higher amount of them both in skin and muscle compared to the untreated group (Figure 23).

Also part of the innate immunity, the complement system may have play an important role in the graft rejection as it was already observed in allograft rejection and IRI studies. In the study from Zhu and al., the showed that C3 and C5 receptors deficient mice but also mice given C3 inhibitor treatment had less skin and muscle damage following VCA transplantation suggesting that these complement proteins do indeed play a role in tissue injury following transplantation and the start of the rejection response (Zhu et al., 2017). In our research, the complement system was investigated in the circulation and in tissues. Following transplantation, there was an increase of sC5b-9 in the blood from all groups (Figure 18). This systemic increase may be due to the IRI which is caused by the surgery. In the skin, as we can observe in the figure 28, this complement protein is significantly increased in the control group. Based on this data from the blood and tissue, it suggests that C5b-9 may be important in the rejection mechanism in the untreated pigs. As for C3b/c and C1q in all transplanted animals, they are present both in skin and muscle and it seems that there is a higher number of complement deposition in the grafts at endpoint compared to the baseline without reaching statistical differences between groups (Figures 20 and 30).

In VCA, the main cause of rejection in the clinic is due to cell-mediate, antibody-mediate or chronic rejection mechanism. In our study, since the animals were treated over a short period of time, we could expect to have either T-cell-mediate or antibody mediated rejection. Therefore, we analyzed different markers that could enlighten us on which rejection mechanism was used. First, we focused on T cells as the drug tacrolimus specifically inhibit the production of cytokine important for T cell proliferation. IL-4 and IL-2 are two of those cytokines. In the graphs from Figure 16, we can observe that the TGMS-TAC group has higher levels systemically of both cytokines compared to the systemic group. It is not surprising since, the TGMS-TAC acts only locally, thus where it is injected, while the oral treatment given with systemic tacrolimus is distributed in the whole body. In the control group, it is not expected to have especially high level of these cytokine since the rejection would not be caused specifically by T cells from the adaptive immune system but by pathway from the innate immune system. Overall, T cell modulating cytokines such as IL-2, IL-12 and IL-18 could not be linked to rejection episodes neither from the untreated nor untreated groups. Only IL-4 shows a significant increase in the TGMS-TAC group at endpoint compared to baseline (p=0,0379). In the tissue, we could evaluate the amount of T and B cells and they seems that they are primary located in the skin. T cells were present in all groups, but it appears that T helper cells were significantly more numerous in the skin of the systemic tacrolimus compared to baseline (p=0,0374) and also in the muscle compared to the baseline (p=0,0115), untreated (p=0,0099) and the TGMS-TAC (p=0,0150) (Figure 26). As we did find working antibodies to differentiate the subclasses of T cells such as $CD8^+$, Th1 or Th2 cells, it is hard to conclude on which T cells subsets are actually more present in the grafts and would therefore promotes rejection in VCA settings. It would need to be further investigated.

The TGMS-TAC treatment appears to promote B cells population in the skin when compared to baseline (p=0,0469) (Figure 27). On the other hand, antibody-mediated rejection does not seem to be the mechanism of rejection either in the untreated or treated groups. Indeed, the analyze of immunoglobulin presence in the skin and muscle shows that there are present in all groups and also in the baseline suggesting that the antibodies were already present before the rejection response started (Figure 31).

We also faced some complications during our research. Four pigs, including three untreated and one receiving systemic tacrolimus had to be taken out of the study due to vascular complications, where the grafts experience internal bleeding or vascular anastomosis failure. Then, one subject with the TGMS-TAC treatment had a local infection in the catheter area which was detected at POD23. A cocktail of systemic antibiotics was then given to treat the infection. Therefore, blood collection was not possible after this timepoint on this pig. As a possible result to this complication, cytokines levels

from C5a, IL-18, MCP-1, IL-8, IL-6 and IL-1α, in this pig greatly increased at POD14 and POD21 and thus complicates the interpretation of the results.

6 Conclusion

In our study we were able to validate the efficacy and the safety of the TGMS-TAC system in a clinically relevant swine model of VCA. The TGMS-TAC prolonged the survival of the graft compared with the untreated graft without causing off-target toxicity. With the local injection of the TGMS-TAC, the DDS reached a high drug concentration in the graft. The amount of tacrolimus needed to avoid rejection was greatly reduced when compared to the systemic application of the drug. As for the mechanism of rejection in the different groups, our preliminary data suggest in the TGMS-TAC group has a higher expression level of IL-1β and IL-4 at endpoint, thus grade III rejection, and an increased EC activation, neutrophils, and B cell infiltration in the skin. In the systemic group, there was an increase in IL-1β as well and increase in MCP-1 expression at endpoint, elevated number of neutrophils and T helper cells in the skin and muscle. As for the untreated animals, rejection leaned towards a C5b-9 complement mediated immune response. However, the exact mechanisms leading to graft rejection in the treated and untreated groups remains still unclear and needs to be further studied.

7 Future perspective

Vascular composite allotransplantation as seen above is a promising approach to restore extensive tissue lost. With a single administration of the immunosuppressive drug Tacrolimus, via the TGMS-TAC, we were able to greatly improve grafts survival without generating off-target toxicity. Based on our preliminary data, the TGMS-TAC system shows promising results and bring us one step closer to its clinical use. However, there are still some aspects of the treatment that need improvement.

There is an initial burst in the release of the drug at POD3 in the TGMS-TAC group, which in the long-term result in low levels of tacrolimus available in the graft. It is not surprising based on the release system of this DDS. Indeed, the surgery itself triggers an inflammatory response due to tissue trauma and injury which results in upregulation of inflammatory mediators that initiates the release of tacrolimus from the hydrogel. Therefore, a second dose of TGMS-TAC could be re-administered at POD14, when the inflammatory response is completely over, to promotes a stable and sufficient amount of tacrolimus within the graft. Another solution to avoid this initial burst of tacrolimus from the hydrogel is the use of a short systemic administration of oral tacrolimus, from POD1-14 as it was shown to maintain the graft rejection free without causing off-target toxicity, followed by a single injection of TGMS-TAC in the graft.

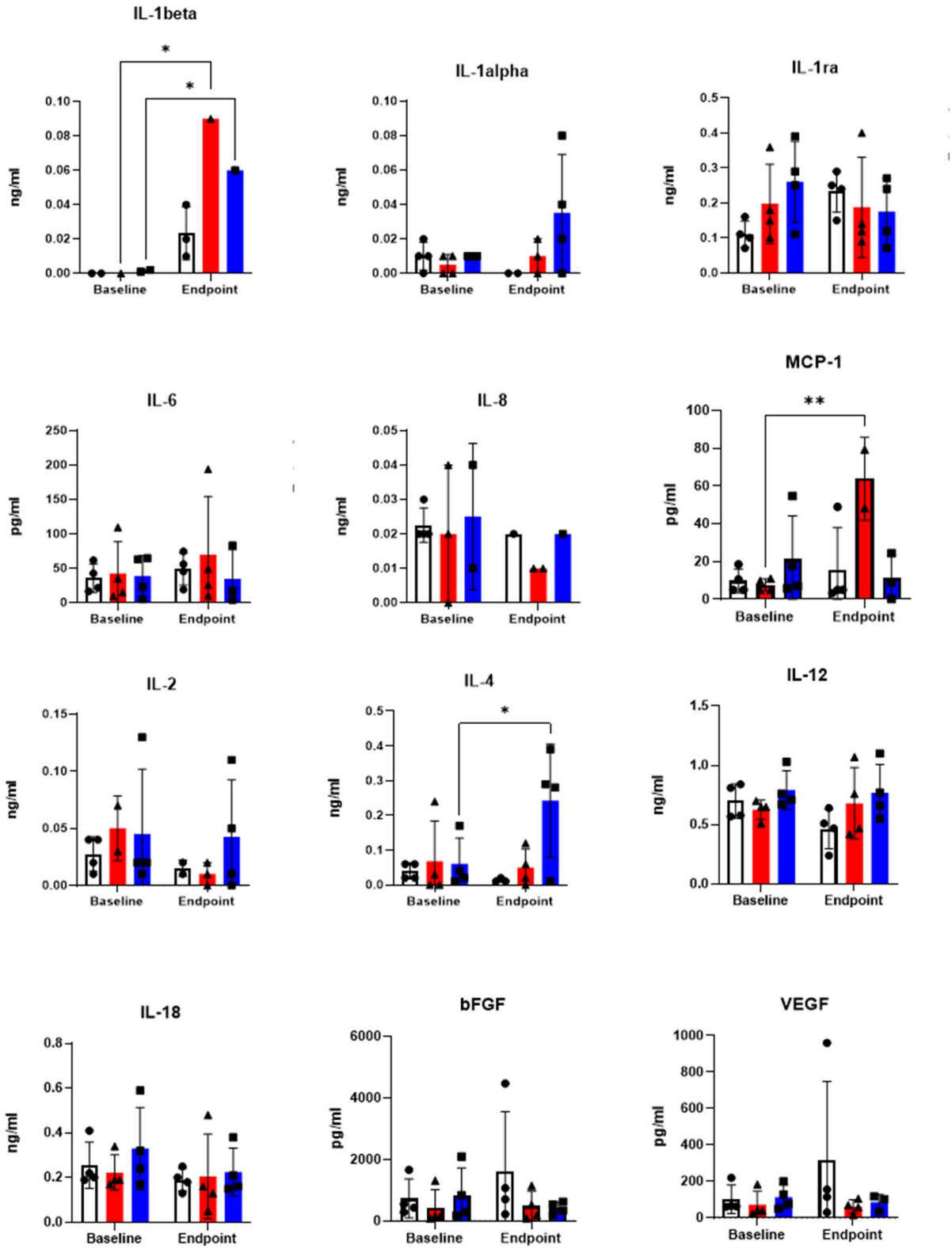
It is also known that graft survival in transplantation is affected by the immune cell population and more precisely by the balance between allo-aggressive and allo-specific T_{reg}. To achieve this equilibrium, a solvent-induced phase inversion in situ forming implant (ISFI) using the FDA-approved polymer poly (D,L-lactic-co-glycolic acid) (PLGA) loaded with rapamycin, named RAPA-ISFI, was designed. Rapamycin is a macrolide that could be used as an immunoregulator in transplantation. It was recently discovered that rapamycin can promote the differentiation of T_{reg} and downregulate Th17 differentiation and function both *in vitro* as *in vivo.* In this study, they used a rat model of VCA and were able to increase levels of chimerism and T_{reg} frequency both systemically and in the VCA graft treated with RAPA-ISFI (Saunders et al., 2001; Sutter et al., 2019). Based on this discovery, the RAPA-ISFI DDS could be also investigated and validated on a clinical large animal model of VCA, thus the porcine model we worked on.

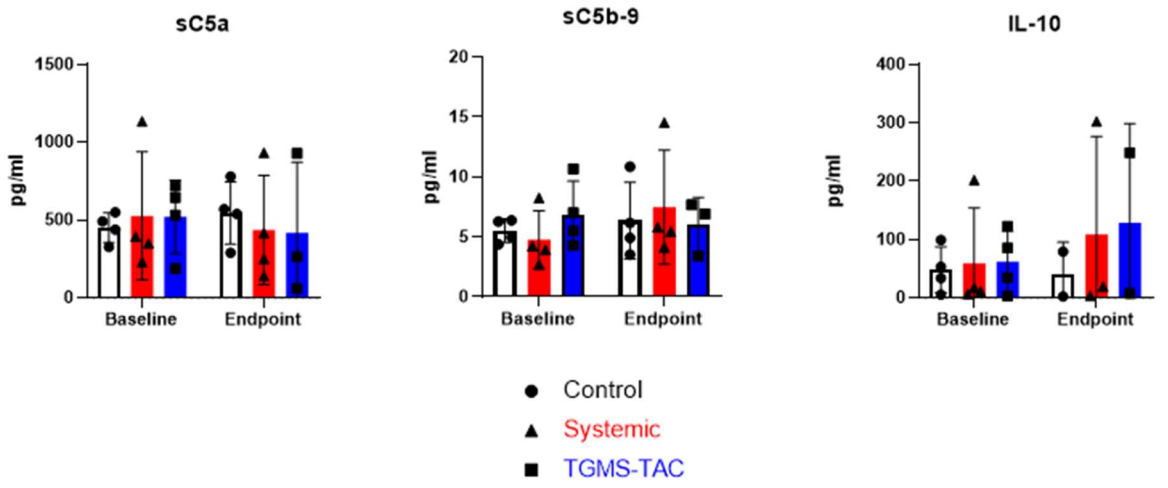
A treatment combining both drugs could also be ideal. Tacrolimus with its ability to deplete T cell mediated response and Rapamycin by promoting essential chimerism and T_{reg} population systematically and within the graft, makes them a perfect duo. Since rapamycin can impair wound healing, low dose systemic tacrolimus can be administered during the first initial phase after surgery, from POD1-14, and then be replace by the local injection of RAPA-ISFI.

Furthermore, in the clinic, an induction therapy is given to patients undergoing VCA which is then followed by a life-long immunosuppression treatment. The induction therapy consists normally of corticosteroids and T cell depleting agents which aims to prohibit the strong immune response after donor tissues and recipient blood come in contact. Until now, the induction therapy was not investigated in combination with TGMS-TAC or RAPA-ISFI treatments. It would therefore be interesting to evaluate the efficacy of localized immunosuppression and immunomodulation combined with induction therapy and subclinical immunosuppression on graft survival.

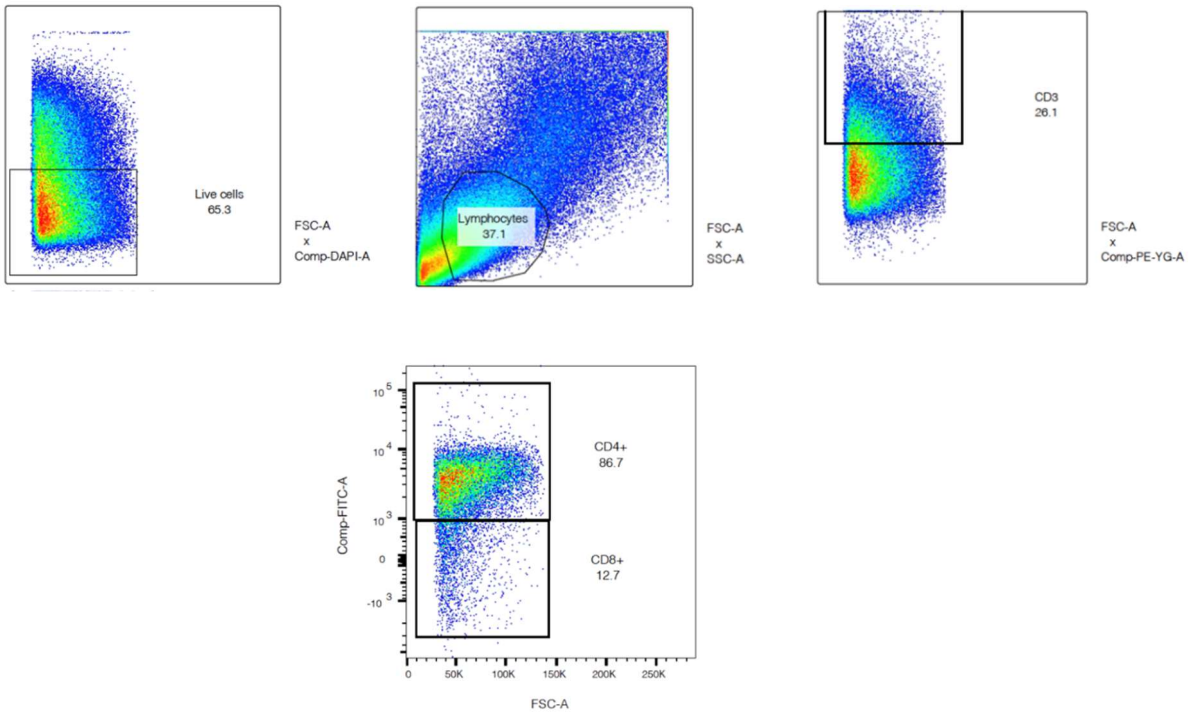
Nowadays, there is no standardized therapy protocols for VCA patients. With this undergoing study and the future perspective given with TGMS-TAC and RAPA-ISFI and the induction therapy, we could uncover the best combination of drugs and treatment for VCA patients which would guaranty long-term graft survival while having no off-target toxicity.

8 Supplementary Figures





Supplementary figure 1: Cytokine comparison of Baseline vs Endpoint. The cytokines were measured in the plasma from the control (N=4), systemic (N=3) and TGMS-TAC (N=4) groups. IL-6, IL-10, C5a, C5b-9, bFGF, VEGF and MCP-1 were analysed with Bio-Plex (pg/ml). IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-8, IL-12 and IL-18 were analysed with Milliplex (ng/ml). Statistical analysis were done with a two-way ANOVA with Šídák's multiple comparisons test. P value >0.005 was considered as significant (* p ≤ 0.05, ** p ≤0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Data are presented showing the mean values ± SD.



Supplementary figure 2: Gating strategy for CD4⁺ and CD8⁺ T cells.

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12 Declaration of consent

Declaration of consent

on the basis of Article 18 of the PromR Phil.-nat. 19

Name/First Name: Yerly Anaïs Elodie

Registration Number: 16-100-919

Study program: Molecular Life Science

Bachelor ☐ Master ☒ Dissertation ☐

Title of the thesis: "Local immunosuppression using self-assembled hydrogel drug delivery system in vascularized composite allotransplantation and its effect on rejection in a swine model."

Supervisor: Prof. Dr. Robert Rieben

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of September 5th, 1996 and Article 69 of the University Statute of June 7th, 2011 is authorized to revoke the doctoral degree awarded on the basis of this thesis.
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