Univeristät Bern

Molecular Life Sciences

Tacrolimus inhibits neutrophils and neutrophil extracellular traps that are involved in vascularized composite allograft rejection

Master Thesis handed in by

Anja Sophie Helmer

Bern, Switzerland

2022

Supervisor

Prof. Dr. Robert Rieben

Department for Biomedical Research, University of Bern, Switzerland

Co-Supervisor

Dr. Med. Isabel Arenas Hoyos

Department for Biomedical Research, University of Bern, Switzerland

Department of Plastic and Hand Surgery, Inselspital, University Hospital of Bern, Switzerland

Abstract

Background: Vascularized composite allotransplantation (VCA) provides a unique treatment option for patients with extensive tissue loss. Currently, the biggest throwback for this promising treatment option is the high rate of acute rejections and the required life-long intake of a triple-drug therapy which is associated with a variety of side effects such as kidney failure, cancer, opportunistic infections, and diabetes. A novel treatment approach consists of an on-demand drug delivery system that is injected locally into the graft, where tacrolimus (TAC) is linked to a triglycerol-monostearate hydrogel (TGMS). This TGMS-TAC has already been tested *in vitro* and *in vivo* in a rodent model and showed promising results in prolonging graft survival.

The mechanism of rejection in VCA has mainly focused on T-cell-mediated rejection. Nevertheless, it has been shown that neutrophil extracellular traps (NETs) to play an important role in transplantation-related ischemia-reperfusion injury which downstream damages transplanted tissue and reduces early graft function. Hence, the aim of our study is to reveal the involvement of neutrophils and neutrophil extracellular traps in vascularized allotransplantation. In addition, we aim to acquire differences in neutrophil and neutrophil extracellular trap responses to site-specific immunosuppression with TGMS-TAC, and in this context, we also aim to assess the efficacy of TGMS-TAC as a novel treatment approach in prolonging VCA graft survival.

Methods: Outbred pigs underwent heterotopic hind limb allotransplantation and were randomly divided into three groups: 1) an untreated group, 2) a TGMS-TAC group where they received a single subcutaneous injection of 140mg of tacrolimus per kg of graft at postoperative day (POD) 0, and 3) a TGMS-TAC (R) group where the animals also received a subcutaneous injection of 140mg of tacrolimus per kg of graft at post-operative day (POD) 0 and every 30 days afterward until endpoint. The endpoint of the experiment was set once the graft reached grade III rejection or survived up to POD90. Following transplantation blood and tissue samples were collected at different time points. Graft survival, cytokine secretion, and innate and cellular immune response including neutrophil and NET deposition were assessed.

Results: Single and multiple injections of TGMS-TAC significantly prolonged graft survival compared to untreated animals. All TGMS-TAC reinjected animals survived until endpoint (POD90) whereas the mean survival time for the group of untreated animals was 7.75 days

and 56 days for the group of animals that were only once injected with TGMS-TAC. T-cell infiltration was significantly reduced in graft skin in both TGMS-TAC single and TGMS-TAC reinjected animals compared to the untreated control group. In both the single and reinjected group, the amount of infiltrating T-cells in the skin graft was reduced about 20% compared to the untreated group. The measured amounts of circulating NETs during graft rejection were negatable low in all treatment groups. Nevertheless, in rejected skin grafts of the untreated animals, we detected a significant increase of infiltrating NETs compared to the healthy control skin. The levels of infiltrating NETs in animals that received multiple injections of TGMS-TAC, were significantly lower compared to the levels in the untreated group. Accordingly, in TGMS-TAC single and reinjected animals, skin neutrophil infiltration was significantly lower compared to the untreated control groups. Finally, we were able to show that TAC is able to inhibit NETosis *in vitro*.

Conclusion: In the current study we were able to show that the reinjection of TGMS-TAC prolonged graft survival significantly and is therefore an effective treatment method for VCA graft recipients.

Further, we were able to show that local in graft T-cell infiltration in TGMS-TAC treated animals was significantly reduced compared to the untreated group. We could also show that neutrophils and NETs were present in rejected skin grafts upon VCA graft rejection and that NET and neutrophil infiltration in rejected skin of animals that were reinjected with TGMS-TAC was significantly reduced compared to untreated animals. Finally, we demonstrated that Tacrolimus is able to inhibit NET formation *in vitro* and that it seems to dampen NET formation *in vivo* locally in the rejected VCA graft skin. Therefore, we propose that TGMS-TAC is an effective tool in preventing local T-cell proliferation and neutrophil activation in VCA grafts but does not interfere with the efficiency of T-cell activation and proliferation as well as NETosis systemically.

Table of Content

1 Introduction

1.1 Vascularized Composite Allotransplantation, Current Challenges and Future Perspective

Vascularized composite allotransplantation (VCA) is a transplantation method where the transplanted graft consists of multiple tissue components. The different components are transplanted as a single unit from the donor to the recipient. An example of such a graft is a whole arm which consists of muscles, skin, bone, vessels and nerves.

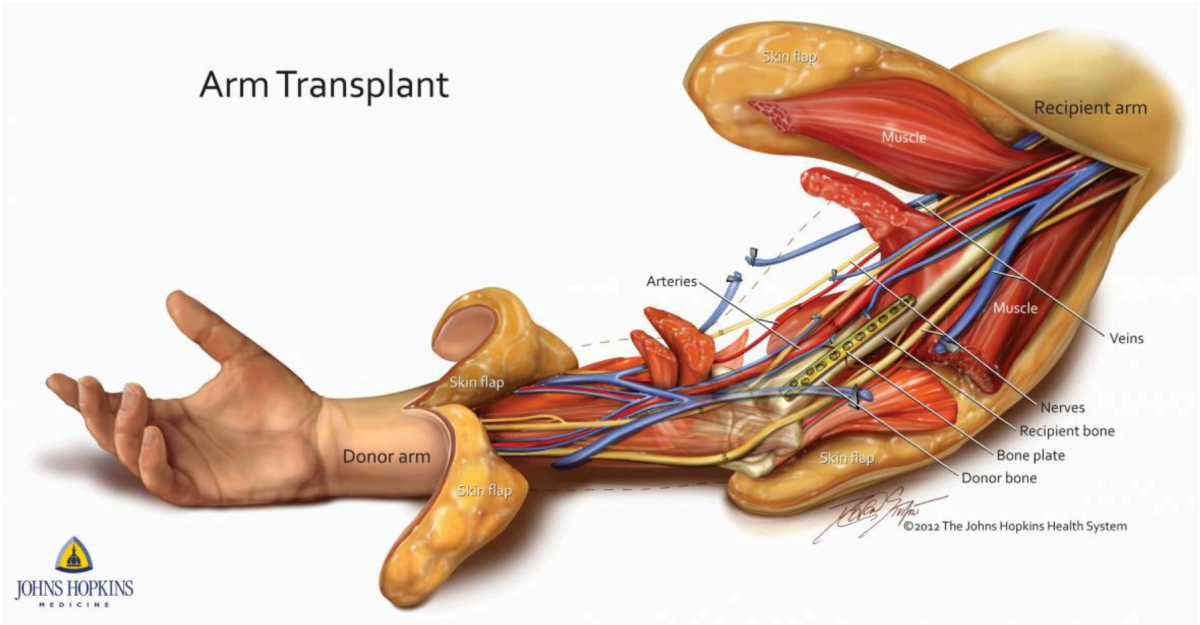


Figure 1: Depiction of an arm transplant consisting of multiple tissue components. Adapted from Hopkins Journal of Plastic and Reconstructive Surgery, 2014 [1]

In contrast to solid organ transplantation like the transplantation of a heart or liver, VCA is considered a life-enhancing but not life-saving procedure. Therefore VCA is facing different challenges and concerns, such as the high immunogenicity of the skin and the high rate of acute rejection of the VCA graft, in the field of transplantation.[2] Nevertheless the number of patients who receive a transplant consisting of multiple tissue components is increasing.[3] The first try at performing a vascularized composite allotransplantation was attempted in the 1960s. Technically the attempt was successful but an acute rejection of the graft occurred soon after the transplantation which soon after led to the loss of the VCA graft. The first successful upper extremity transplantation in humans was performed in 1998 in France where

the patient tolerated the immunosuppressive treatment well and signs of rejection disappeared after some time which enabled the patient to experience progress in motor and sensory functions.[4,5]

The most commonly performed VCA is the transplantation of an upper extremity and the face. These transplants, each unique, interact directly with the physical environment of the recipient. There are different advantages that can be associated with VCA. One of those advantages is the possibility of direct observation of the graft and therefore the opportunity to diagnose graft rejection at an early stage just by observing the macroscopic changes. Nonetheless, the procedure also faces many disadvantages. In solid organ transplantation, different assays are established which can be used for monitoring the graft similar to the observation of creatinine levels in kidney transplantation. Currently, there are no noninvasive established assays available that can be used to monitor VCA grafts, such as a biomarker that can be measured in the patient's blood. Anyhow, the majority of transplant recipients appreciate the good function of their graft and the feeling of having a vivid extremity instead of a prosthesis.[6]

VCA allows the reconstruction of body parts that are malformed because of an accident, congenital malformations or surgical excisions of malignant tumors when the conduction of conventional surgery is not possible or prostheses are not considered the best option to substitute an upper extremity. Based on nerve regeneration and sensorimotor recovery of upper extremity allotransplants, patients who underwent VCA surgery, are enabled to perform most daily activities and challenges. [7] Regardless of the many advantages VCA has to offer there are risk factors that have to be taken into account when considering a VCA. The transplanted graft's long-term survival is only possible in combination with lifelong immunosuppressive therapy.[8] Typically the immunosuppressive therapy starts with an antibody-based induction therapy like anti-thymocyte globulin for T cell depletion followed by maintenance consisting of a calcineurin inhibitor such as tacrolimus, an antimetabolic agent such as mycophenolate mofetil and a corticosteroid like prednisolone.[9] This lifelong, high-dose, multi-drug immunosuppression is associated with a wide range of side effects including kidney failure, diabetes mellitus, leukopenia, hypertension, opportunistic infections and a higher risk of cancer among others.[10] In 2018 there were 66 hand transplant recipients registered worldwide, 26% of patients suffered from elevated creatine values, which indicates

kidney damage, 32.3% suffered from opportunistic bacterial infections and 3 of the patients developed malignancies. Therefore the demand for a modified and improved immunosuppression is high, which can on the one hand possibly increase the number of patients applicable for VCA and on the other hand improve the life quality of patients that already received a vascularized composite allotransplant.[11] One possibility to overcome the side effects associated with systemic immunosuppressive drug application would be the application of an on-demand drug delivery system that releases the drug in response to the nature and intensity of inflammation present during inflammation. This approach leads to lower off-target toxicity because of the local injection into the graft which is preventing the systemic distribution of the active pharmaceutical compound and therefore reduces the side effects associated with systemic immunosuppression and at the same time increases graft survival. One proposed on-demand drug delivery system is a triglycerol monostearate hydrogel which is loaded with Tacrolimus (TGMS-TAC). This hydrogel releases Tacrolimus locally, only in response to inflammatory stimuli and prolongs VCA survival with a single injection.[12]

One of the major challenges that come along with VCA is graft rejection. Usually, the transplanted grafts in VCA include the skin which is considered to be highly immunogenic[13] as it contains various immunologically active cells and extracellular components in combination with skin-specific antigens which makes it a highly immunologic site in the transplanted graft. Therefore, it is considered the primary site of VCA acute rejection.

Various types of immune cells are involved in VCA graft rejection. [9] Until now studies about transplant rejection mainly focused on the adaptive immune system. Especially B and T cells were the main focus of many transplant rejection studies in solid organ transplantation and vascularized composite allotransplantation. Although the cells of the adaptive immune system naturally play an important role in VCA graft rejection, the role of the innate immune system is at least as important. Innate immune cells like dendritic cells, monocytes, and natural killer (NK) cells are responsible for alloantigen recognition and are responsible for the activation of adaptive immune cells. Therefore a better understanding of the innate immune system in allorecognition is of great importance.[14]

In addition to the mentioned cells of the innate immune system, neutrophils need also to be considered very important players in inflammation and rejection. They are often seen as non-

specialized effector cells which indicates tissue inflammation. Recent evidence has shown that neutrophils can be divided into subsets that have specific effector functions like the formation of extracellular traps or other subtypes that are also able to stimulate angiogenesis. They can release molecules that inhibit T cell activation and therefore damper graft rejection as well as release oxidative and proteolytic effectors that can lead to sterile tissue damage. [15] Therefore the effector functions of neutrophils in transplantation have to be considered a double-edged sword.

The role of neutrophils and neutrophil extracellular traps (NETs) in VCA is not well known. Hence the aim of this study is to evaluate the role of neutrophils and neutrophil extracellular traps in rejected porcine VCA allografts.

1.2 Immunological Processes in VCA

Transplantation is a process where a non-functioning or non-existing organ is replaced with a graft. This graft can originate from different sources. It can either come from the same induvial (autogenic transplantation), from a different individual of the same species with different genetical background (allotransplantation), or from a different species (xenotransplantation). When the graft is placed into its normal anatomical location the transplantation is referred to as orthotopic transplantation. If the graft is placed in a different site from its normal anatomical location it is referred to as a heterotopic transplantation. In most cases, the graft comes from a donor with a different genetical background but from the same species, therefor an allotransplantation is performed in the majority of incidences. The immune system of the recipient will therefore be activated as it is recognizing the graft as foreign. This recognition can lead to an inflammation which is called rejection.[6] In transplantation rejection can be categorized as hyperacute, acute, and chronic rejection. Acute rejection is considered the most common complication in VCA with the skin being considered the main target.[8] Around 88% of VCA patients experience at least one acute rejection episode in the first year after the transplantation.[12] The antigens that are found in donated grafts that are able to stimulate the adaptive immune response of the recipient are called alloantigens, in the setting of an allotransplantation. These antigens are usually major histocompatibility complex (MHC) proteins, which are also known in humans as human leucocyte antigen (HLA). They are specifically and uniquely encoded by every individual. In a normal setting, the HLA allows the

immune system to distinguish their cells between foreign and self. The MHC molecules can be divided into two classes: MHC class I and MHC class II. MHC I molecules are expressed on most nucleated cells and only interact with CD8+ T-cells through the T-cell receptor complex (TCR). MHC class II molecules are expressed on B-lymphocytes and dendritic cells and only interact with CD4+ T-cells. The normal function of the MHC is to present peptides derived from protein antigens in a way so they can be recognized by T-cells. In the setting of an allotransplantation they act as antigens that cause graft rejection. Other than the MHC antigens there are also other polymorphic antigens present in allotransplantation against which the recipient can elicit an immune response. The immune response against these molecules is not as severe as the immune response opposed to MHC molecules and only induces a weak and slow rejection. Accordingly, these antigens are called minor histocompatibility antigens. These antigens are usually presented by APCs to T-cells similar to any protein.

1.2.1 Immunological Processes in VCA: Innate Immune Response

The human immune system can be divided into two major parts: innate and adaptive immunity. The interaction of both systems plays an important role in the rejection of grafts in transplantation.

Innate immune cells can recognize not only structures that are produced by microbial pathogens but can also be activated in sterile inflammation. Self and non-self-molecules can activate the host's innate immune defense and thereby innate immune cells are recruited to the site of infection. [6,14,16] The innate immune system is the first line of defense in every individual and can be found in all multicellular organisms. It is composed of myeloid cells such as dendritic cells, macrophages and neutrophils. By using their non-rearranged receptors, known as pattern recognition receptors (PRRs), they are able to detect non-host-derived and host-derived molecules that are released upon damage and stress from the tissue. In transplantation endogenous molecules which arise from ischemic and surgical trauma are released that subsequently are capable of activating PRRs. An important subgroup of those receptors is Toll-like receptors (TLRs). They are very important in activating the innate immune response and downstream the adaptive immunity in transplantation. TLRs are expressed in a variety of cells such as dendritic cells, mast cells and T-cells. Their expression is regulated by the presence of inflammatory mediators and other danger signals. As soon as a TLR is

stimulated, an intracellular cascade is activated that leads to downstream activation of the transcription factor NF-κB. The activated transcription factor migrates into the nucleus and thus activates the rapid transcription of genes that are associated with inflammation. Subsequently, proinflammatory cytokines and chemokines as well as antimicrobial peptides and adhesion molecules are produced. More antigen-presenting cells (APCs) are recruited to the site of infection and enhance their antigen presentation and up-regulate costimulatory molecules.

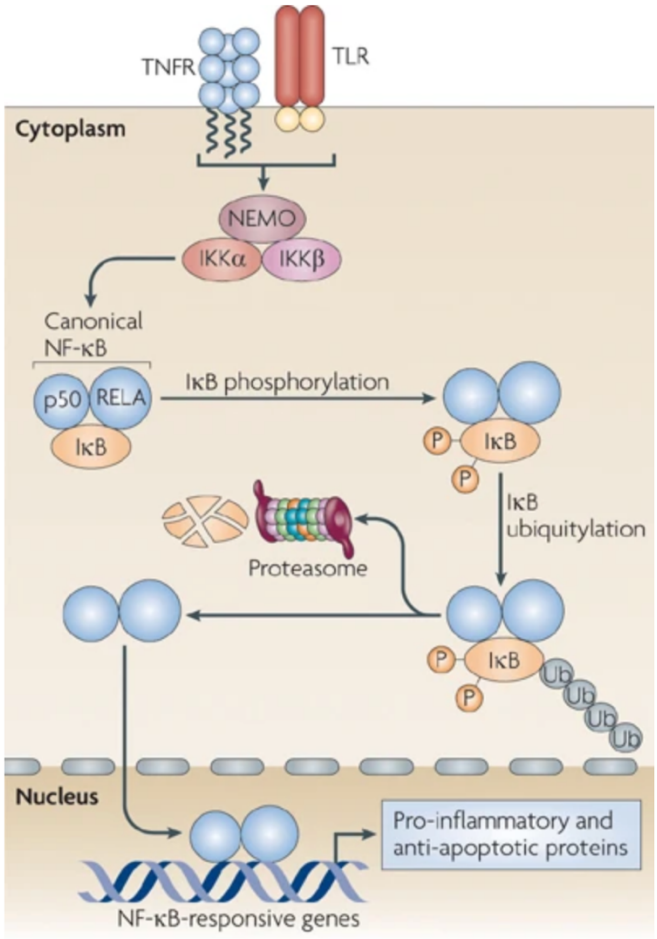


Figure 2: Activation of the transcription factor NF-κB. The dimers of NF-κB are kept in their inactivated form by the inhibitor of NF-κB (IκB). Once the TLR is stimulated IκB gets phosphorylated which activates it and subsequently triggers its polyubiquitylation and therefore degradation. The NF-κB dimers accumulate to the nucleus and activate the transcription of NF-κB dependent genes. Adapted from Pasparakis et al., 2009 [17]

The activated APCs are then able to activate alloreactive T- and B- cells. [18,19] Nevertheless the adaptive immune response is not only important in transplantation because of its ability to trigger T- and B-cell response. Certainly, other immune factors of the innate immune system are also important in vascularized composite allotransplant graft rejection. Ischemia-reperfusion injury (IRI) is considered to be a major risk factor for graft dysfunction and rejection.[20] The influence of IRI in VCA graft rejection is not very well known but it is suspected

that composite tissue allografts are more susceptible to IRI than solid organ grafts because of their high heterogenicity which inherits different immunogenicity in combination with the high sensibility of the muscle component that is included in the VCA graft. The complement system plays an important role in IRI post-transplantation through the promotion of inflammatory responses and the provision of an immunostimulatory environment. The transplanted graft is exposed to recipient complement proteins as soon as it is reperfused during transplantation. Complement proteins and fragments that are produced within the graft also enter the recipient's systemic circulation. The complement system is an important mechanism in antibody-mediated cytotoxicity, but it can also be activated independently of antibodies. It is also known that complement fragments are able to moderate T-cell differentiation and B-cell response to alloantigens. Complement fragments can also work as chemotaxins and activators of neutrophils and macrophages. The complement system can be activated through three different pathways: the classic pathway, the lectin pathway, and the alternative pathway. These pathways can be activated by distinct pathological processes in the graft like IRI and antibody-mediated rejection. The classical pathway of the complement cascade is activated by antibodies that are bound to their target ligands. Antibodies that are directed against HLA which are expressed on endothelial cells of the allograft are important mediators in this complement cascade. The lectin pathway is usually activated when activated proteins bind to sugars that are expressed on the surface of bacteria. In the context of an allotransplantation, this pathway can also be activated by binding ligands that are expressed on injured cells. The alternative pathway in contrast to the classical and lectin pathway can be activated non-specifically in plasma through a process referred to as tick-over. Eventually, the activation of all three pathways leads to a common pathway, the cleavage of the C3 protein into C3a and C3b/c. Downstream this cleavage leads to the cleavage of C5 which generates soluble C5a and C5b fragments. C5b then leads to the formation of the membrane attack complex (MAC) which is also referred to as C5b-9. This complex forms a pore in target cells which causes the activation of cell lysis.[21]

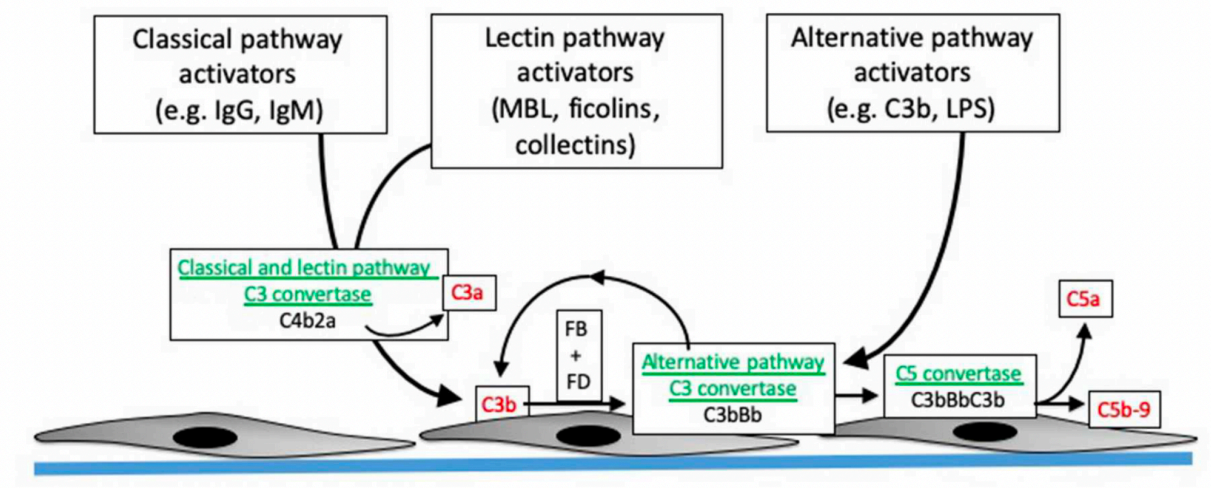


Figure 3: Pathways of complement activation. The classical pathway, lectin pathway and alternative pathway all lead to the formation of the membrane attack complex (C5b-9). Convertases are depicted in green and pro-inflammatory-molecules, which are generated during the complement activation, are depicted in red. Adapted from Grafals et al., 2019 [21]

1.2.2 Immunological Processes in VCA: Adaptive Immune Response to Alloantigens

The adaptive immune system is composed of different lymphocytes such as B- and T-cell which carry out the adaptive immune response. T-cells are responsible for the cellular immune response as part of the adaptive immune response to alloantigens.

In the context of an allotransplantation, T-cells can be activated by two pathways. The first one is the already mentioned conventional, indirect pathway. When considering the number of mismatched major and minor histocompatibility antigens that are contained within a graft, a high number of these allopeptide epitopes could be generated for recognition via the indirect pathway of T-cell activation. In this process, the alloantigens are processed by host antigen-presenting cells and presented to self T-cells. Even though in theory both CD4+ and CD8+ T-cells can recognize a processed alloantigen on MHC complexes, in the context of the indirect pathway the CD8+ T-cell response is not considered relevant for the early onset rejection of vascularized allografts since the differentiation of naïve CD8+ T-cells to cytotoxic effectors directed against specific alloantigens requires the help of already activated allo-specific CD4+ T-cells. The direct pathway of T-cell activation by alloantigens is considered the direct interaction of infiltrating recipient T-cells with allogenic MHC molecules that are recognized as intact proteins on the surface of donor antigen-presenting cells by both CD4+ and CD8+ T-cells. The recipient CD4+ and CD8+ T-cells recognize both, intact MHC I and MHC II molecules on donor cells as alloantigens. Recently a third, pathway was proposed in which

T-cells are activated through a semi-direct pathway. Here the T-cells recognize the intact alloantigen on host dendritic cells. This theory implies that an alloantigen transfer between antigen-presenting cells is possible. Since the T-cells in the semi-direct pathway are activated through the recognition of intact alloantigens they are likely to respond in the same way as T-cells that are activated through the direct pathway.[22,23]

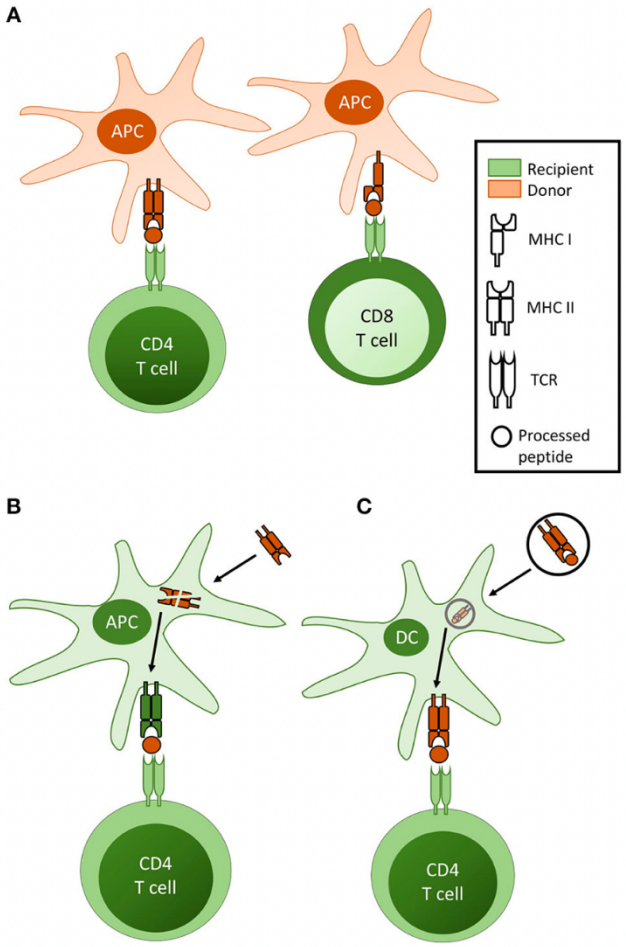


Figure 4: The pathways of T-cell allorecognition. ***(A)*** *Direct allorecognition of CD4+ and CD8+ T-cells. The alloantigens are recognized as intact MHC I and MHC II complexes on the surface of donor APCs.* ***(B)*** *Indirect alloantigen recognition by CD4+ T-cells. MHC processed fragments are presented on recipient APCs.* ***(C)*** *Semi-direct allorecognition by CD4+ T-cells. Intact MHC complexes are presented on recipient dendritic cells and recognized by CD4+ T-cells. Adapted from Siu et al., 2018* [23]

It is well established that cell-mediated immunity is based on T-cells. These cells can be divided into different subsets. The two major subsets are CD8+ and CD4+ T-cells. In the context of allograft rejection CD8+ T-cells, once activated, differentiate into cytotoxic T lymphocytes (CTLs) and kill the cells in the graft that express the allogeneic class I MHC molecules. CTLs can also secrete pro-inflammatory cytokines that can contribute to graft damage. CD4+ T-cells can be subdivided into different groups. They are all referred to as T-helper cells. These cells can for example help in the activation of T-cell-dependent alloreactive B-cells.[24] In the last decades

five major CD4+ T-helper subsets have been identified: Th1, Th2, Th17, regulatory T-cells (Tregs), and follicular T-helper-cells (Tfh). Each subset of T-helper-cells is defined by the expression and production of lineage-specific cytokines and transcription factors. For Th1 cells for example the production of IFN-γ, a pro-inflammatory cytokine, and the transcription factor T-bet are specific. Foxp3 on the other hand is a specific transcription factor only found in Tregs. Each T-helper-cell subset is important and plays a critical role in defending organisms from infections and other threats by coordinating the actions of the immune cells as well as non-immune cells.

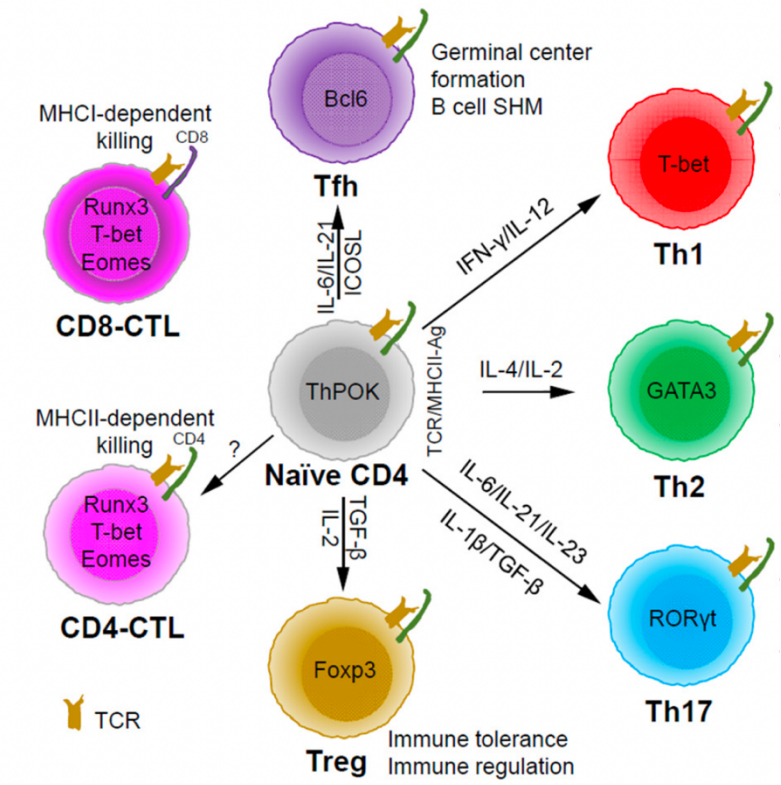


Figure 5: Development of CD4+ T helper cell subsets with their linage specific transcription factors and cytokines. Adapted from Zhu et al., 2020 [25]

Especially Th1, Th2 and Th17 cells are known to play an important role in allograft rejection.[25,26] Th17 cells are associated with many autoimmune and inflammatory conditions. They have also been associated with acute and chronic rejection of allograft transplants. The function of self-reactive effector Th17 cells can be controlled by Tregs. Regulatory T-cells are important in the regulation and suppression of an alloreactive T-cell response and are able to induce graft tolerance.[27]

The humoral immunity is not considered a major factor in VCA graft rejection. In VCA the antibody-mediated rejection (AMR) is caused by the binding of alloantibodies to alloantigens

like graft HLA molecules on graft vascular endothelial cells. The binding of the antibodies activates the complement cascade which then leads to the lysis of the cells, recruitment and activation of neutrophils and the formation of thrombi which in the end causes graft rejection.[8]

Since AMR in VCA is not considered a major factor in VCA by itself, it is rather considered as a supporter to T-cell mediated rejection. Nevertheless, the presence of alloantibodies is associated with a poorer outcome in graft tolerance. Donor specific antibodies (DSA) are considered a high-risk factor in long-term graft survival, and they are also able to activate the classical pathway of the complement cascade. B-cells are contributing to allograft rejection by differentiating into antibody-secreting-plasma cells, shaping the T-cell immune response against the allograft and cytokine production.[28]

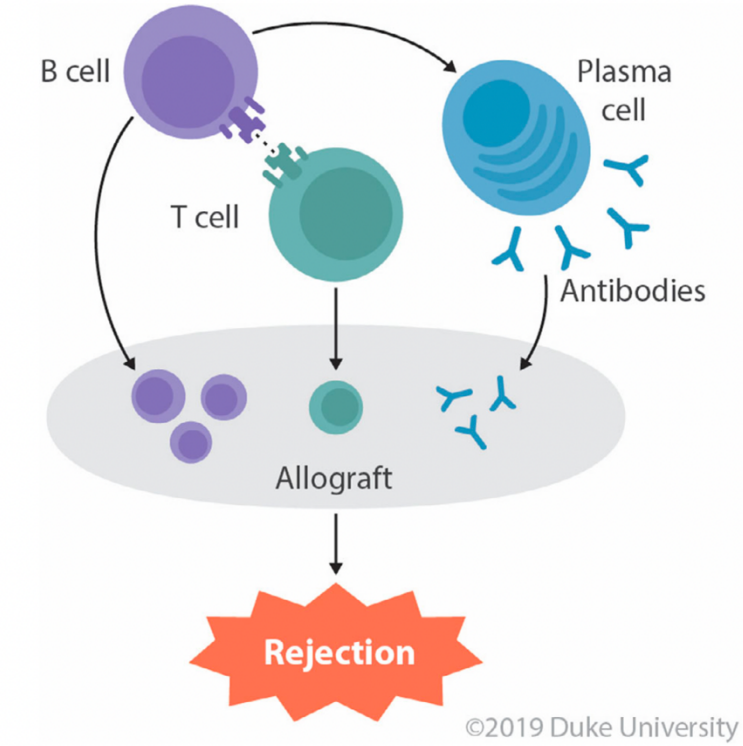


Figure 6: Pathways in which B cells contribute to allograft rejection. B cells are contributing to rejection by differentiating into plasma cells that produce donor specific antibodies and by co-stimulation of effector T-cells. Adapted from Schmitz et al.,2020 [28]

1.2.3 The skin as an Immunogenic Organ in VCA

As mentioned before acute chronic rejection is considered the most common complication in VCA, with the skin being the major target. On the one hand, the skin is a highly immunogenic organ and therefore a high-risk factor for graft rejection but on the other hand, it gives the

advantage of visual monitoring and therefore early detection of acute rejection. The high immunogenicity of the skin might be the greatest challenge in VCA graft rejection. In VCA it is usually the first and sometimes also the only tissue that is rejected. The skin can be looked at as a microenvironment that is able to activate the host's immune system. This microenvironment contains many elements which increase the susceptibility of the skin to rejection. It is a highly vascularized organ and a big variety of tissue-resident immune cells such as T-cells, Langerhans cells, NK cells, macrophages and dendritic cells are embedded in it. The number of immune cells that are present within the skin is dependent on different parameters such as the host's immune status or the presence of an infection and can therefore vary accordingly since many circulatory immune cells can be recruited into the tissue. A variety of danger signals such as ischemia can activate these resident immune cells. This unique form of external activation of the immune system is only possible since the skin works as a barrier tissue. As mentioned before acute rejection in VCA is mainly cellular mediated whereas antibody-mediated rejections in VCA grafts are very limited. Chronic rejection is not very often experienced in VCA since the field is not enough to identify the specific cases but the more years pass, chronic rejections of VCA grafts are described more often.[4,7,29]

1.3 Neutrophils and Neutrophil Extracellular Traps in VCA

Neutrophils are usually the first cells to infiltrate the site of inflammation which in the case of VCA is the transplanted graft. They are the first line of defense of the innate immune response. When they reach the site of inflammation they execute their function by phagocytosis, degranulation and the formation of NETs. In transplantation, the role of Neutrophils is mainly focused on the destructive role of neutrophils during ischemia-reperfusion injury (IRI). In this sterile inflammation, neutrophils release a lot of oxidative and proteolytic effector activity which damages the tissue of the transplanted allograft. Neutrophil infiltration and activation of the infiltrated tissue lead to the release of damage-associated molecular patterns (DAMPs) from necrotic cells and the extracellular matrix (ECM). These molecules then induce the production of inflammatory cytokines and the recruitment of other cells of the innate immune system like macrophages. Neutrophils also express PRRs like macrophages and other myeloid cells. Through the activation of these receptors by DAMPs, reactive oxygen species (ROS) and

other hydrolytic enzymes are generated which increase allograft damage. Nevertheless, neutrophils also play an important role in the communication and activation of the adaptive immune system. Neutrophils are capable to leave peripheral sites of inflammation and deliver antigens to draining lymph nodes. Under specific conditions, neutrophils display a dendritic cell-like phenotype and are able to present antigens in an MHC-II-dependent manner. Thereby they are able to induce the proliferation of antigen-specific T-cells. It has also been shown that cytokines which are produced by neutrophils are able to influence B-cell survival, maturation and differentiation.[15,30,31]

T-cells express a variety of co-inhibitory receptors like programmed death receptor 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In viral infections it has been suggested that neutrophils, by the expression of programmed death ligand 1 (PD-L1), can also mediate the immune suppression in PD-1-expressing T-cells.[32] However this migration and activation abilities of neutrophils have only been shown in non-sterile infections but not in the setting of transplantation.

Neutrophils are the most abundant immune cells in the human body, but they only have a short half-life of six to eight hours. To extend their effector function neutrophils, once activated, they undergo a process that is called NETosis. NETosis is the process in which neutrophils release neutrophil extracellular traps (NETs), a structure of chromatin filaments that are coated with histones, granular and cytosolic proteins and proteases. In the setting of an unsterile inflammation NETs help the neutrophils to immobilize bacteria, fungi and viruses. This catching process leads to a more efficient elimination of pathogens. There are different stimuli that can activate NETosis. Antibodies or cholesterol crystals for example are able to induce the so-called late suicidal NETosis which occurs after hours of stimulation. Early vital NETosis can be induced by the binding of platelets or other damage-associated molecular patterns to the TLR4 that is expressed on neutrophils. Both, the late suicidal and the early vital induction of NETosis result in activation of protein arginine deiminase 4 (PAD 4). This activation leads to chromatin decondensation. Following the decondensation of chromatin, neutrophil elastase (NE) in the early vital NETosis and NE and myeloperoxidase (MPO) in the late suicidal NETosis translocate into the nucleus and promote further unfolding of chromatin which leads to the disruption of the nuclear membrane. In the late suicidal NETosis the chromatin is then released into the cytosol where it is decorated with granular and cytosolic

proteins. Subsequently, NETs are released through the disruption of the plasma membrane and the neutrophil dies. In the early vital NETosis the protein-decorated chromatin is expelled out of the neutrophil via vesicles and the neutrophil stays alive.

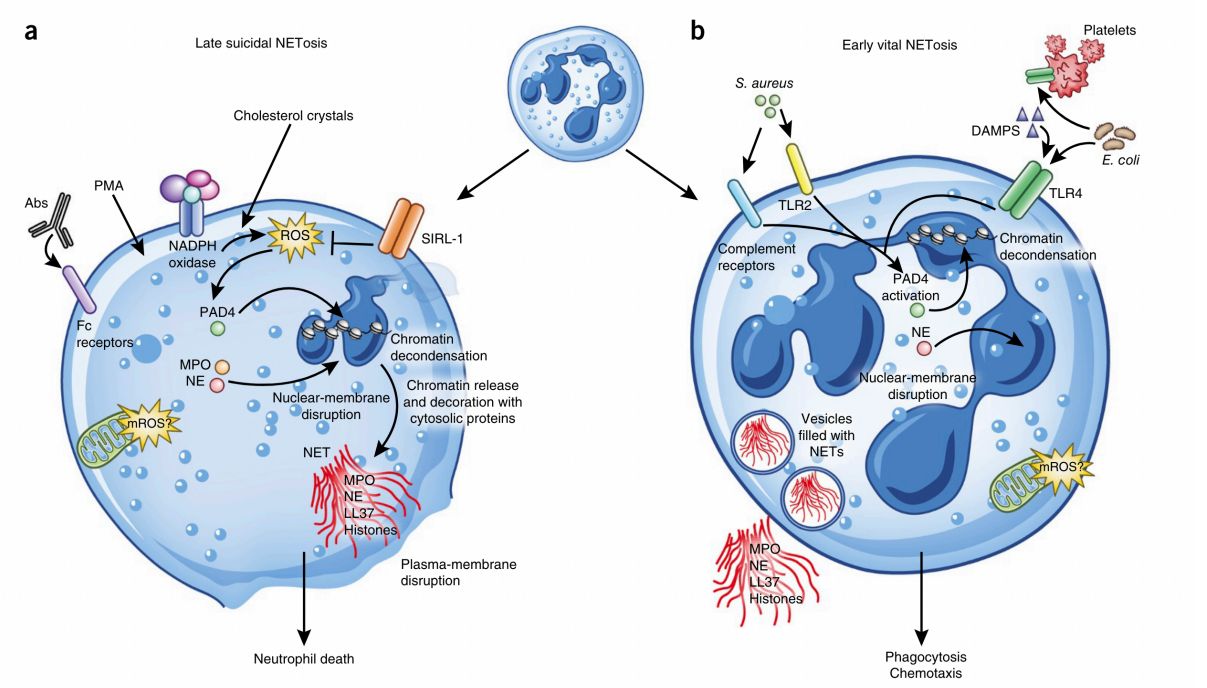


*Figure 7: NETosis.**(a)** Late suicidal NETosis which leads to neutrophil death. **(b)** Early vital NETosis without neutrophil death. Adapted from Jorch et al., 2017* [33]

Vital NETosis might be more abundant in infections than in sterile injury, whereas suicidal NETosis is occurring in sterile and non-sterile injury.

In noninfectious diseases, NETs play an important role. Upon the release of intracellular proteins, which are usually not in contact with extracellular space, the host's immune system can release DAMPs as a result of the presentation of potential autoantigens. This amplifies the ongoing immune response and increases the intrinsic cytotoxic activity of neutrophils. Therefore, NETosis plays an important role in many pathophysiological conditions such as rheumatoid arthritis, diabetes, cancer, and wound healing. NETs can also play an important role in thrombotic events such as vaso-occlusions where they increase the damage which occurs during ischemia-reperfusion injury. Further, NETs are able to help resolve inflammation by eliminating dead cells and cell debris which supports eliminating one source of sterile inflammation.[33–35]

In transplantation, NETs are known to cause extensive endothelial cell injury. This endothelial cell damage is especially happening during IRI through platelet activation and subsequent NET

formation which can also lead to vascular endothelial dysfunction.[34] It has been shown that NETs, because of their intrinsic cytotoxic activity, can stimulate an innate immune response in lung transplantation and therefore prevent transplant tolerance.[36] Recently it has been described that NETs can influence the antibody-mediated rejection of grafts in solid organ transplantation. As mentioned before NETs can cause extensive endothelial cell damage and are able to activate the functions of B and T-cells. It is assumed that NETs can be seen as a source of auto-antigens, which could contribute to the antibody-mediated rejection of grafts in transplantation.[34] However, in vascularized composite allotransplantation underlying processes of neutrophils and NETs are not well understood.

1.4 Current Treatment Options in VCA

In the setting of a vascularized allotransplantation, the immune system of the recipient will recognize the graft as foreign. Therefore, the host's immune system will be activated and without treatment, the graft will be rejected. To avoid graft rejection and minimize the strength of specific allogeneic recognition, the fully functional immune system of the recipient has to be damped with immunosuppressive medication. The common clinical treatment for VCA is a lifelong intake of systemic immunosuppressive medication.[37] Typically the treatment following a VCA starts with an antibody-based induction therapy where T-cells are depleted using an anti-thymocyte globulin. Some induction agents are Alemtuzumab or Basiliximab which are monoclonal antibodies that can block IL-2 receptors on T-cells as well as CD52 receptors on B-cells. Both are important for their respective proliferation. Following the T-cell depletion, life-long intake of a triplet drug combination is taken by the patient that typically consists of an antimetabolic agent such as mycophenolate mofetil, a corticosteroid like prednisolone and a calcineurin inhibitor such as tacrolimus.[9]

Tacrolimus is the most commonly used calcineurin inhibitor used in the immunosuppression following VCA.[4] Calcineurin is a calcium-dependent serine/threonine protein phosphate that modulates the cellular response to calmodulin, a protein that binds to Ca2+ and is important in many cellular processes like Ca2+-dependent intracellular signal transduction pathways. Calcineurin is an important player in T-cell activation and regulation of cellular processes in T-cells. In activated T-cells there is a large influx of Ca2+ which activates calcineurin. The activated calcineurin dephosphorylates the transcription factor which is called nuclear factor

of activating T-cells (NF-AT). Once dephosphorylated NF-AT is activated, it translocates into the nucleus. This process activates the gene that is responsible for IL-2 expression of the cell. Interleukin 2 is an important cytokine that is needed for T-cell proliferation and the generation of effector and memory cells. Calcineurin inhibitors such as tacrolimus inhibit the activation. of calcineurin and therefore the production of IL-2 which inhibits the proliferation of T-cells. [38]

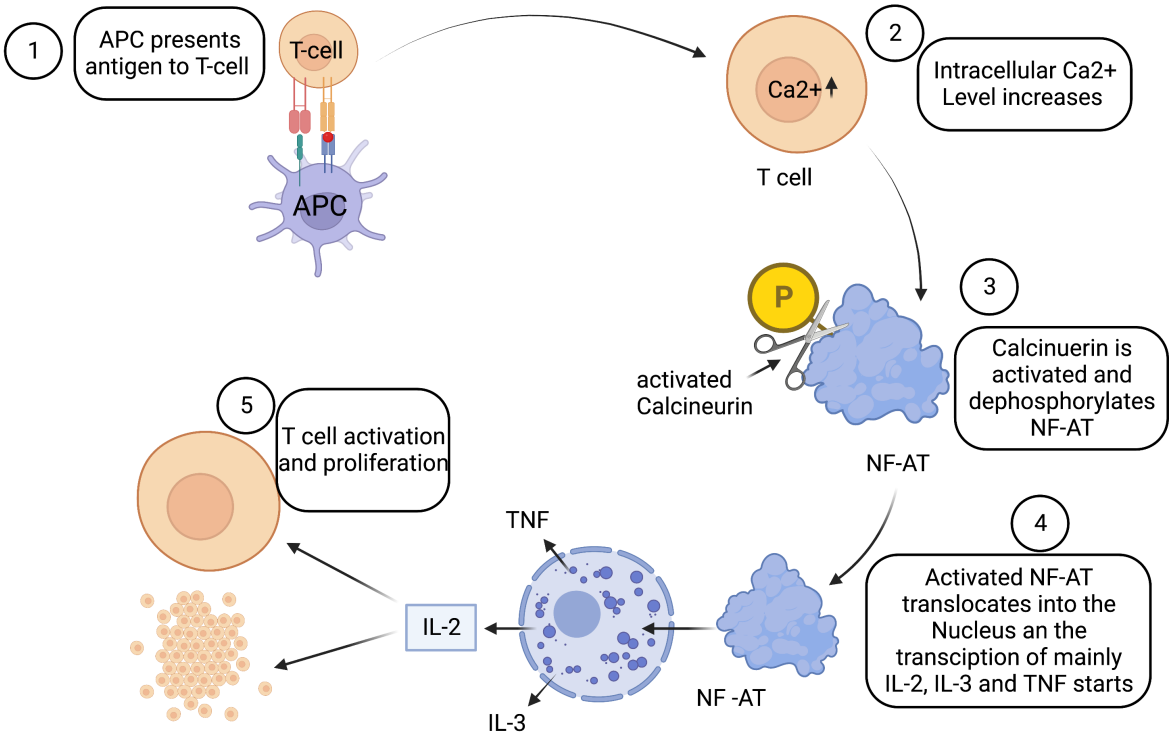


Figure 8: Mode of action of calcineurin. To be activated T-cells require not only the presentation of the antigen via MHC and interaction of it with the TCR, also co-stimulation by the APC is needed. Co-stimulatory molecules on the APC, CD80-86 and CD40 and the corresponding molecules on T-cells, CD28 and CD154 are necessary for T-cell activation. Once the T-cell gets activated, there is a largen influx of Ca2+ into the cell which leads to the activation of calcineurin. Activated calcineurin then dephosphorylates NF-AT which translocates into the nucleus and initiates IL-2, IL-3 and TNF production. Created with BioRender.

However, the lifelong intake of immunosuppressive drugs goes along with side effects. Diabetes mellitus, leukopenia, hypertension, opportunistic infections, higher cancer risk and increased financial burden are associated with chronic immunosuppression.[10] Recently it was shown that 32.2% of hand transplant recipients experienced opportunistic bacterial infections and 41.5% showed signs of hyperglycemia.[12] Therefore several strategies have been investigated that could reduce off-target toxicity and graft survival. Nevertheless, one major problem in VCA management that always has to be taken into account is that VCA, in contrary

to solid organ transplantation is a life-enhancing and not a life-saving procedure. Hence the risk-to-benefit ratio of the transplantation always has to be considered.

1.5 Novel treatment approach in VCA: TGMS-TAC

Compared to solid organ transplantation VCA offers a unique opportunity for local delivery of immunosuppressive medication which can be monitored and applicated easily.[39] Therefore, several research groups have tried to develop an on-demand drug delivery system that can be applicated locally.[12] In our experiments we tested this on-demand drug-delivery system that only releases the pharmaceutically active compound in response to inflammatory stimuli. This localized immunosuppression should decrease systemic-related immunosuppressive off-target toxicity and at the same time increase the local concentration of the active pharmaceutical in charge of immunosuppression, thus leading to prolonged graft survival. The system we used is composed of a self-assembling triglycerol monosterate (TGMS) hydrogel which is loaded with the immunosuppressive tacrolimus (TAC). The hydrogel loaded with the active pharmaceutical compound TAC can be injected subcutaneously into the VCA graft. Tacrolimus should then only be released in response to elevated levels of inflammatory-related enzymes in the graft.

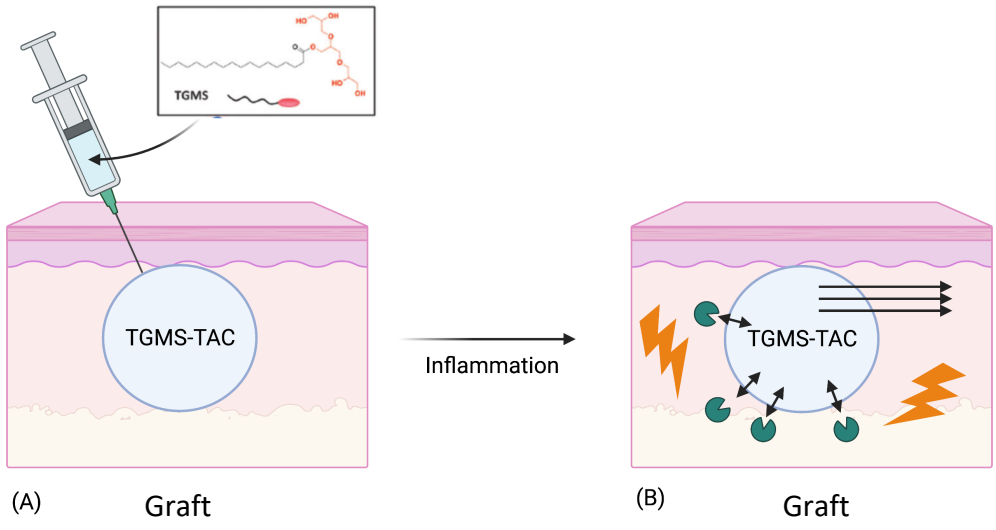


Figure 9: Mode of TGMS-TAC. ***(A)****TGMS-TAC is injected subcutaneously into the grafts skin.* ***(B)*** *Upon inflammation different enzymes that are associated with rejection trigger the release of the active pharmaceutical compound, tacrolimus, into the graft. Created with BioRender.*

This local on demand immunosuppression should be able to decrease systemic tacrolimus levels and therefore its related off-target toxicity, while at the same time increase local

tacrolimus levels which in the end should lead to extended graft survival.[12,40] In rodent models the use of TGMS-TAC already showed promising results in terms of decreased off-target toxicity and graft survival. In our group we are now trying to verify these findings in a porcine model.

2 Aim

The aim of our study is to reveal the involvement of neutrophils and neutrophil extracellular traps in a model of porcine heterotopic limb transplantation. In addition, we aim to acquire differences in neutrophil and neutrophil extracellular trap responses in site-specific immunosuppression with TGMS-TAC single and reinjection and this context we also aim to assess the efficacy of TGMS-TAC as an alternative for systemic TAC treatment.

3 Material and Methods

3.1 Ethical statement

All experiments were planned and carried out in agreement with current 3R guidelines and approved according to the Swiss animal protection laws by the Veterinary Authorities of the Canton Bern, Switzerland, approval number: BE48/19. Anesthesia during hind limb transplantation was performed according to the animal permit with state-of-the-art treatment. Pain management was used subsequently to reduce the exposure of the animals to stress during the follow-up of the transplantations until and including euthanasia.

3.2 Study design

In our study, we aimed to reveal the involvement of neutrophils and neutrophil extracellular traps in porcine vascularized composite allotransplantation. In addition to testing the role of neutrophils and neutrophil extracellular traps in VCA we were also interested if there is any difference in neutrophil and NET response if animals were treated with a single injection or were reinjected with a local immunosuppression post transplantation. To achieve our goal we preformed heterotopic hind-limb-transplantations in Swiss landrace pigs. Donor (N=6) and recipient pigs (N=12) were matched based on their highest incompatibility in swine leukocyte antigens (SLA) to achieve the best mismatch possible. Male and female pigs were included. Following transplantation animals were divided randomly into three different groups. Group 1 (N=4) was used as a control group where animals did not receive any immunosuppressive

treatment. Group 2 (N=4) animals were single injected with 140mg/kg of graft weight of TGMS-TAC subcutaneously into the graft on the day of the transplantation. Group 3 (N=4) animals were injected the same way as group 2 with 140mg/kg of TGMS-TAC subcutaneously on the day of transplantation and in addition, the same dose of TGMS-TAC was reinjected every 30 days post-transplantation until endpoint.

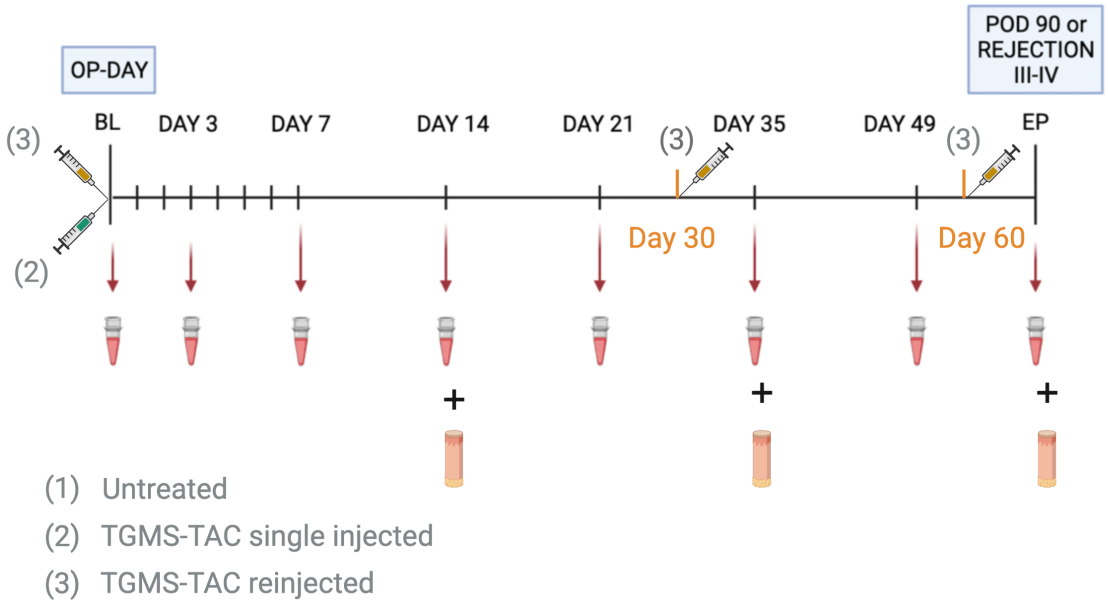


Figure 10: Study design. Group (1) pigs were untreated. Group (2) pigs were singe injected with TGMS-TAC on the day of the operation (OP-Day). Group (3) pigs were injected with TGMS-TAC on the day of operation and every 30 days post operation. Blood and skin samples were taken accordingly. Created with BioRender.

Table 1: Porcine Group allocation

Groups	Pig Number
Group 1	Pig 1
	Pig 2
	Pig 3
	Pig 4
Group 2	Pig 5
	Pig 6
	Pig 7
	Pig 8
Group 3	Pig 9
	Pig 10
	Pig 11
	Pig 12

3.2.1 Heterotopic swine hindlimb transplantation

Six outbred land pigs between 18 to 30kg were selected to serve as donors for 12 recipient pigs according to the highest mismatch in SLA. In both the donor and recipient groups female and male pigs were included. The selected pigs were sedated with Propofol 1-4mg/kg, ketamin 1mg/kg and inhaled isoflurane and afterwards put under general anesthesia. Osteomyocutaneous limb allografts containing lymph nodes were harvested from each donor pig and transplanted on the contralateral side of each recipient pig. One donor donated two hind limbs for two recipient pigs. The grafts were transplanted into a subcutaneous pocket previously made from the groin to the dorsolateral abdominal wall. Donor vessels from the graft were ligated and then donor and recipient were linked by an arterial end-to-end anastomosis. To enable better post-transplantation monitoring of the grafts, the skin paddles from the donor grafts were exteriorized on the dorsolateral side of the recipients. To facilitate post-operative venous blood collection, a port-a-cath was inserted into the external jugular vein and then placed subcutaneously in the posterior neck area.

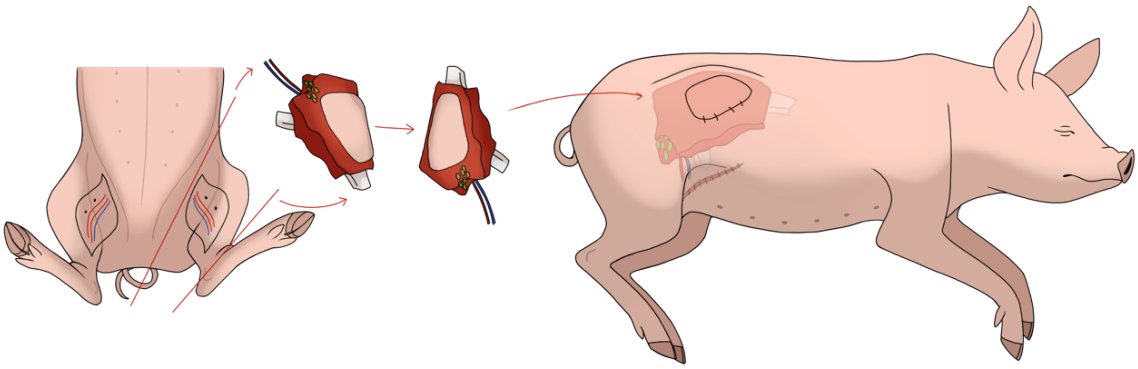


Figure 11: Heterotopic model of swine hind limb transplantation. Modified from Ibrahim et al, 201.

3.2.2 Follow-up post-transplantation

Following transplantation, the pigs were monitored for 24 hours in the intensive care unit in the experimental surgical facility (ESF), where the previous transplantations were performed. Subsequently, the pigs were transferred to the Clinic for Ruminants and Pigs, Faculty of Veterinary Medicine, University of Bern and maintained there for a monitory period of 10-15 days. According to their individual health status, the pigs were transported to a local farm in Bern until endpoint. The pig's health status was monitored daily in the first two weeks after transplantation and then twice a week till endpoint based on a predefined score sheet.
In addition to the health status, the graft rejection status was monitored and graded according to the visible criteria of skin evaluation.

Table 2: Macroscopic criteria of skin evaluation

Grade	Macroscopic skin criteria
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 determined when Grade III-IV rejection or graft survival until post-operative day (POD) 90 was reached.

3.3 Sample collection and euthanasia

Pig samples were collected at different time points post-transplantation. Blood samples were taken on the day of the transplantation, which were defined as baseline samples. Following transplantation, blood samples were taken on POD 1, 3, 7, 14 and afterwards weekly until endpoint was reached. Skin punch biopsies were taken from the graft and contralateral side at POD 7, 14, 35 and then every two weeks until endpoint. Euthanasia was performed at endpoint under general anesthesia and samples were collected in sterile conditions. Skin samples from graft, and contralateral side, blood samples, muscle samples from graft, close to graft and contralateral, lymph node samples from graft, close to graft and contralateral side, thymus samples, liver samples, kidney samples, spleen samples were taken during euthanasia. From each organ one sample was snap frozen on dry ice, one sample was stored in a cassette in formaldehyde (for histopathology analysis) and one sample was stored in a cryo mold covered with Tissue-Tek O.C.T (for tissue immunofluorescence staining analysis) (Sakura Finetek, 4583).

3.4 Blood and Plasma collection

Blood was taken as described in the previous chapter via the port-a-cath on the posterior neck area. Blood was collected in EDTA and Serum tubes. Both tubes were centrifuged 15 minutes at 10.000G at room temperature (RT). Plasma was collected an aliquoted from the EDTA tubes and serum from the serum tubes. All aliquots contained 500µL and were stored at -80°C.

3.5 Peripheral blood mononuclear cell (PBMC) collection

Blood samples were taken via the port-a-cath on in 3.3 defined time points in EDTA tubes. Following blood collection tubes were centrifuged 15 minutes at 10.000G at RT. Plasma was extracted and stored as described in 3.4. The cellular part of the blood was re-suspended in 1x phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) (Sigma, F7524) in a 1:1 dilution. Then the dilution was carefully layered on density gradient media solution (Ficoll-Paque PLUS® 17144002) and centrifuged at 400G for 20 minutes at RT. PBMCs were collected from the density gradient and re-suspended in 90% FBS + 10% Dimethyl Sulfoxide (DMSO) (Sigma, D4540) for storage at -150°C until use.

3.6 Histopathology

Skin and muscle samples were collected at endpoint and stored in cassettes submerged in formaldehyde. Samples were sent to the institute of pathology of the University of Bern. There they were fixed in 4% formaldehyde before being embedded in paraffin and stained with hematoxylin and eosin for microscopic quality evaluation. The skin samples were scored according to the Banff score which defines different stages of swine skin VCA rejection.

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)

VCA, vascularized composite allotransplantation.

*Figure 12: **(A)** Modified Banff criteria for swine VCA rejection classification, adapted from Etra et al., 2019*[41]

3.7 Flow cytometry

PBMCs were thawed and re-suspended in warm complete cell culture medium (RPMI (Gibco Ref 31870-025), penicillin streptomycin (P/S) (Gibco, 15140-122) and FBS (Sigma, F7524)). Following, cells were centrifuged for 5 minutes with 400G at RT and distributed into a 96 well plate with a concentration of $1x10^6$ cells/100µL. Then cells were incubated for 2 hours in the incubator at 37°C with 5% $C0_2$ to recover the epitopes. Next, dead/live mix (Fixable Yellow Dead Cell Stain Kit 405nm, Thermofisher, Ref.: L34959) was added to each well in a 1:1000 dilution. After washing, FACS buffer (1xPBS with 2% FBS [Sigma, F7524]) was added to each well and cells were centrifuged at 400G for 5 minutes at RT, PBMCs were resuspended in FACS buffer with FcR blocker (FcR Blocking Reagent, human, Macs, Ref.: 130-059-901) (dilution 1:12,5) and incubated for 10 minutes. Subsequently, the according antibodies (see Table 3, Panels for Flow Cytometry) were added and incubated for 30 minutes together with the cells. For intracellular staining, cells were fixed and permeabilized with Foxp3 / Transcription Factor Staining Buffer Set (eBioscience®, Ref.: 00-5523-00) and stained with the corresponding antibody (See Table 3, Panels for Flow Cytometry). Following, the cells were resuspended in

FACS buffer. Data was acquired with the flow cytometry analyzer Cytoflex S2(Beckman Coulter) and analyzed using Flow-Jo software V.10 (Tri-Star, Ashland, United States).

Table 3: Panels for Flow cytometry

Panel	Intracellular/Extracellular	Color/Origin/Type	Target	Dilution	Company	Reference Number/Clone
1	Extracellular	FITC/Mouse anti Pig/IgG1	CD3	1:100	Biorad	Ref.: MCA5951F Clone: PPT3
	Extracellular	PE Cy5/Mouse anti Pig/IgG2b	CD4	1:100	Thermofisher	Ref.: 28734 Clone: 74-12-4
	Intracellular	eFluor 450/Rat anti human, Pig/ IgG2a	FoxP3	1:300	Thermofisher	Ref.: 48-5773-82 Clone: FJK-16s
2	Extracellular	RPE/Mouse anti Pig/IgG1	CD3	1:100	Biorad	Ref.: MCA5951PE Clone: PPT3
	Extracellular	PE Cy5/Mouse anti Pig/IgG2b	CD4	1:100	Thermofisher	Ref.: 48-5773-82 Clone: FJK-16s
	Extracellular	FITC/Mouse anti Pig/IgG1	CD8	1:100	Biorad	Ref.: MCA5954F Clone: PPT23

3.8 Skin homogenization and Protein Quantification

For further analysis skin samples needed to be homogenized. Snap-frozen samples were taken from -80°C storage. From each sample around 50mg were weighted and incubated with RIPA lysis and extraction buffer (Thermofisher, Ref.: 89900) containing protease inhibitor cocktail (Halt™ Protease and Phosphatase Inhibitor Single-Use-Cocktail, Thermofisher, Ref.: 1861280).

Then tissue was homogenized with a plastic pestle around 40-50 passages on ice. The samples were then lysed again in a tissue lyser for 30 seconds at 20Hz with a magnetic bead included in each sample tube. After 1 hour of incubation on a rotator at 4°C, samples were centrifuged 15 minutes at 13.000 rpm at 4°C. The supernatant was collected and a protein quantification was performed to ensure that the tissue homogenization worked. For this protein quantification the Coomassie Bradford Protein Assay Kit (Thermoscientific, Ref.: 23200) was used. Bovine serum albumin (BSA) standards were prepared with mqH_2O and 5µL each were added to the appropriate wells in a 96 well NUNC flat bottom transparent well plate, in duplicate. Skin samples were diluted 1:10 and muscle samples 1:50 with mqH_2O. Following, 250µL of Coomassie Protein Assay Reagent (Thermoscientific, Ref.: 1856209) were added to each standard and sample well. After a 30 second incubation absorption was measured at 595nm in the Varioskan Lux Microplate Reader. Skin and Muscle samples with a sufficient number of protein were stored at -20°C for further analysis.

3.9 Cytokine Level measurements in Skin, Muscle and Plasma

Cytokine and chemokine levels in skin, muscle and plasma samples were measured using the Milliplex immunoassay (MILLIPLEX®Porcine Cytokine/Chemokine Magnetic Bead Panel, Millipore. PCYTMAG-23K). The Miliplex technology is based on the Luminex xMAP® technology and uses fluorescent-coded magnetic beads microspheres. We performed a 6-plex assay for the following cytokines and chemokines: IFNµ, IL-1α, IL-1β, IL-1ra, IL-10, TNFα. The procedure was performed according to the kit`s instructions. Homogenized skin and muscle lysate and plasma samples were diluted 1:1 in 200µL of Assay buffer. All samples were tested in duplicates. The plate was run according to the instruction guidelines with the FLEXMAP 3D® (LUMINEX) system. Results are depicted in pg/mL.

3.10 Immunofluorescence staining

To visualize specific cellular structures and proteins, an immunofluorescence staining of skin, muscle and lymph node tissue was performed. Skin, muscle and lymph node samples were collected at baseline and at endpoint from pigs in all groups and stored at -80°C in tissue cryo

molds and covered with Tissue-Tek O.C.T (Sakura Finetek, 4583). 5μm thick sections were cut using the Leica Cryostat CM1950 and placed on superfrost glass slides and stored at -20°C until they further use.

For staining samples were airdried and then fixed with ice cold acetone methanol 1:1 for 5-8 minutes. Then the slides with fixed tissue were rehydrated in glass jars in 1x Tris-Buffered Saline (TBS) for 5 minutes at RT. Afterwards slides were dried and marked around with a Dako pen (Dako Pen-Delimiting Pen, Aligent Dako, Ref.: s-200230-2). Tissue samples were then blocked with blocking solution containing 10% Donkey/Goat Serum, 0,5% BSA and 0,05% Tween (AppliChem, A4974) in 1xTBS. After 1 hour of incubation at RT blocking solution was removed and specific primary antibody mix was added (See Table 4, Antibodies for Immunofluorescence). Samples were incubated over night at 4°. Then samples were washed for 1,5 hours in 1x TBS with change of TBS every 30 minutes. Following, specific secondary antibody mix including DAPI (4`6 Diamidino-2-phenylindol, in1:1000 dilution, Sigma, Ref.: 10236276001) (See Table 4, Antibodies for Immunofluorescence) was added to each sample. After 1,5 hours of incubation, samples were again washed for 1,5 hours in 1x TBS with change of TBS every 30 minutes. Afterwards, slides were mounted with prolong gold antifade reagent. (Thermofisher, Ref.: P36934). Samples were covered with cover slides and dried for 30 minutes before they were sealed with nail polish. Images were acquired with the confocal microscope LSM Zeiss 980 using the 20x objectives. Pictures were analyzed with Fiji, ImageJ software.

Table 4: Antibodies for Immunofluorescence

Primary Antibodies						
Target	**Species**	**Clone**	**M/P**	**Dilution**	**Reference Number**	**Company**
CD31	Rat anti Pig IgG1	377537	M	1:100	MAB33871	R&D Systems
CD163	Mouse anti Pig	2A10/11	M	1:100	mCA2311	Biorad

Target	Species	Clone	M/P	Dilution	Reference Number	Company
H4Cit	Rabbit anti H3Cit	/	P	1:100	07-596	Millipore
MPO	Mouse anti MPO IgG	/	M	1:100	ab25989	abcam
E-Selectin	Mouse anti Pig IgG	1.2B6	M	1:100	sc-18852	Santa-Cruz
CD3	Mouse anti Pig Cy5	/	M	1:100	4510-13	Biotech
IgG	Goat anti Pig FITC	/	P	1:100	6050-02	Southern Biotech
C3b/c	Rabbit Anti Human FITC	/	P	1:100	F0201	DAKO
Secondary Antibodies						
Target	**Species**	**Clone**	**M/P**	**Dilution**	**Reference Number**	**Company**
	Donkey anti Rat AF488 IgG	/	P	1:500	A21208	Invitrogen
	Donkey anti rabbit AF488	/	P	1:500	A32790	Invitrogen
	Goat anti Mouse AF546	/	P	1:500	A11030	Invitrogen
	Goat anti Rabbit AF568	/	P	1:500	A11036	Invitrogen

Target	Species	Clone	M/P	Dilution	Reference Number	Company
	Goat anti Rat AF568 IgG	/	P	1:500	A11077	Invitrogen
	Goat anti Rat AF680 IgG	/	P	1:500	A21096	Invitrogen

(M) = Monoclonal, (P) = Polyclonal Antibody

3.11 Western Blot

Homogenized tissue (skin or muscle) samples or plasma samples were thawed. The sample loading solution included: 50µg of sample, DTT sample reducing agent (Blot™ Sample Reducing Agent DTT, Invitrogen, Ref.: B0009), Lämmli loading buffer (Blot™ LDS Sample Buffer, Novex, Ref.: B007) and dH_2O. The sample mixes were heated at 95°C for 10 minutes before loading into mini Gels (Mini-Gel Blot™ 4-12% Bis-Tris Plus, Invitrogen, Ref.: NW04120BOX). Mini Gel Tanks (Invitrogen, Ref.: A25977) were prepared according to instructions and filled with running buffer (MES SDS Running Buffer Blot™, Novex,B0002). 12µL of Protein Ladder (Spectra™ Multicolor Broad Range Protein Ladder, Invitrogen, Ref.: 26634) was added into the appropriate well. Protein separation electrophoresis was performed using a PowerPac™ Basic power supply (Biorad, Ref.: 1645050). Following proteins were transferred onto PVDF membranes (iBlot 2 PVDF regular stack, Invitrogen, Ref.: IB24001) using the iBlot 2 Transfer machine (Invitrogen, Ref.: IB21001). Proteins were transferred at a constant voltage of 25V for 6 minutes. Following, PVDF membranes were blocked for one hour with Intercept Blocking Buffer PBS, (Licor, Ref.: 927-70001). Afterwards, the appropriate primary antibody was added (see Table 5, Antibodies for Western Blot) diluted in blocking buffer. Gels were incubated overnight at 4°C with primary antibody. The next day membranes were washed with PBS-0.05% Tween (AppliChem, A4974) for 1.5 hours, rotating on a tube roller with a change of washing buffer after 30 minutes and one hour. Following secondary antibody diluted in blocking solution was added to the membrane and incubated for one hour. Afterwards, membranes were washed again with PBS-0.05% Tween for 1.5 hours with change

of the wash buffer every 30 minutes on a tube roller at RT. Membranes were developed in the Li-Cor Odyssey Infrared Image System. Pictures were analyzed using Image Studio software. If needed membranes were striped for additional staining. 0.2M NaOH stripping solution was added to each membrane in a 50mL falcon tube. Membranes were incubated for 30 minutes on a tube roller at RT with stripping solution with a change of striping solution after 15 minutes. Following the membranes were washed with PBS-0.05% Tween for at least 30 minutes with a change of wash buffer after 10 minutes. Following the membranes were blocked again with blocking buffer for one hour on a tube roller at RT. Afterwards, the membranes were either stained again or stored at 4°C.

Table 5: Antibodies for Western Blot

Primary Antibodies				
Target	**Species**	**Dilution**	**Reference Number**	**Company**
MPO	Rabbit anti human	1:500	A0398	DAKO
B-Actin	Mouse anti Human, Pig IgG	1:500	926-42212	Licor
Secondary Antibodies				
	Goat anti Mouse IgG IRDye 800cW	1:5000	926-32210	Li-Cor
	Goat anti Rabbit IgG IRDye 800cW	1:5000	926-32211	Li-Cor

3.12 Home Made DNA-MPO-ELISA

Home Made DNA MPO-ELISA was performed with skin, muscle and lymph node lysate as well as with plasma. A 96 Well flat bottom, transparent, nunc MaxiSorb plate (Invitrogen, Ref.:44-2404-21) was precoated with MPO Antibody (Mouse anti Human MPO Antibody, Clone 4A4, IgG2b, Biorad, Ref.: 0400-0002) diluted in PBS 1x without calcium- and magnesium-chloride.

After incubation over night at 4°C the antibody mix was removed and the wells were washed three times with 5%BSA in 1xPBS (without calcium- and magnesium-chloride) Following, wells were blocked with 5% BSA in 1xPBS (without calcium- and magnesium-chloride). Afterwards 60µL of sample was added to each well in PBS 0.3%BSA. All Samples were added in a 1:4 dilution in duplicates. As positive control, neutrophils that were stimulated but not digester were also added in a 1:16 dilution. After 30 seconds of shaking at RT, DNAse digestion was performed by adding 2U/mL of DNAse I (DN25-100MG). The digestion was stopped by adding 2.5mM of EDTA. After 2 hours of incubation at RT, 1:250 diluted Peroxidase-conjugated anti-DNA antibody (Monoclonal Antibody from cell death detection ELISA Kit, Monoclonal, Clone MCA-33, Roche, Ref.:11544675001)in 1x PBS (without calcium- and magnesium-chloride) 0.5% BSA was added to each well. Following a 90-minute incubation at RT the wells were washed five times with 1xPBS (without calcium- and magnesium-chloride) 0.1% Tween. Then 100µL of TMB substrate solution (Thermoscientific, Ref.: N301) were added to each well. Afterwards, the plate was incubated for approximately 20 minutes in the dark at RT. Then the reaction was stopped by adding stop solution (0.5M Sulfuric Acid Solution, Fluka Analytical, Ref.: 35276-1L). After 5 minutes incubation at RT, the absorbance was measured at 450nm in a Varioskan Lux Microplate Reader.

3.13 C3a -ELISA

For C3a analysis of pig plasma the Porcine C3a Kiit (MyBiosource, Ref.: MBS2509360) was used. Plasma samples were taken out of the -80°C freezer and thawed. Standard working solutions were prepared according to kit instructions and added in duplicates to the appropriate wells. Samples were diluted 1:50 with sample solution and added in duplicate to the appropriate wells. The procedure was performed according to the kit`s instructions. The optical density was measure at 450nm with a Varioskan Lux Microplate Reader.

3.14 NETosis inhibition by Tacrolimus

Porcine neutrophils were isolated from EDTA-blood from healthy pigs by density centrifugation with Histopaque-1119 (MERCK, Ref: 11191) and a density gradient centrifugation with Percoll (Merck, Ref: P4937). Following, the purity of the isolated neutrophils was confirmed with the Sysmex KX21N Hematology Analyzer (Sysmex

Corporation. Hyogo, Japan). To be able to define the effects of tacrolimus on NET formation the neutrophils were cultured in RPMI 1640 medium (Gibco, Ref.: 72400047) and stimulated with 5µM Ionomycin (ThermoFischer Scienific, Ref.: I24222) with different concentration of Tacrolimus (R&S Pharmachem, Ref.: 104987-11-3). For the definition and visualization of NETs, cellular DNA was stained with DAPI (Invitrogen, Ref.: D1306) and visualized using a Zeiss LSM 980 confocal microscope.

3.15 Statistical analysis

Data was analyzed with Prism software version 9.4.1 (Graphpad software). Statistical analysis between different groups were acquired with ordinary one-way ANOVA with Tukey`s multiple comparisons test. When statistical analysis with comparing multiple parameters within and between groups were performed, a two-way ANOVA with Šídák`s multiple comparisons test was used. P values ≤ 0.05 were considered as significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4 Results

4.1 TGMS-TAC injection into the graft prolongs graft survival

The macroscopic evaluations of the transplanted limb grafts were evaluated via the skin paddle of the graft. The paddles were observed for signs of rejection such as erythema, edema, epidermolysis, necrosis, and mummification which all indicate different stages of rejection (see Table 2). The grafts from all groups showed edema and erythema, which are signs of grade I rejection, at POD 4. This early onset of rejection suggests that the inflammatory trauma that was caused by the surgery triggers an inflammatory response, and the animals need time to recover from this surgical trauma. As expected and in contrast to the other groups, the untreated control group 1 did not recover from surgical trauma and showed additional signs of inflammation such as necrosis and epidermolysis shortly after the transplantation with reaching grade III rejection around POD 8 since they did not receive any form of immunosuppressive therapy. The other groups including the single and reinjected TGMS-TAC group animals showed almost no sign of rejection. At POD 14 the skin grafts from TGMS-TAC treated animals showed no more signs of rejection and appeared similar to healthy

skin. Animals that were single injected with TGMS-TAC reached grade III rejection around POD 60. Animals that were reinjected with TGMS-TAC all reached the defined endpoint POD90 with one pig showing grade IV rejection and three showing grade II rejection.

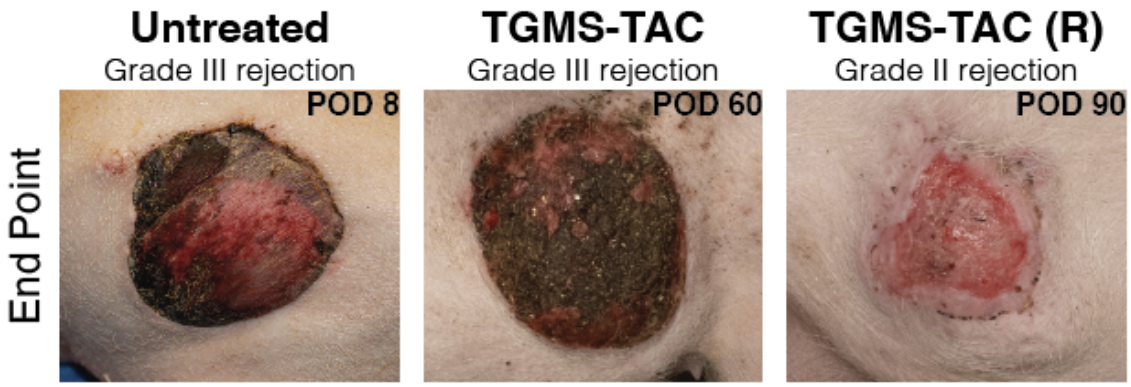


Figure 13: Representative macroscopic evaluations of VCA grafts at Endpoint

In addition to the macroscopic evaluation of the VCA grafts, rejected skin and muscle were histologically evaluated. Each sample was assessed by certified pathologist from the Institute of pathology of the University of Bern. The skin of each pig was evaluated and according to epidermal involvement in terms of infiltrating inflammatory cells, keratinocyte necrosis epidermal necrosis and full-thickness necrosis respectively, a Banff score was given to each pig.[42] Most of the animals which were untreated showed signs of moderate neutrophilic inflammation and some also showed signs of dermal edema. As for the pigs which received a single injection of TGMS-TAC, 50% showed severe signs of dermal infiltration whereas the other 50 % only showed moderate signs. 75% of the animals which were reinjected with TGMS-TAC showed milder to moderate dermal infiltration whereas one pig showed signs of full thickness necrosis. Muscle samples were also histologically evaluated and received a score for necrotic or atrophic damage and leukocyte (lc) infiltration. In the untreated group, the lc infiltration was the same for all pigs and showed extensive damage including necrosis accompanied by neutrophilic inflammation. As for the pigs that received a single injection of TGMS-TAC, the animals showed no signs of necrosis and only mild to moderate perivascular infiltrates. 75% of the animals which received multiple injections of TGMS-TAC showed only very little signs of lc infiltration and necrosis, whereas only one showed signs of extensive necrosis.

Overall, there was no significant difference in the pathological skin score between the groups detectable, nor in the scores that the animals received for pathological muscle scoring. Nevertheless, we detected a favorable trend showing a decrease in lc infiltration in the TGMS-TAC and TGMS-TAC reinjected animals compared to the untreated group.

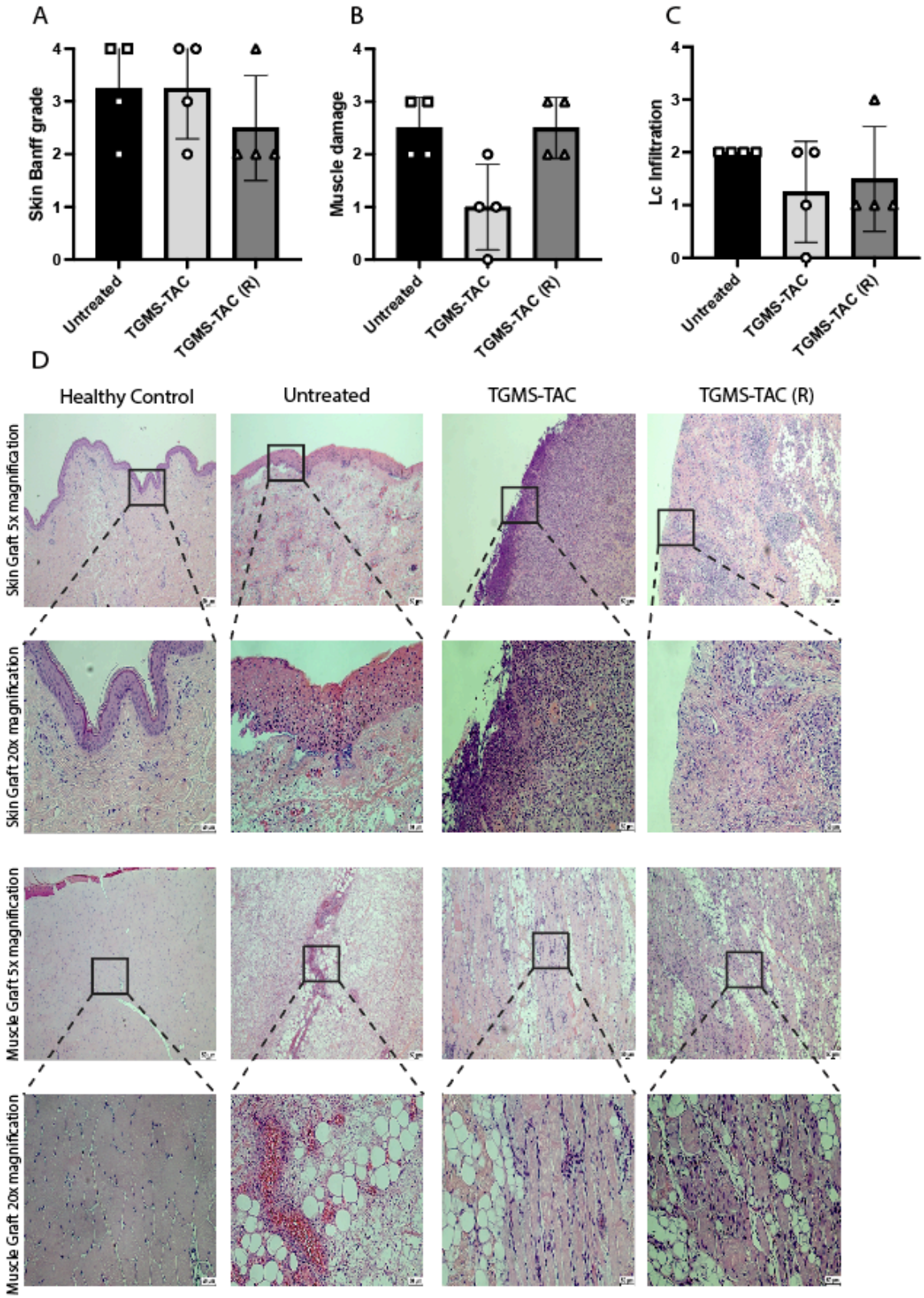


Figure 14: ***Histological evaluation of skin and muscle. (A)*** *Skin from transplanted grafts after rejection were graded based on swine VCA skin rejection classification criteria (modified Banff criteria).* ***(B)*** *Muscle grafts after rejection were evaluated based on extended necrosis and atrophy and* ***(C)*** *lc infiltration.* ***(D)*** *Representative pictures of histological evaluations of VCA graft skin and muscle at endpoint. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

In the group of untreated animals, the grafts survived for a maximum of 9 days. Two of the four animals in this group reached grade III rejection at POD7 whereas one animal reached rejection on POD8 and the last on POD9. The mean survival time of the grafts in this group is 7.75 days.

The animals that were single-injected with TGMS-TAC had increasingly higher and significantly longer graft survival compared to the untreated animals (p=0.0062). The mean survival time of the grafts in this group was 46 days. Animals that received multiple injections of TGMS-TAC survived significantly longer compared to untreated (p= 0.0025) animals. The pigs from this double injected group also survived significantly longer than the animals that were only single injected with TGMS-TAC (p= 0.0027). Pigs that received multiple injections, survived all until the defined endpoint POD90. Our findings suggest that reinjection of TGMS-TAC is the most efficient method for prolonging graft survival in a VCA setting.

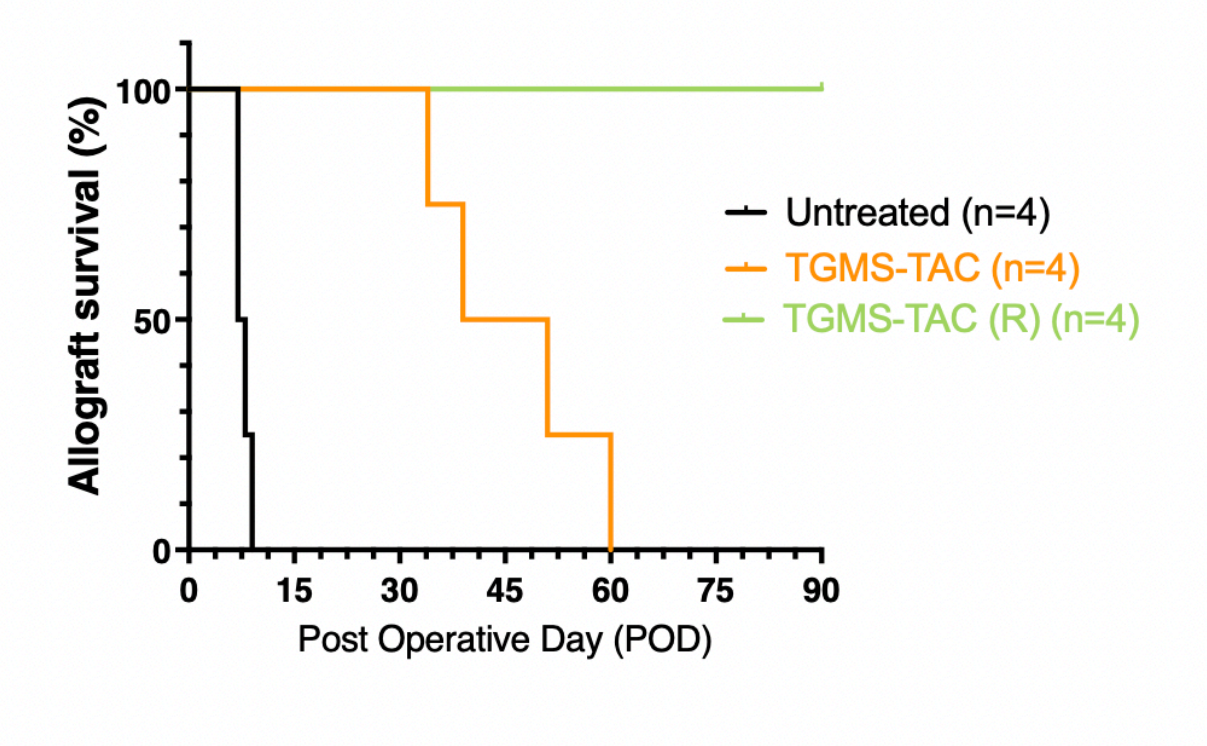


Figure 15: Kaplan-Meier Graf of allograft survival of different groups. Grafts of group 1 (untreated group, n=4) reached grade III rejection around POD8. Grafts of group 2 (TGMS-TAC single injected, n=4) reached grade III rejection around POD46. The grafts of animals that were reinjected with TGMS-TAC (group 3, n=4) all reached endpoint.

Table 6: Summary of graft survival of all 4 groups

Group	Median Graft-Survival Time (days)	P value test of different survival times	n
Untreated	7.75		4
TGMS-TAC	46	0.0062 vs. untreated	4
TGMS-TAC (R)	90	0.0025 vs. untreated 0.0027 vs. TGMS-TAC	4

4.2 Cytokine levels in plasma, skin and muscle tissue during VCA graft rejection

In graft rejection many cytokines and chemokines play an important role. Many of them are related to neutrophil attraction in inflammation and respectively some are also produced by neutrophils and exert their pro-inflammatory functions through autoregulation. To assess the role of neutrophils in VCA graft rejection we were interested in systemic processes during rejection. In addition to their relevance as chemoattractant proteins we were also interested in measuring the systemic levels of specific cytokines since a majority of them are produced by T-cell subsets that may also be effected by TGMS-TAC injections. Therefore, we measured cytokines in plasma and compared the folds increase and decrease respectively between baseline and endpoint levels and see if there are any differences detectable between the different treatment groups. Since all pigs are showing a natural difference between all cytokine concentrations in plasma, we wanted to assure that this bias is not included in our results. Hence, we compared the folds increase and decrease of the specific cytokine at the time of rejection (endpoint) to the specific baseline value they individually showed at baseline. In addition, we controlled for any local differences in cytokine levels detectable in the VCA graft during rejection. To do so, we measured and compared the concentrations of cytokines in graft tissue between treatment groups and between skin and muscle samples. The following cytokines were tested: Pro-inflammatory, neutrophil activating cytokines such as IL-1α, IL-1β, TNF-α and IFN-μ and the receptor antagonist IL-1ra of IL-1α and IL-1β. All cytokines were measured using a Milliplex immunoassay.

4.2.1 Systemic Cytokine Plasma Levels during rejection

The neutrophil stimulating, pro-inflammatory cytokine IL-1α does not show any significance in folds increase or decrease to the individual baseline. In the untreated group, the cytokine concentration of IL-1α seems to stay the same. In the TGMS-TAC single injected and TGMS-TAC reinjected groups we were able to detect a slight increase of IL-1α at endpoint compared to baseline. Nevertheless, we were not able to detect any differences between the different groups in changes of IL-1α at endpoint compared to baseline levels. For IL-1β we were not able to detect high enough readouts in plasma of all treated groups suggesting that systemic IL-1β levels are too low systemically at endpoint to be detectable. The pro-inflammatory cytokine TNF-α shows a tendency of increase in groups of pigs which were either single or double injected with TGMS-TAC towards endpoint compared to the other group. However, we were not able to detect a significant difference between the groups. Furthermore, we detected that IFN-γ levels in all treatment groups but significant increase or decrease was detected. The receptor antagonist IL-1ra levels increased the most in the group of animals that received multiple injections of TGMS-TAC at endpoint compared to the other groups, but the differences were not significant. Finally, the anti-inflammatory cytokine IL-10 showed the highest amount of increase in the animals that were reinjected with TGMS-TAC compared to all other treatment groups. Regardless, these differences were not significant either. (see Figure 16).

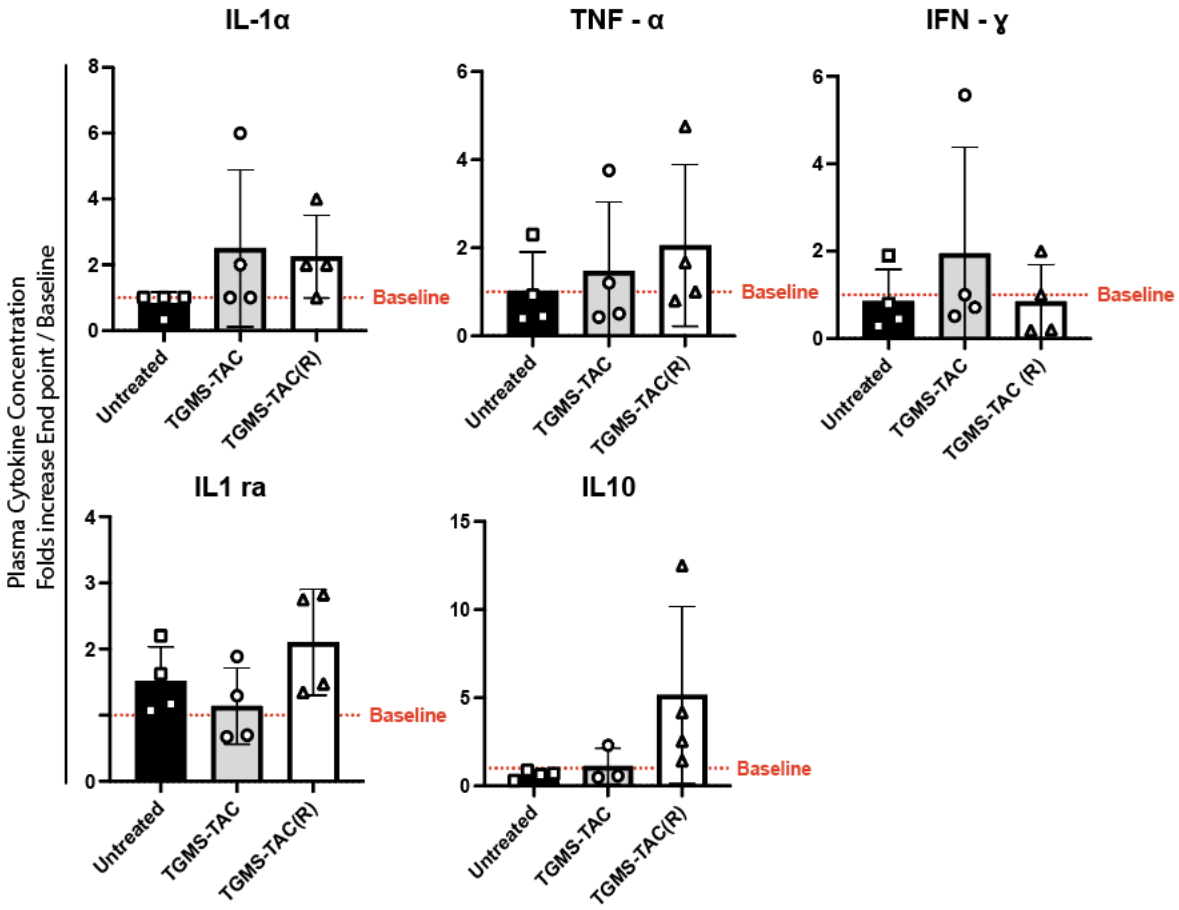


Figure 16: ***Plasma Cytokine Levels increase respectively decrease endpoint compared to Baseline.*** *Plasma samples of transplanted animals, which were either untreated or treated with a local injections of TGMS-TAC (single or reinjected), were tested for folds increase or decrease of cytokine levels endpoint compared to Baseline. Concentrations were acquired with Milliplex immunoassay. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test and two ANOVA with Šídák multiple comparisons test was used for statistical analysis. p value <0.05 was considered as significant.*

4.2.2 Local levels of cytokines in skin and muscle samples during graft rejection

In skin and in muscle we were not able to detect any significant difference of IL-1α levels between the different groups. IL-1β levels in skin and muscle of rejected grafts also showed no significant differences between the different treatment groups. In muscle, the detectable levels of IL-1β of the single and reinjected group were both very small (161 ± 55.15 ng/ml TGMS-TAC; 172 ± 83.27 ng/ml TGMS-TAC (R)) and comparable to healthy donor muscle (122 ng/ml) while the levels in skin were much higher (6468 ± 7471 ng/ml TGMS-TAC; 11936 ± 8725 ng/ml TGMS-TAC (R)) compared to the healthy control (122 ng/ml).

Furthermore, we were only able to detect measurable values of TNF-α in skin but not in muscle. The values for muscle were most likely too small to be measurable by the

immunoassay. However, we were not able to detect any significant difference between the groups in skin. Further, IFN-μ values in both skin and muscle samples of all groups neither showed any significant differences between the treatment groups nor between skin and muscle sample of the same group. Nevertheless, IFN-μ values in muscle were in general higher when compared to skin values, whereas IL-1α and IL-1β values were generally higher in skin compared to muscle.

IL-10 were only detectable for skin tissue since the values for muscle were under the measurable range. The anti-inflammatory cytokine showed a tendency of increase in the skin of animals which were reinjected with TGMS-TAC (R) (91 ± 89.54 ng/ml) compared to the other groups (24 ng/ml healthy control; 55 ± 26.46 ng/ml TGMS-TAC; 42.50 ± 9.75 ng/ml TGMS-TAC (R)).

The receptor antagonist of IL-1α, IL-1ra also did not show any significant differences between the different treatment groups (see Figure 17)

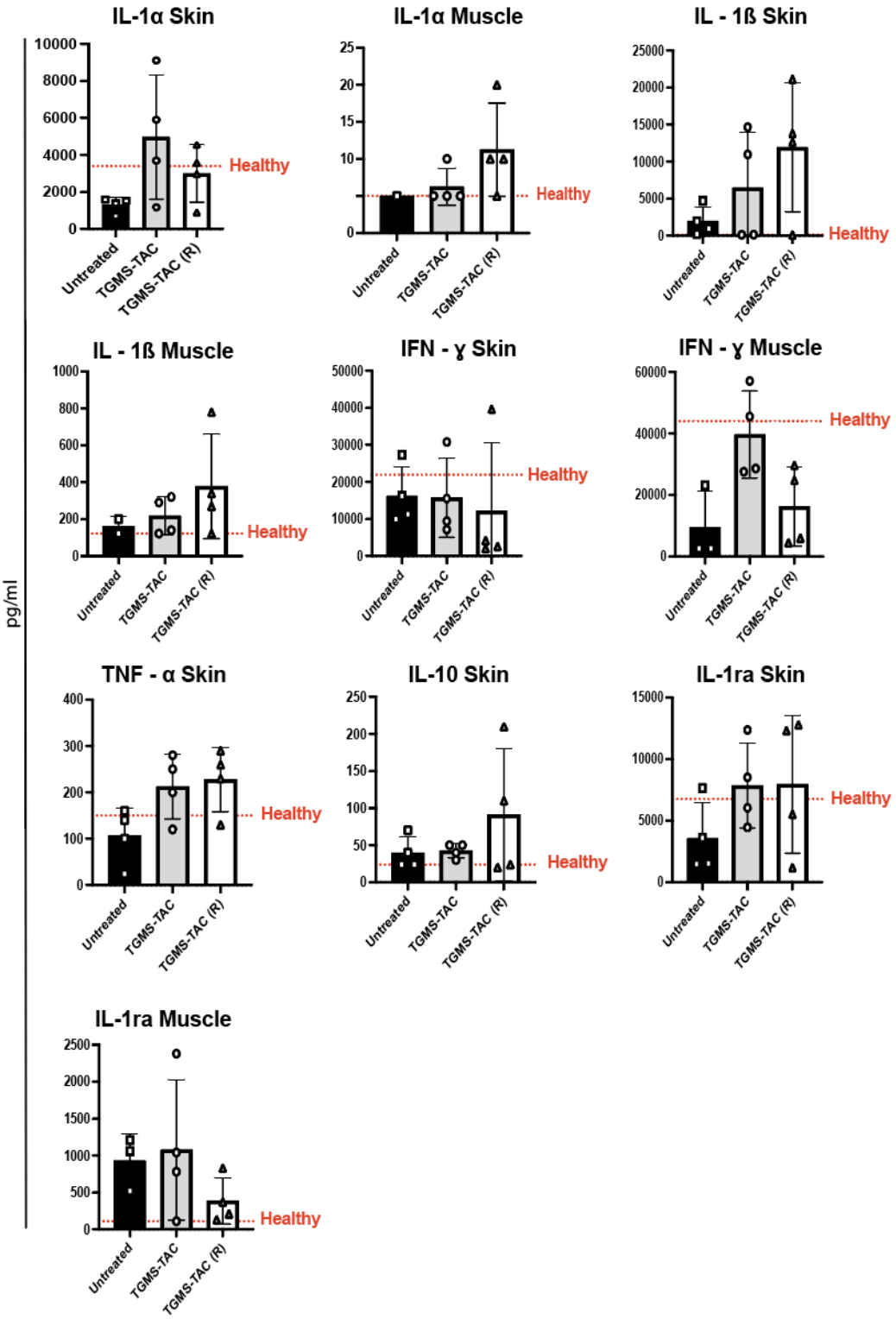


Figure 17: ***Local Skin and Muscle Cytokine Levels from rejected VCA grafts.*** *Skin and Muscle samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were taken and local cytokine levels were measured with a Milliplex immunoassay to quantify local cytokine concentrations during rejection. Data are presented as mean ± SD (n=1 for healthy control, n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.3 TGMS-TAC reduces endothelial cell activation in VAC grafts upon rejection

The upregulation of endothelial cell adhesion molecules such as E-Selectin can lead to an upregulation of the recruitment of leukocytes such as neutrophils into inflamed tissue during graft rejection. Hence, we were interested to determine if there were any significant differences in endothelial cell activation detectable in the tissue of rejected VCA grafts from differently treated animals. To characterize the local endothelial cell activation, we performed an immunofluorescence staining of skin tissue of rejected VCA grafts. We used E-Selectin as a marker for endothelial cell activation. We were able to detect significantly higher levels of endothelial cell activation in the untreated animals compared to the animals that were reinjected with TGMS-TAC. The group of animals that were only injected once with TGMS-TAC also showed a tendency towards a smaller level of endothelial cell activation compared to the untreated animals. (see Figure 18).

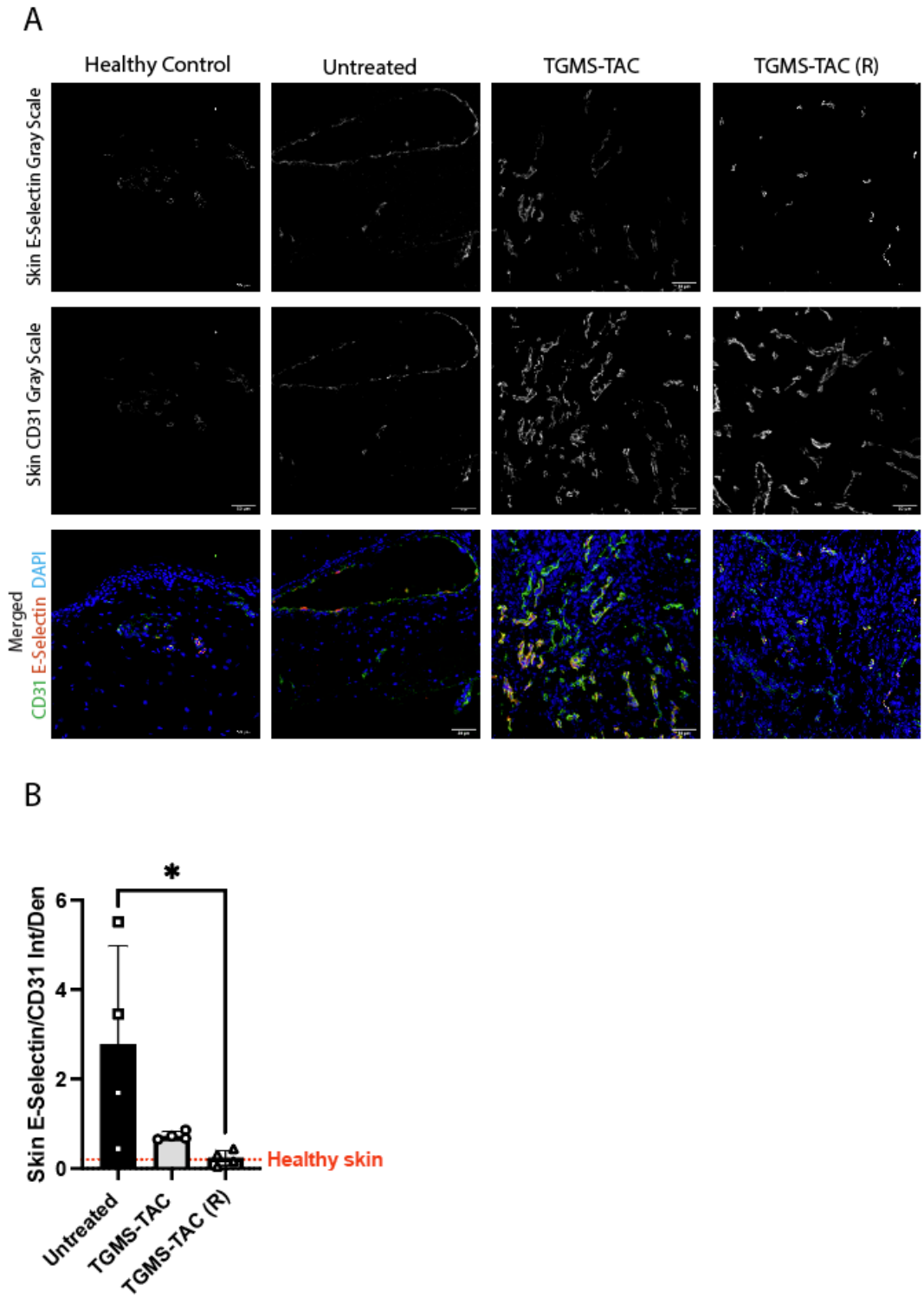


Figure 18: ***Immunofluorescence staining of E-Selectin in Skin tissue or rejected grafts.*** *Skin samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for E-Selectin to visualize the level of endothelial cell activation.* ***(A)*** *Representative pictures of immunofluorescence staining of E-Selectin in VCA graft skin samples.* ***(B)*** *Immunofluorescence quantification of E-Selectin in ratio to CD31 positive staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each data point represents the mean integrated density from 4 pictures randomly taken of each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC, and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant (* p≤0.05).*

4.4 Complement activation is dampened by TGMS-TAC reinjection in VCA graft muscle

Complement factors can work as chemotaxins for a variety of leukocytes. Neutrophils for example can be activated by different complement factors such as C1q, C3bc and C3a. C3a and C3b/c both play an important role in all three of the known activation cascades of the complement system. We were interested in significant differences detectable in systemic levels of complement factors, which subsequently are able to activate neutrophils during graft rejection, between different treated groups. To determine differences of systemic complement factor levels we measured the concentration of complement factor C3a, which is not attached to inflamed tissue but rather stays in circulation upon activation, in the plasma of differently treated animals at baseline and endpoint using a C3a ELISA kit. Since all pigs are showing a natural difference between C3a levels we wanted to assure that this bias is not included in our results. Hence, we compared the folds increase respectively decrease of the plasma samples at the time of rejection (endpoint) to the specific baseline value they individually showed at baseline.

We were not able to detect any significant differences in C3a changes compared to baseline between the different treatment groups in the pig plasma. In all treatment groups, the measured levels of C3a were even smaller compared to the levels of C3a in the plasma of healthy pigs. Therefor the amounts of circulating complement factor C3a can be considered as negligibly low during VCA graft rejection.

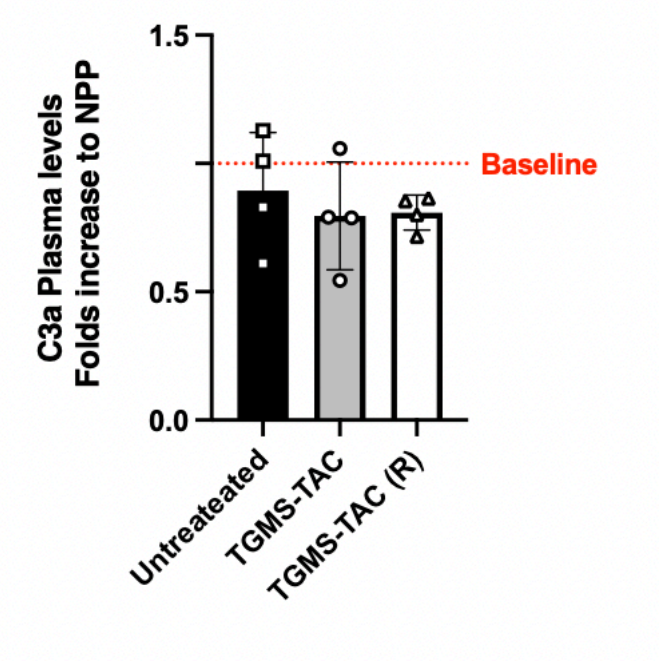


*Figure 19: **C3a plasma levels as folds increase compared to Baseline**. C3a Plasma levels of untreated, TGMS-TAC single injected and TGMS-TAC reinjected animals were measured with a C3a ELISA. Data are presented as mean ±SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

In addition to complement activation systemically we were also interested in differences detectable in local complement deposition between differently treated animals. To characterize the

local complement deposition in tissue we performed an immunofluorescence staining of skin and muscle tissue of rejected VCA grafts. We used C3b/c as a universal marker for complement activation because it is a common protein for all three distinct complement pathways and the first merged protein for the three activation pathways. We were able to detect C3b/c deposition in the skin of all groups but there was no significant difference of C3b/c deposition detectable between the differently treated groups. Nevertheless we were able to detect that C3b/c deposition in rejected graft skin of animals that were treated with TGMS-TAC was considerably higher compared to the levels in the untreated group (see Figure 20).

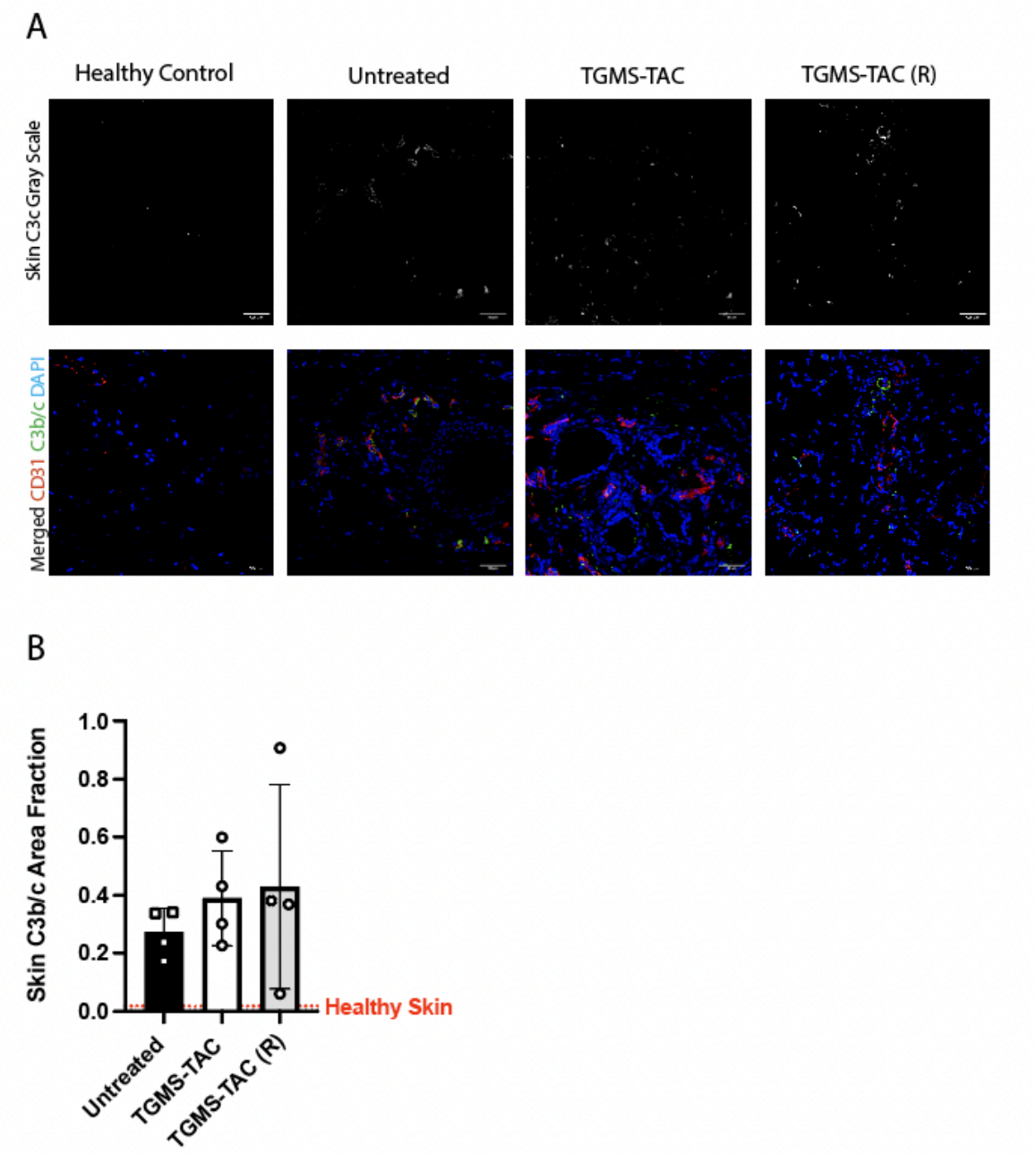


Figure 20 ***Immunofluorescence staining of C3b/c in Skin tissue or rejected grafts.*** *Skin samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for C3b/c to visualize the level of complement factor deposition in tissue.* ***(A)*** *Representative pictures of immunofluorescence staining of C3b/c in VCA graft skin samples.* ***(B)*** *Immunofluorescence quantification of C3b/c staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the area fraction. Each data point represents the mean integrated density from 4 pictures randomly taken from each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

In muscle we were able to detect that the untreated animals tend to have the highest amount of C3b/c deposition compared to the other groups. The animals that were injected only once with TGMS-TAC also tended to show higher amounts of C3b/c deposition in rejected muscle tissue compared to the healthy control. The C3b/c deposition in animals that received multiple injections of TGMS-TAC, was considerably lower compared to the untreated and single injected group of animals. (see Figure 21).

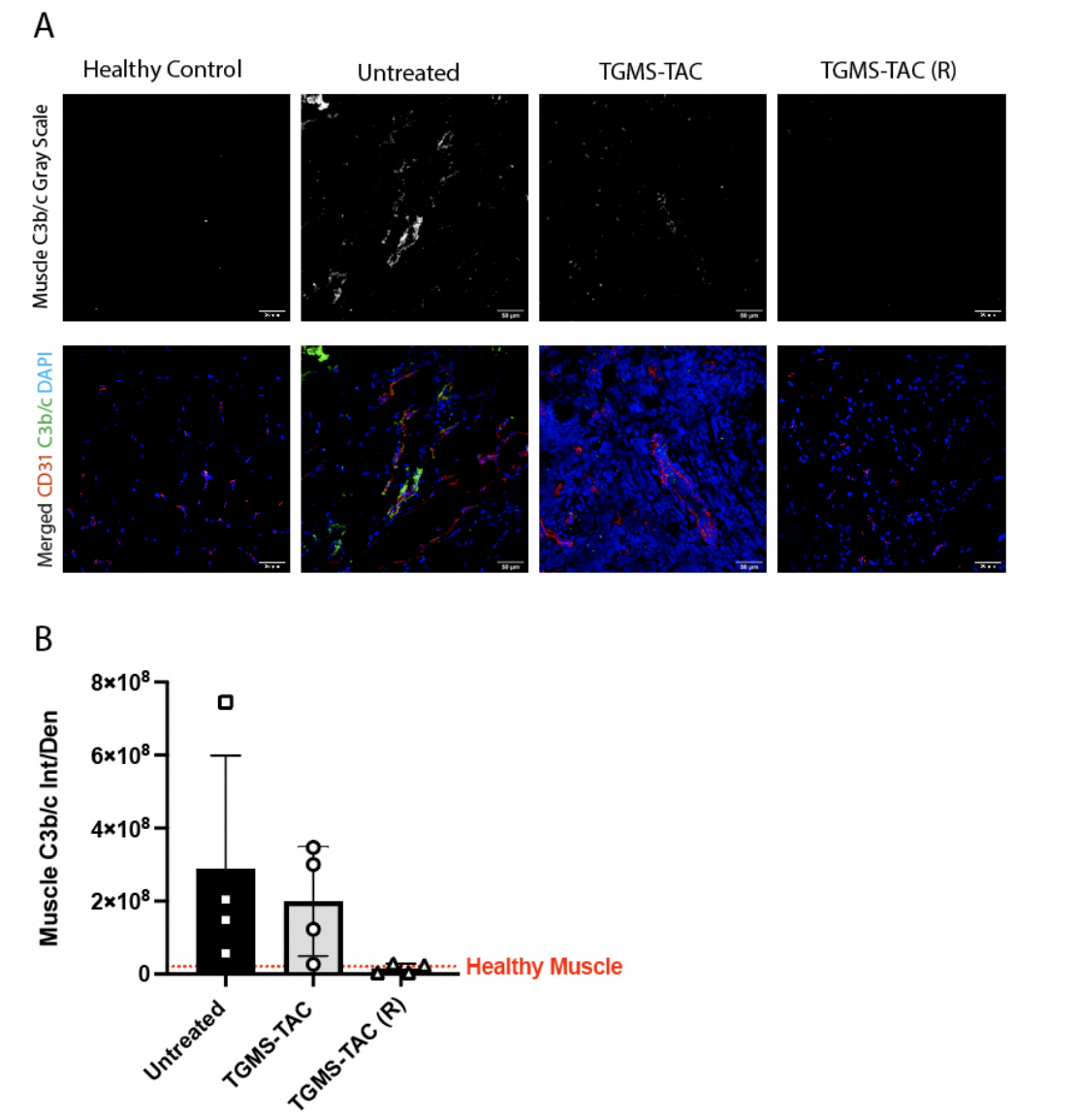


Figure 21: ***Immunofluorescence staining of C3b/c in Muscle tissue of rejected grafts.*** *Muscle samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for C3b/c to visualize the level of complement factor deposition in tissue.* ***(A)*** *Representative pictures of immunofluorescence staining of C3b/c in VCA graft muscle samples.* ***(B)*** *Immunofluorescence quantification of C3b/c staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each data point represents the mean integrated density from 4 pictures randomly taken of each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.5 Local macrophage infiltration in the skin is dampened upon TGMS-TAC reinjection

Macrophages and neutrophils work together as part of the innate immunity and can complement each other in their effector functions during graft rejection. Hence, we wanted to investigate the infiltration of macrophages in rejected skin and muscle tissue of VCA grafts and compare the macrophage deposition between the different groups. We used CD163 as a marker for macrophages.

We were not able to detect any significant differences in macrophage deposition in skin between the different groups of pigs. We saw a tendency for smaller macrophage deposition in the healthy control compared to the other groups. The amount of macrophage deposition in the skin of animals that were reinjected with TGMS-TAC also tended to be smaller compared the animals which only received a single injection of TGMS-TAC (see Figure 23).

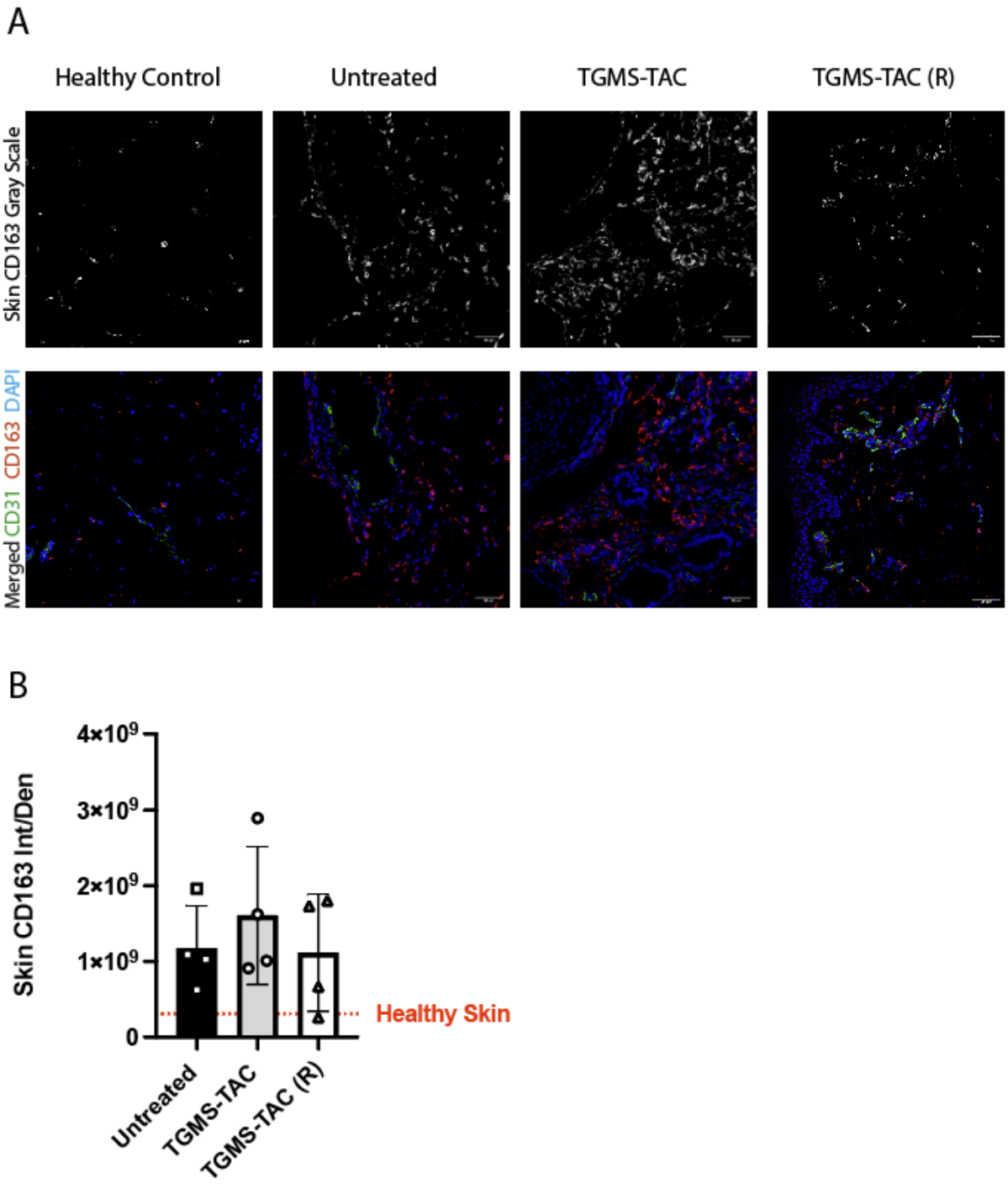


Figure 22: ***Immunofluorescence staining of CD163 in skin tissue of rejected grafts.*** *Skin samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for CD163 to visualize the level of macrophage infiltration in tissue* ***(A)*** *Representative pictures of immunofluorescence staining of CD163 in VCA graft skin samples.* ***(B)*** *Immunofluorescence quantification of CD163 staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each data point represents the mean integrated density from 4 pictures randomly taken of each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC, and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

In muscle, we were also not able to detect any significant difference of macrophage deposition between the different treatment groups. The amount of macrophage deposition tended to be the lowest in the animals which were treated with a single injection of TAC, which was similar compared to the number of macrophages in the healthy donor muscle (see Figure 24).

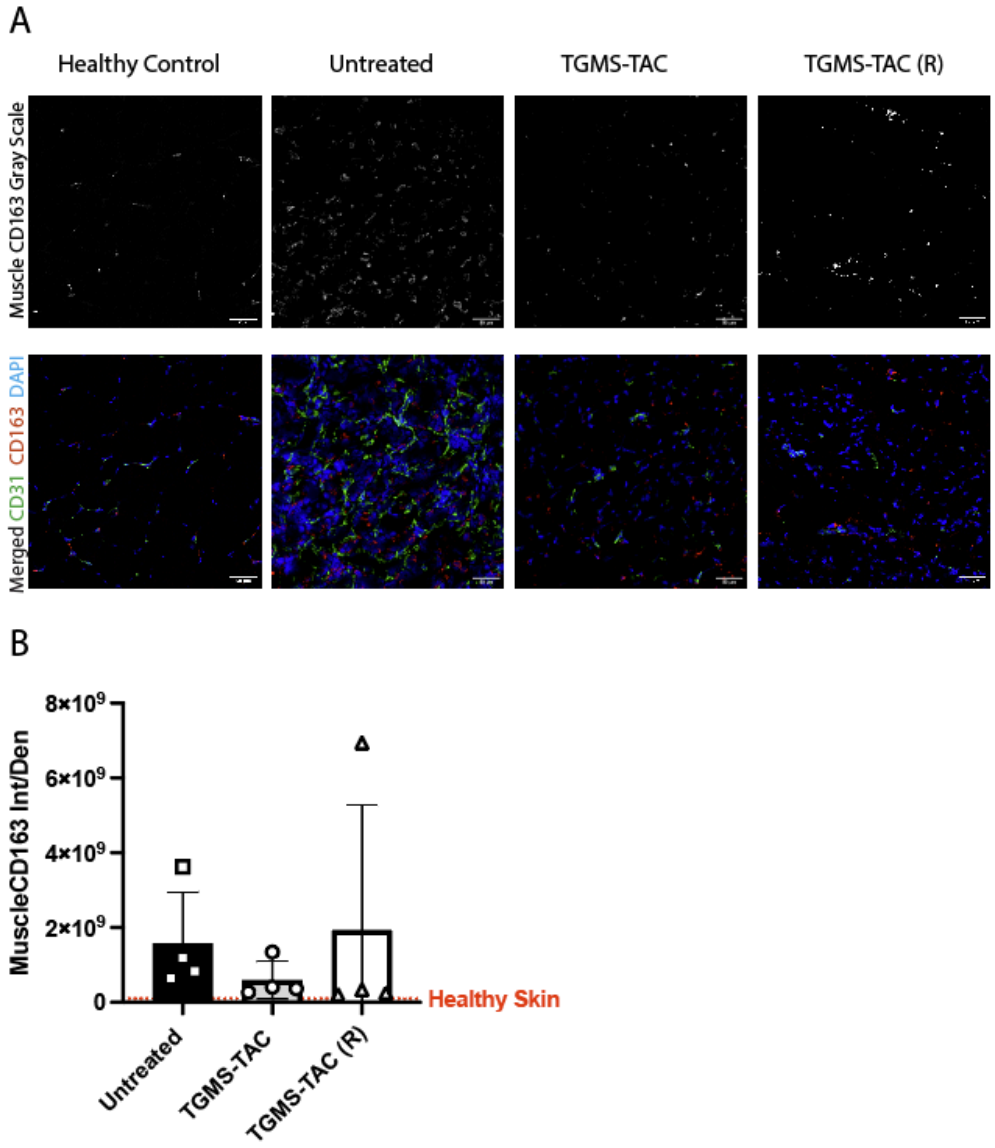


Figure 23: ***Immunofluorescence staining of CD163 in muscle tissue of rejected grafts.*** *Muscle samples from healthy animals (healthy control) and from rejected grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for CD163 to visualize the level of macrophage infiltration in tissue* ***(A)*** *Representative pictures of immunofluorescence staining of CD163 in VCA graft muscle samples.* ***(B)*** *Immunofluorescence quantification of CD163 staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each data point represents the mean integrated density from 4 pictures randomly taken of each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC, and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.6 T-cell activation and proliferation is dampened upon TGMS-TAC reinjection

T-cells are known to play a very important role in VCA graft rejection. They are the major target of immunosuppressive drugs like tacrolimus which are given to patients to prevent graft rejection. T-cells can be divided into different subgroups which all play an important role in VCA graft rejection. Both CD8+ and CD4+ cytotoxic T-lymphocytes are involved directly in acute graft rejection. Tregs, a subgroup of CD4+ T-cells, on the other hand are able to induce tolerance after graft transplantation and are able to dampen the cytotoxic ability of other T-cell subgroups. Neutrophils are able to influence T-cell proliferation and differentiation in the context of VCA graft rejection.

Hence, we were interested to characterize the different circulatory T-cells in the blood to be able to define what is happening systemically during VCA graft rejection in differently treated animals. Thus, we preformed Flow cytometry of blood samples in order to determine the abundance of T-cell subtypes systemically in different treatment groups and if the levels of subgroups increase respectively decreased during rejection at endpoint compared to baseline. Further, we were also interested in what is happening locally in tissue during graft rejection and if there are any differences detectable in T-cell infiltration in skin and muscle of the different treatment groups. Hence, we performed an immunofluorescence staining of rejected VCA skin and muscle tissue using CD3 as a marker for T-cell determination.

4.6.1 Circulatory T-cell levels are reduced by TGMS-TAC reinjection upon rejection

We compared the levels of each T-cell subtype at endpoint to the levels which we measured at baseline for each group of animals that was treated with TGMS-TAC and to an untreated control. Additionally, we were also interested to see how many T-cells of a specific subtype were present in relation to all T-cells present at baseline compared to endpoint and if there are any differences in the frequency of certain subtypes between the different groups detectable.

We were not able to detect any significant difference in increase or decrease of CD4+ T-cell levels between the different treatment groups. The frequency of CD4+ T-cells also did not change significantly between baseline and endpoint of each group. The levels of FoxP3+ T-cells also did not change significantly from baseline to endpoint between the different groups.

Nevertheless, the highest increase of Foxp3+ T-cells at endpoint compared to baseline was measured in the untreated group, whereas a decrease was measured for the group of TGMS-TAC reinjected pigs. Like CD4+ T-cells in general, FoxP3+ T-cells were also the most abundant in the animals which were treated with a single injection of TGMS-TAC. Comparable to the increase of FoxP3+ T-cells, we also measured the highest increase of CD8+ T-cells in the animals which were untreated compared to the other groups where the amount of CD8+ T-cells nearly stayed the same at endpoint compared to baseline. Nevertheless, the differences in changes of CD8+ T-cells at endpoint compared to baseline were not significantly different. Furthermore, the frequency of CD8+ T cells did not change significantly between baseline and endpoint within any of the three groups and also did not show any significant difference at baseline or endpoint between the groups (see Figure 25 A).

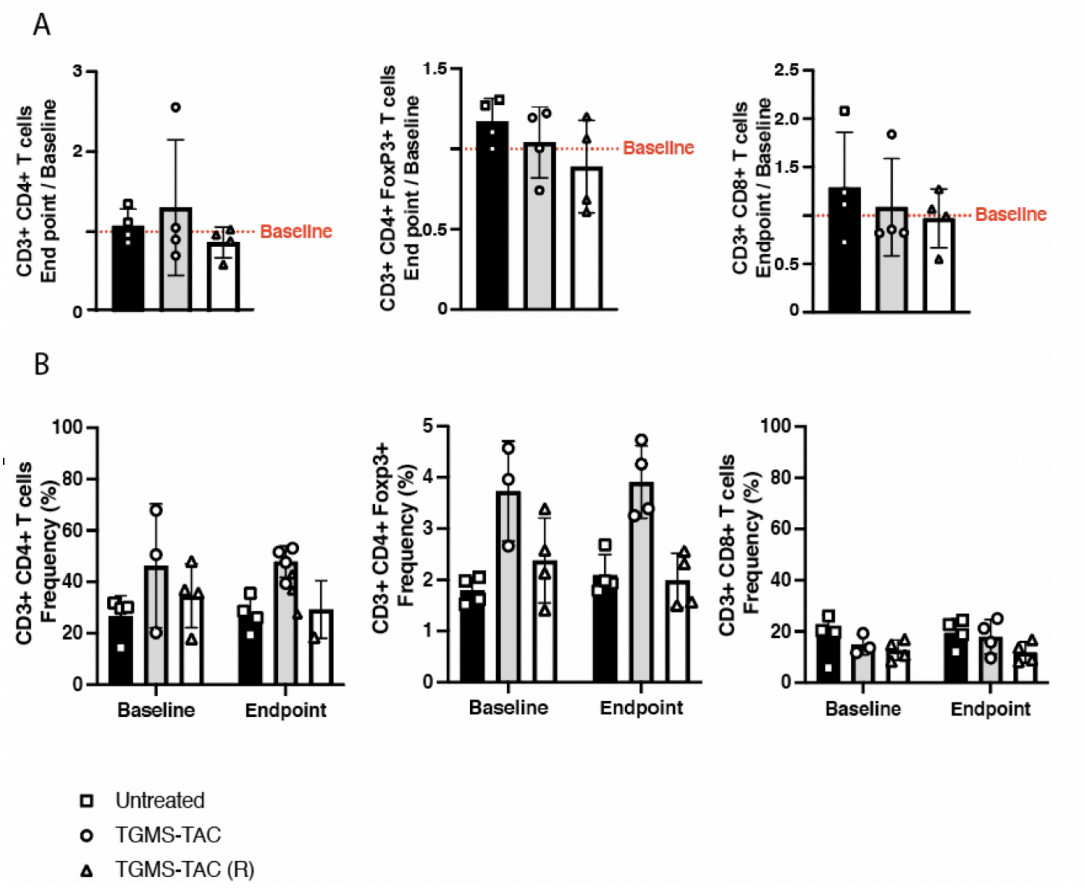


Figure 24: ***Systemic blood levels of T-cell subtypes at endpoint compared to baseline. (A)*** *CD4+, CD4+FoxP3+ and CD8+ T-cell subtypes were measured at baseline and endpoint in pig blood levels. The number of folds increase respectively decrease was measured with Flow cytometry and compared between the untreated, TGMS-TAC single and reinjected (TGMS-TAC (R)) groups.* ***(B)****Frequency of CD4+, FoxP3+ and CD8+ T-cells at endpoint compared to baseline out of all CD3+ measured T cells in blood from untreated, TGMS-TAC single and reinjected (TGMS-TAC (R)) pigs. One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.6.2 In grafts T-cell infiltration is reduced by TGMS-TAC reinjection upon rejection

To characterize the local T-cell infiltration we performed an immunofluorescence staining of skin and muscle tissue of rejected VCA grafts of different treatment groups and healthy skin and muscle tissue. CD3 was used as a general marker for T-lymphocytes. To assess the abundance of infiltrating T-cells we counted the amount of CD3+ stained cells and compared them to the total amount of all graft infiltrating cells which were observed in five representative pictures. We were able to detect that the frequency of CD3+ T-cells in skin in both the TGMS-TAC single ($p=0.0036$) and reinjected ($p=0.0094$) group was significantly lower compared to the untreated group (see Figure 26 B).

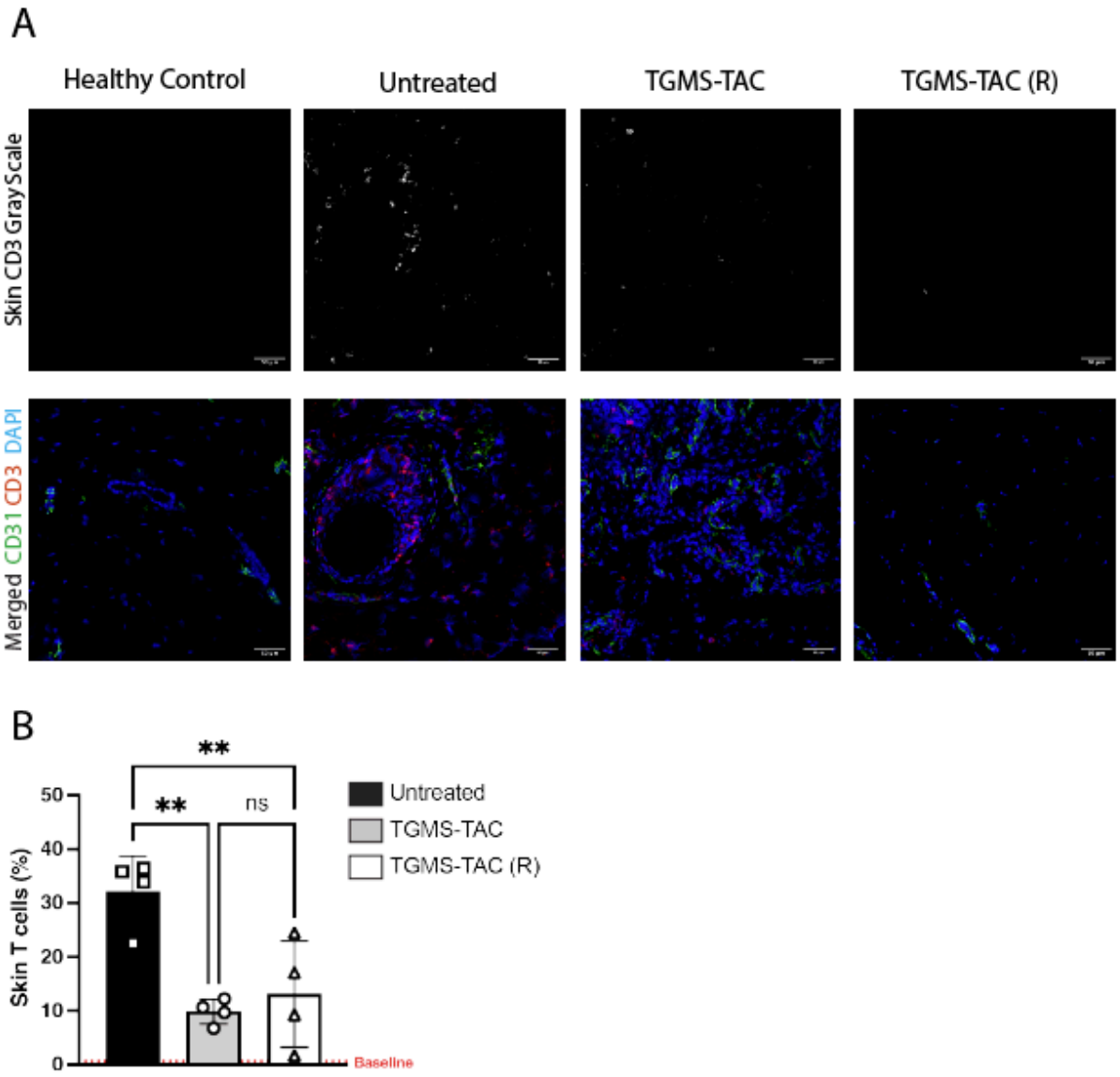


Figure 25: ***Immunofluorescence staining of CD3+ cells in rejected VCA skin grafts. (A)*** *Skin from healthy animals (healthy control) and from grafts of transplanted animals, which were either single or reinjected with TGMS-TAC, were stained for CD3 to visualize the level of T-cell infiltration. Representative pictures of immunofluorescence staining of CD3 in VCA graft skin samples.* ***(B)*** *Frequency of CD3+ cells compared to all cells in rejected VCA skin tissue of untreated and TGMS-TAC single and reinjected animals (TGMS-TAC(R)). Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value >0.05 was considered as significant (* p≤0.05, ** p ≤0.01)*

In muscle tissue of rejected VCA grafts, we were not able to detect any significant differences in T-cell infiltration between the different treatment groups. Nevertheless, similar to the infiltration of T-cells in skin we were also able to detect high amounts of infiltrating T-cells in the systemically treated group which is comparable to the amounts of

CD3+ cells in the untreated group. The amounts of infiltrating T-cells in muscle tissue of TGMS-TAC single and reinjected animals were considerably smaller compared to the untreated and systemic group (see Figure 27).

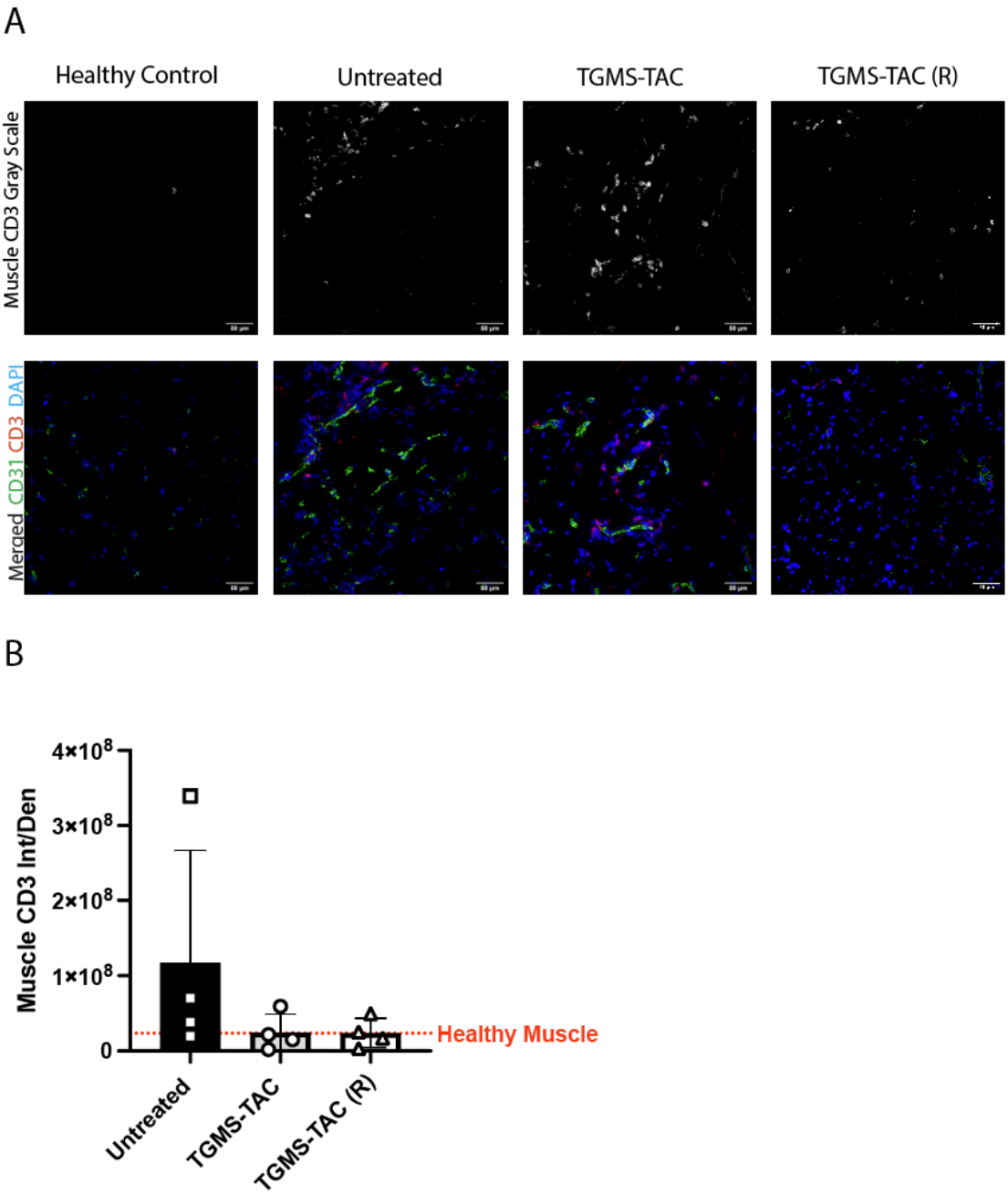


Figure 26: ***Immunofluorescence staining of CD3+ cells in rejected VCA muscle grafts. (A)*** *Muscle from healthy animals (healthy control) and from grafts of transplanted animals, which were either single or reinjected with TGMS-TAC, were stained for CD3 to visualize the level of T-cell infiltration. Representative pictures of immunofluorescence staining of CD3 in VCA graft muscle samples.* ***(A)*** *Representative pictures of immunofluorescence staining of CD3 in VCA graft muscle samples.* ***(B)*** *Immunofluorescence quantification of CD3 positive staining. Immunofluorescence quantification was obtained using ImageJ software by measuring the integrated density. Each data point represents the mean integrated density from 4 pictures randomly taken from each pig. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.7 Neutrophils and NETs in VCA graft rejection

Finally, we wanted to assess if neutrophils and subsequently NETs are present systemically in plasma and infiltrating the tissue upon VCA graft rejection. Therefore we first we performed a western blot to confirm the presence of neutrophils in rejected VCA grafts. Afterwards, we measured the plasma levels of NETs by performing a DNA-MPO ELISA. We measured the plasma levels of pigs after they rejected their transplanted grafts and compared the fold change at endpoint, compared to the levels which we measured in pooled plasma of healthy pigs. Finally, we performed the same DNA-MPO ELISA with skin, muscle and lymph node samples harvested from the rejected grafts and compared the fold increase or decrease of NET levels to pooled healthy skin, muscle or lymph node tissue lysate. In order to reimburse our findings in skin of rejected grafts we subsequently performed a western blot of skin tissue lysate.

4.7.1 Circulating NETs upon rejection in plasma

We were able to detect NETs in porcine plasma during VCA graft rejection in all different treatment groups. Nevertheless, the number of NETs we detected in the untreated and the animals which were reinjected with TGMS-TAC were clearly lower compared to the healthy donor plasma (NPP). Therefore the amounts of circulatory NETs during rejection can be considered as negligibly low in all treatment groups. Only in the group of animals that received one local injection of TGMS-TAC into the graft, the number of NETs in plasma elevated slightly compared to the amount in the NPP (see Figure 28).

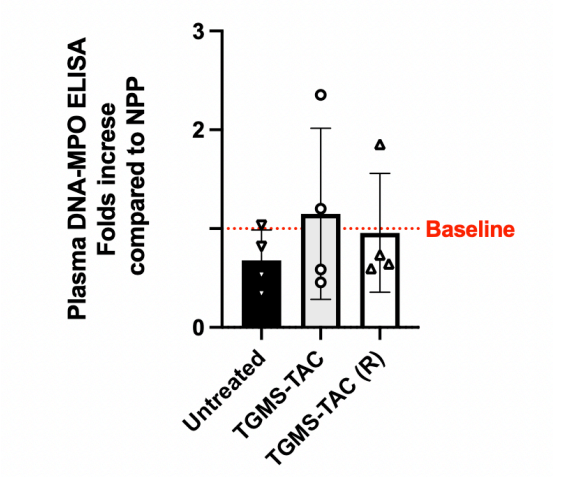


Figure 27: ***Plasma levels of NETs folds increase respectively decease at endpoint compared to normal pooled plasma (NPP) of healthy pigs.*** *Systemic levels of NETs in plasma of untreated and TGMS-TAC single and reinjected (TGMS-TAC (R)) pigs were measured with a DNA-MPO ELISA and compared to NET levels in pooled plasma of healthy pigs. Data are presented as mean ± SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant.*

4.7.2 In skin of rejected VCA grafts, neutrophil and NET infiltration is reduced upon TGMS-TAC reinjection

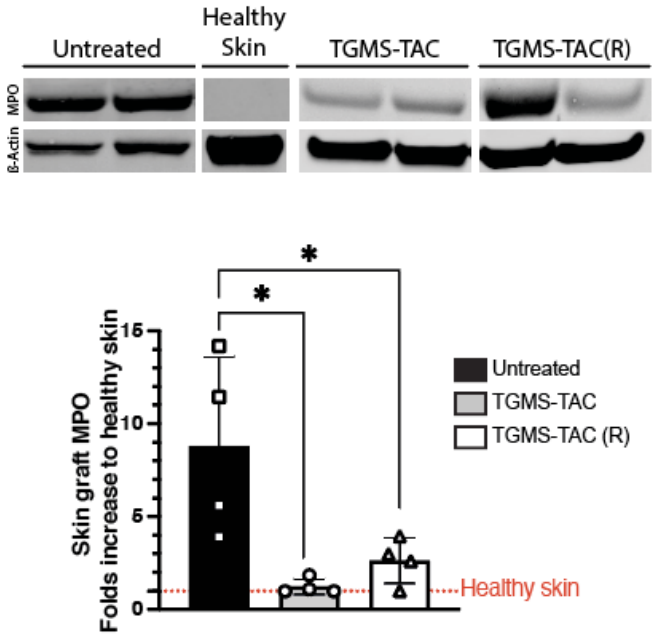


Figure 28: ***Western Blot of rejected VCA skin tissue stained for MPO.*** *Neutrophil levels in untreated and TGMS-TAC single and reinjected (TGMS-TAC (R)) pigs were measured with the western Blot technique. MPO was used as a marker for neutrophils β-Actin was used as a loading control. Data are presented as mean ±SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant (* p≤0.05)*

In transplantation immunology it is already known that neutrophils are present in the graft during rejection.[15] To confirm the presence of neutrophils in the differently treated groups of animals during VCA graft rejection, we performed a western blot of skin tissue of rejected VCA grafts. We stained the skin tissue for myeloperoxidase (MPO) which is a marker for neutrophils. As expected, we were able to detect neutrophils in rejected VCA grafts of all treatment groups. In addition we detected significantly lower levels of MPO in the animals which were reinjected with TGMS-TAC compared to untreated animals, which showed the highest amounts of MPO in skin (p=0.03). Also, in the animals that were only treated with a single injection in TGMS-TAC, the amount of infiltrating neutrophils was significantly lower compared to the untreated group. (see Figure30).

Since we were able to confirm the presence of neutrophils in VCA grafts during rejection, we were interested to assess if NETs are also present. Therefore we performed a DNA-MPO ELISA of graft skin, muscle and lymph node lysate. We were able to detect NETs in skin, muscle and lymph node samples from rejected VCA grafts in all different treatment groups. In skin tissue of rejected VCA grafts we measured the highest increase in NET levels compared to healthy donor skin in the untreated group of pigs. In the TGMS-TAC reinjected group of animals we

measured the highest decrease of NET levels compared to healthy skin. The difference in decrease between the untreated group and the TGMS-TAC reinjected group was significant (p=0.00412). Further, we were not able to detect a significant difference of NET levels in the animals which only received a single injection of TAC compared to healthy skin (see Figure 29 A).In contrast to NET levels in the skin, we detected the highest increase of NET levels compared to healthy muscle, in the group of animals that were reinjected with TAC. The highest decrease of NET levels in muscle was measured in untreated animals. Also in muscle the difference in folds increase between the untreated group and the TGMS-TAC reinjected group was significant (p=0.0318). The levels of NETs in muscle in the single injected TAC group did not change compared to the healthy muscle control (see Figure 29 B).

In lymph node samples from rejected VCA grafts we did not detect any significant difference in NET levels between the different treatment groups and the healthy lymph node sample. In both groups of animals which received TGMS-TAC either once or several times, the number of NETs was increasing compared to the untreated group were NET levels did not change compared to the healthy lymph node control (see Figure 29 C).

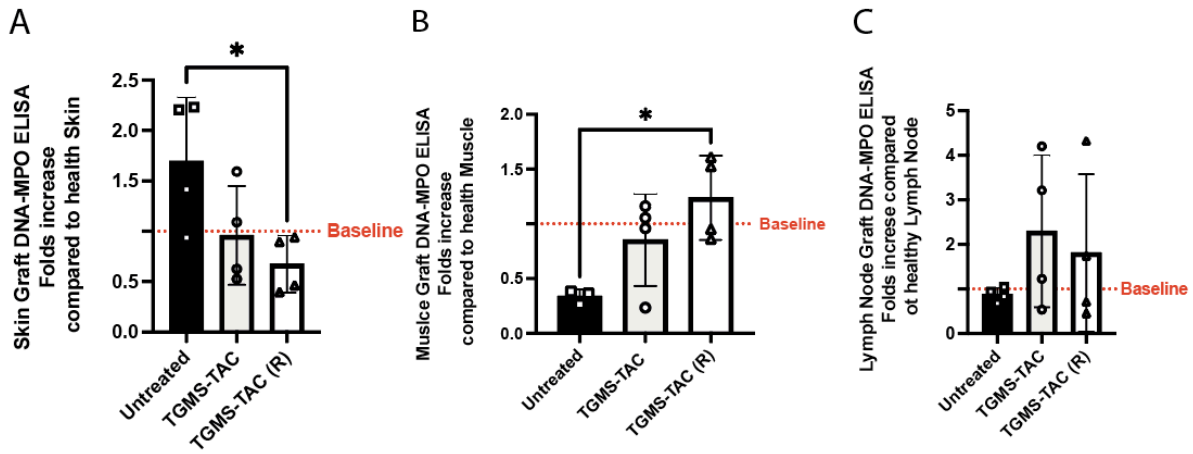


*Figure 29: **Plasma levels of NETs folds increase or decrease at endpoint compared to samples of healthy pigs.** Skin**(A)**, muscle**(B)** and lymph node**(C)** levels of NETs in plasma of untreated and TGMS-TAC single and reinjected (TGMS-TAC (R)) pigs were measured with a DNA-MPO ELISA and compared to NET levels in skin, muscle and lymph node tissue of healthy pigs. Data are presented as mean ±SD (n=4 for untreated, n=4 for TGMS-TAC and n=4 for TGMS-TAC (R) group). One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant (* p≤0.05)*

In addition to the quantitative method of detecting NETs in rejected VCA grafts, we wanted to reconfirm the presence of NETS in skin and muscle using immunofluorescence microscopy. Hence, we performed an immunofluorescence staining of skin, muscle and lymph node tissue and stained for H4Cit in combination with MPO for the detection of neutrophils and NETs.

We detected NETs in all lymph node samples from the healthy control, the untreated animals and the animals which were either single or reinjected with TGMS-TAC. We detected no significant differences in H4Cit or neutrophil infiltration between the different treatment groups. The highest number of infiltrating neutrophils in the lymph node was detected in the animals which received only a single injection of TGMS-TAC whereas the smallest amounts were detected in the healthy control (see Figure 33).

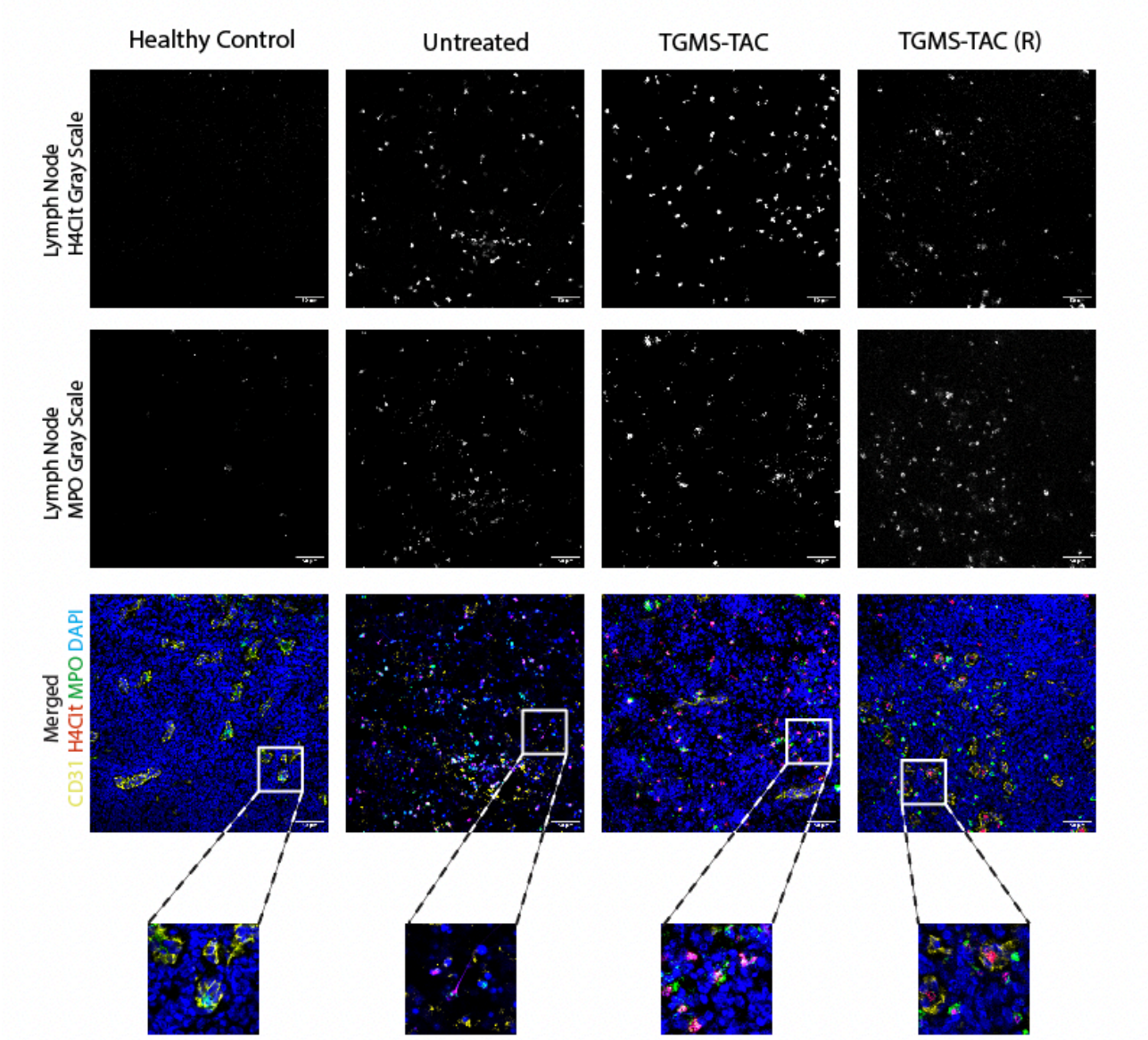


Figure 30: ***Immunofluorescence staining of H4Cit and MPO in lymph Node tissue or rejected grafts.*** *Muscle from healthy animals (healthy control), from grafts of transplanted animals that were either untreated or received local injections of TGMS-TAC (single or reinjected) were stained for H4Cit and MPO to visualize the level of NET infiltration. Representative pictures of immunofluorescence staining of H4Cit and MPO in VCA graft lymph node samples. A representative part of each picture is shown in a bigger magnification to visualize NETs*

Further, in muscle we were also able to detect infiltrating NETs in all treatment groups. The amount of H4Cit in the TGMS-TAC reinjected animals was similar to the amount we detected in the healthy control animals. The highest neutrophil infiltration was detected in the untreated animals. In the TGMS-TAC reinjected and untreated group the amount of neutrophil infiltration in the muscle also tended to be small.

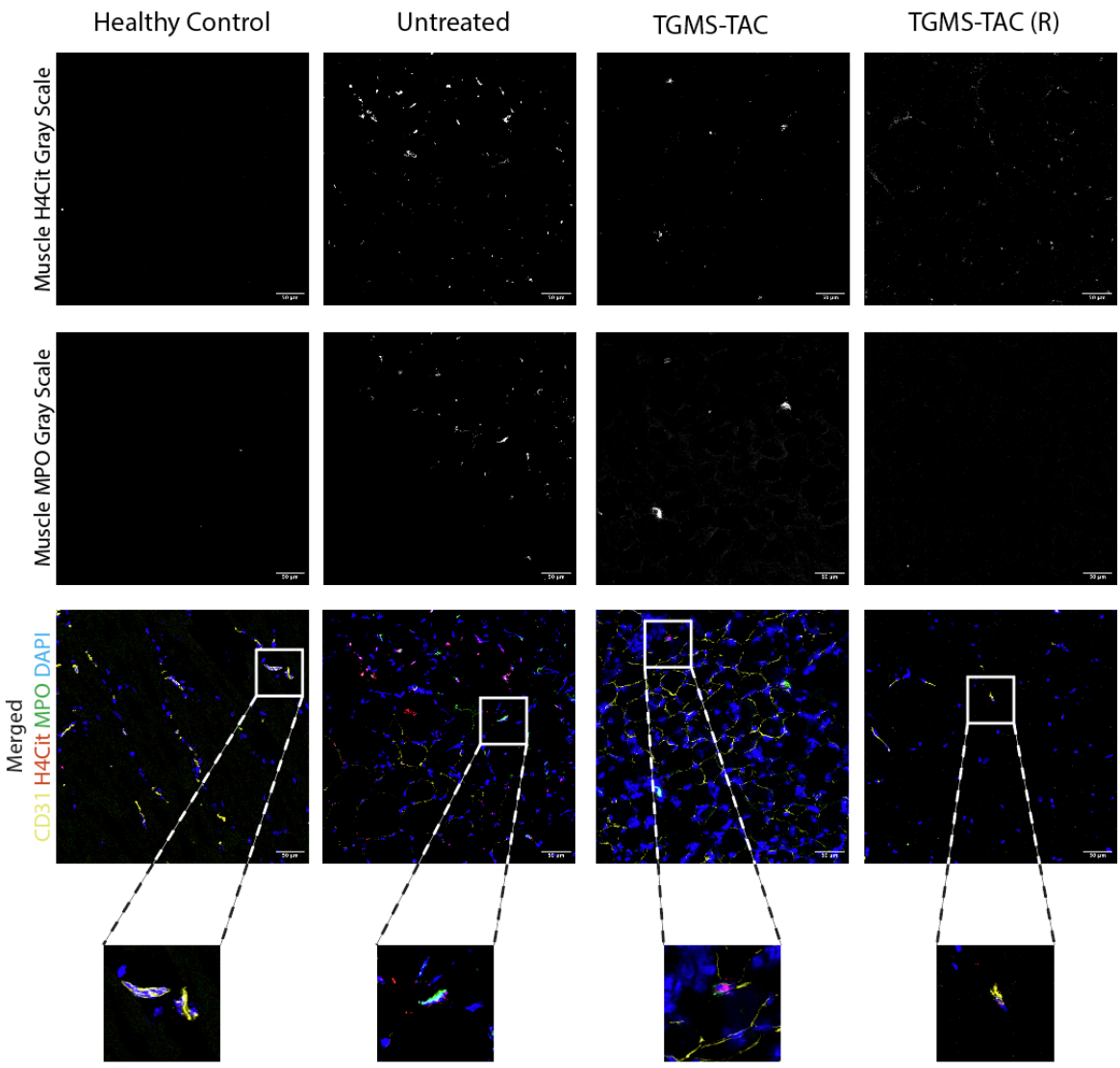


Figure 31: ***Immunofluorescence staining of H4Cit and MPO in muscle tissue or rejected grafts.*** *Muscle from healthy animals (healthy control) and from grafts of transplanted animals, which were either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for H4Cit and MPO to visualize the level of NET infiltration.* ***(A)*** *Representative pictures of immunofluorescence staining of H4Cit and MPO in VCA graft muscle samples. A representative part of each picture is shown in a bigger magnification to visualize NETs.*

Finally, we were able to detect NETs in skin of all different treatment groups. Only if a structure showed both a positive staining for MPO around the cell and a nice colocalization of DAPI with H4Cit in a web-like structure, it was counted as a NET. The lowest NET infiltration was detected in the healthy skin control. In the untreated group of animals, we detected the highest amount of infiltrating NETs in graft tissue. Animals that received a single injection of TGMS-TAC already showed lower amounts of NET infiltration than the untreated group. The levels of infiltrating

NETs in tissue of animals that were reinjected with TGMS-TAC were significantly lower compared to the untreated group and comparable to the levels in healthy skin.

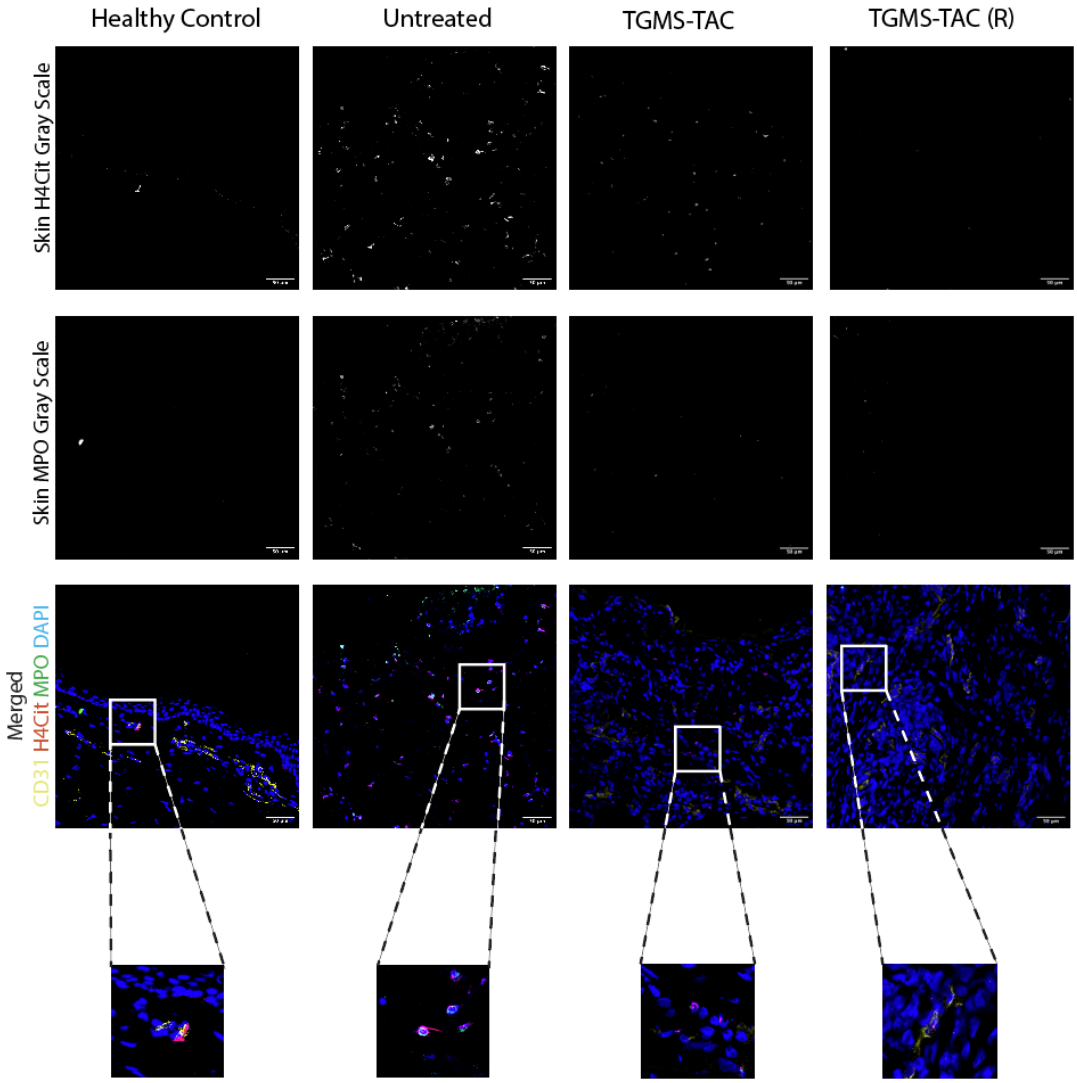


Figure 32: ***Immunofluorescence staining of H4Cit and MPO in Skin tissue or rejected grafts.*** *Skin from healthy animals (healthy control) and grafts of transplanted animals, either untreated or treated with local injections of TGMS-TAC (single or reinjected), were stained for H4Cit and MPO to visualize the level of NET infiltration.* ***(A)*** *Representative pictures of immunofluorescence staining of H4Cit and MPO in VCA graft skin samples. A representative part of each picture is shown in a bigger magnification to visualize NETs.*

4.7.3 Tacrolimus inhibits NET formation

Since we were able to detect that NET infiltration in rejected VCA graft skin and muscle was reduced upon TGMS-TAC injection, we wanted to asses *in vitro* if not only T-cell proliferation is reduced by TAC but also NETosis. Therefor we isolated neutrophils from healthy porcine

blood and assessed the effect of Tacrolimus on NET formation to define if other than T-cell activation and proliferation, TAC is also able to inhibit neutrophil activation and therefore NET formation. As expected, without neutrophil stimulation with Ionomycin, no NETs were formed. If the neutrophils were activated by Ionomycin and no tacrolimus pre-treatment was added to the cells, we were able to detect a high amount of NET formation. When we added increasing amounts of tacrolimus we were able to detect that the NET formation was significantly decreased up to 20%. Already at the smallest amount of 25 ng/ml of TAC, the NET formation decreased by 15% compared to the sample where no TAC was added). We detected the smallest amount of NET formation in the probe where we added 100 ng/ml (see Figure 34). These results show that NET formation can be inhibited by the addition of TAC.

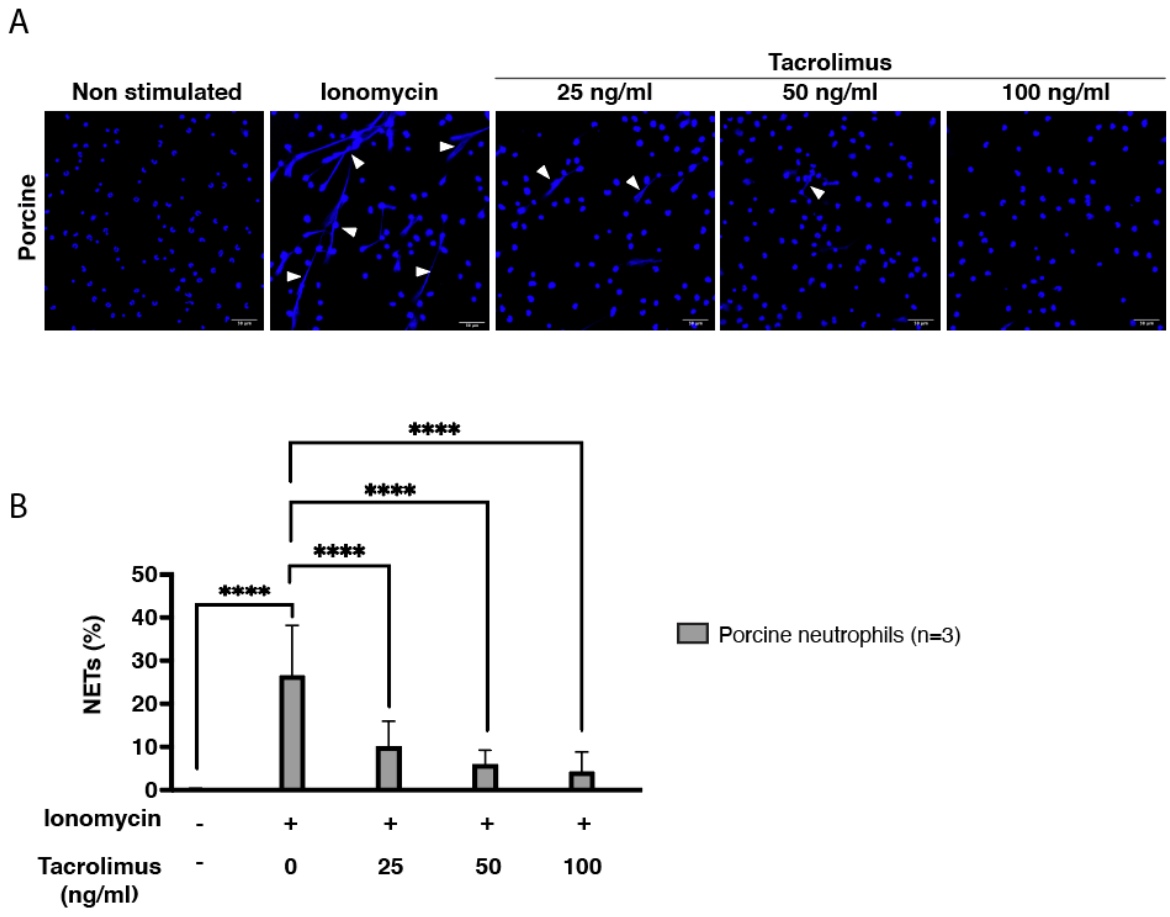


Figure 33: ***NETosis inhibition assay. (A)*** *Four representative pictures of each treatment condition with either no stimulation, Ionomycin stimulation without TAC, Ionomycin stimulation with 25 ng/ml TAC, Ionomycin stimulation with 50 ng/ml TAC and Ionomycin stimulation with 100 ng/ml TAC. (n=3)* ***(B)*** *% of NETs present in each representative picture. One way ANOVA with Tukey`s multiple comparison test was used for statistical analysis. p value <0.05 was considered as significant (* p≤ 0.05, ** p ≤0.01, *** p≤0.001, **** p≤0.0001).*

5 Discussion

Vascularized composite allotransplantation provides a unique opportunity for the reconstruction of lost extremities and organs. Nonetheless, VCA is facing multiple challenges which are preventing the wider use of this restorative surgical technique. The high incidence of acute rejection episodes as well as the life-long intake of immunosuppressive medications, which can cause life-threatening side effects, are considered the major challenges that withdraw VCA as a standard technic in the treatment of extensive tissue loss. Replacing the unspecific, systemic immunosuppressive therapy, which is currently used to prevent VCA rejection with a site-specific on-demand drug delivery system, provides a promising opportunity to decrease off-target toxicity and increase graft survival. In addition, a better knowledge of the immune response could help to understand the mechanisms of graft rejection better and might even provide a new treatment approach, especially of the innate immunity where the underlining reactions in VCA graft rejection are not well understood,

In this study, we were able to evaluate the efficacy of TGMS-TAC on-demand drug delivery system in a porcine model and in this context the presence of neutrophils and NETs in VCA graft rejection.

In comparison to earlier results in rodents[11], we were able to demonstrate that the use of an enzyme-responsive drug delivery system for localized immunosuppression in VCA results in long-term graft survival in a porcine model. In addition, we were able to show that the reinjection of TGMS-TAC increased graft survival significantly compared to the untreated group. All four pigs of the reinjected groups reached the defined endpoint POD90, and 75% of the animals only received a defined Banff score rejection of 2. Also, animals that only received a single injection of TGMS-TAC only rejected around POD60 whereas untreated animals already reached grade III rejection around POD7.

Those findings suggest that a local injection of the on-demand drug delivery system TGMS-TAC is a promising method of prolonging graft survival in a VCA setting.

Further, we were able to show a tendency of increased levels of the anti-inflammatory cytokine IL-10 present in plasma and also in the skin of rejected grafts in animals that received multiple injections of TGMS-TAC. IL-10 is known to work as an anti-inflammatory cytokine and can dampen neutrophil recruitment to the site of inflammation. These findings also match to the findings we assessed by western blot, where we detected significantly lower levels of

MPO, which is a marker for neutrophils, in the skin of reinjected animals. The number of T-cells that we measured in the grafts of the reinjected group was also significantly lower compared to the untreated control group. The assessed amounts of IL-1ra, which is a receptor antagonist for the pro-inflammatory cytokines IL-1α and IL-1β, systemically and in the skin of the TGMS-TAC (R) group also were higher than in the other groups. These findings also correspond to the increased levels of IL-1α and IL-1β which are dampened by the high levels of their antagonist IL-1ra. All these findings indicate that the rejection of the graft was dampened by the local injection TGMS-TAC. Our results are supported by significantly smaller amounts of endothelial cell activation in the skin of the TGMS-TAC reinjected group. Compared to the untreated group, the amount of endothelial cell activation is significantly lower and comparable to the amount of endothelial cell activation in healthy skin. In addition, the amount of endothelial cell activation in the animals which only received a single injection of TGMS-TAC was considerably lower compared to the untreated. Hence, the amount of infiltrating immune cells to the site of inflammation in the graft is also considerably lower in those groups. We were able to detect a tendency of decreased levels of infiltrating macrophages in the skin of the animals which were reinjected with TGMS-TAC compared to the other groups.

The complement system is also an important player in graft rejection, especially in terms of IRI-induced complications and the ability of downstream activation of neutrophils.[43]

The amounts of circulating C3a in all three treatment groups were considerably lower compared to the levels of the complement factor we detected in the plasma of healthy pigs. Since the complement factor C3a is considered a marker for all three pathways of complement activation, we suspect that during VCA rejection, systemically the complement system is not involved in graft rejection significantly. Nevertheless, in skin samples we were able to show that C3b/c deposition in rejected skin grafts was higher compared to the levels of C3b/c in healthy skin. We were able to show that the highest amounts of C3b/c infiltration were detectable in the animals that received multiple injections of TGMS-TAC. Also, animals that only received one injection of TGMS-TAC showed considerably higher levels of C3b/c infiltration compared to the untreated group. In contrast to the results we got for C3b/c infiltration in skin samples, we were able to detect that the number of C3b/c depositions in

the TGMS-TAC reinjected group was considerably lower compared to the levels in healthy muscle tissue compared to the animals.

It has already been shown that calcineurin inhibitors such as tacrolimus can increase complement activation.[44] Since C3/b can be used as a universal marker for complement activation we suspect that locally in rejected skin grafts the concentration of tacrolimus is sufficient enough to increase the levels of local complement activation whereas in muscle it is already decreased as much as that the effects are not sufficient enough to influence the activation of the pathways.

T-cells, as part of the adaptive immunity, play a central role in acute graft rejection as well as in the induction of donor-specific tolerance to the transplant.[45] Therefore we assessed the amounts of CD4+, CD8+ and Tregs in circulation at endpoint and compared them to the levels at baseline. The amounts of CD4+ T-helper cells were slightly lower at endpoint compared to baseline in the animals which received multiple injections of TGMS-TAC whereas the amounts of circulating CD4+ T-cells in the animals which only received a single injection of TGMS-TAC increased compared to both other groups. These findings suggest that the once-injected TGMS-TAC was already used up by the high amounts of inflammatory enzymes which were produced right after the transplantation because of the local damage that was induced due to the surgery and the following rejection. On the other hand, these findings also suggest that the on-demand drug delivery system is working locally in the graft and is able to damper T-cell proliferation only locally as it is intended to do. Therefore, the systemic efficiency of the immune system is not affected as much as it would be during a systemic immunosuppressive treatment and the possible side effects that are associated with the life-long intake of systemic immunosuppressive treatment are most likely suppressed. Subsequently, we assessed the local amounts of T-cell infiltration in the skin of both TGMS-TAC treated animals and compared them to an untreated control group. We were able to detect significantly lower amounts of skin infiltrating CD4+ T-cells in both groups which received either a single or multiple injections of TGMS-TAC. In muscle samples, we detected lower amounts of infiltrating T-cells in both with TGMS-TAC treated groups of animals. Therefore, we believe that the amounts of usable, hydrogel-bound TAC were still sufficient in the local inhibition of T-cell proliferation. However, we detected the strongest decrease of CD4+FoxP3+ Tregs in the TGMS-TAC reinjected group. Therefore, the tolerance induction that those lymphocytes provide might also be reduced in

this group. Nevertheless, these findings suggest that the reinjection of TGMS-TAC offers an effective method for CD4+ and CD8+ T-cell reduction.

It has already been shown that the formation of neutrophil extracellular traps and subsequently the activation of neutrophils can stimulate the innate immune response and prevents transplantation tolerance.[36] Further it has also been suggested that neutrophils are able under specific conditions to present antigens in a MHC-II-dependent manner to T-cells and thereby activate them. [31] Hence we assessed if neutrophils and neutrophil extracellular traps are present in rejected VCA grafts and if there are any significant differences in neutrophil and NET deposition between different treatment groups.

Locally, in the rejected VCA grafts, we were able to detect that the levels of infiltrating neutrophils were significantly lower in the animals which received either a single injection of TGMS-TAC or were reinjected, compared to the untreated group.

In circulation, we were able to detect NETs in all different treatment groups. Nevertheless, the number of NETs in the treatment groups were considerably lower compared to the amount of NETs in healthy pig plasma. Therefore, the number of circulating NETs during rejection can be considered as negligibly low during VCA graft rejection.

In the rejected skin graft of the untreated group, on the other hand, we were able to detect a significant increase in infiltrating NETs compared to healthy control skin. In the group of animals that received multiple injections of TGMS-TAC, the number of infiltrating NETs in the rejected skin graft was significantly lower compared to the number of NETs in the untreated animals. These findings suggest that locally TGMS-TAC can decrease not only T-cell proliferation but also NETosis in the skin. Similar to T-cell proliferation inhibition, we suspect that TGMS-TAC is inhibiting the local activation of neutrophils but does not interfere with systemic neutrophil activation.

Additionally, we were able to detect NETs in the lymph node of all different treatment groups, suggesting that activated neutrophils might be able to transfer into the draining lymph node and assist the antigen-presenting structures like dendritic cells with antigen presentation to T- and B-lymphocytes. Finally, we were able to show that TAC is indeed able to significantly decrease NET formation if added directly to activated neutrophils (already with 25 ng/ml $p<0.0001$). Therefore, we suspect that it is able to significantly inhibit the activation of

neutrophils locally in the skin and is not interfering with the systemic recruitment and activation of neutrophils.

6 Conclusion

We were able to show that the reinjection of TGMS-TAC significantly prolongs VCA graft survival. The grafts of this group showed lower levels of endothelial cell activation as well as decreased macrophage infiltration in rejected skin. Further, we were also able to show that the T-cell deposition of CD3+ T-cells was significantly lower both in the animals that received multiple injections of TGMS-TAC as well as in the animals which only received a single injection. Therefore, we propose that TGMS-TAC is an effective tool in preventing local T-cell proliferation in VCA grafts but does not interfere with the efficiency of T-cell activation and proliferation systemically. Finally, we were able to detect NETs systemically and locally in rejected VCA grafts suggesting that they are involved in graft rejection. We were able to detect significantly lowered levels of NETs at endpoint in the skin tissue of animals that received multiple injections of TGMS-TAC compared to animals that were untreated.

All these findings suggest that TGMS-TAC can dampen VCA graft rejection in terms of local T-cell proliferation inhibition and the inhibition of NET formation.

To further test the efficiency of TGMS-TAC and the role of NETs in VCA graft rejection further studies with a later-defined endpoint and a larger number of test subjects need to be conducted.

7 Acknowledgment

First of all I would like to thank Prof. Dr. Robert Rieben for the opportunity of conducting my Master Thesis under his supervision in his group. I would like to especially thank Dr. Isabel Arenas Hoyos for her extraordinary great supervision during this project. I had the pleasure of working with her on a daily basis where she provided me with excellent supervision in all aspects of the project. She taught me much in terms of laboratory techniques, transplantation immunology and scientific writing. I very much appreciated the daily exchange about our project where she encouraged me to contribute my own ideas which lead to a significant increase of my scientific knowledge. I also want to thank Lei Zhang for helping me with the conduct of a lot of experiments and also Valentin Zollet and Anastasia Milusev for sharing their knowledge about experimental techniques. Finally, I want to thank all lab members for their help during my master thesis. I very much enjoyed being part of this group and would not want to miss this amazing experience.

SCORE SHEET PIG WELL-BEING				**Animal #**			**Date of surgery**				**Graft side** Left__ Right__				**Sex:**		**Age:**		**Page #**	
Date of evaluation																				
POD																				
Evaluator:																				
Weight																				
Temperature																				
HR / RR																				
Solitary Performance																				
Lameness (0-3)																				
Appearance (0-1)																				
Lying and restlessness (0-3)																				
Food interest (0-3)																				
Social Performance																				
Aggression with co-mates (0-3)																				
Isolation (Co-mates) (0-1)																				
Agitation at human approaching (0-3)																				
Wound Palpation																				
Gentle palpation at approximately 3 cm from the suture line (0-3)																				
Total:																				
Intervention needed? (Yes/No)																				
Wound care (Cross)																				
Flunixin Meglumine (2.2 mg/kg) Indicate AR																				
Graft evaluation (0-4)																				

Solitary performance

Lameness (0-3):

0. Normal walking
1. Slight limping
2. Moderate limping with kicking-like movements
3. Severe limping/immobile

Appearance: (0-1)

0. Normal appearance
1. Salivation

Lying and restlessness (0-3):

0. Normal lying
1. Lying guarding the wound/ moves without external stimulation
2. Move often/ poor wake sleep times
3. Continuous pacing around the pen/ in the box

Food interest (0-3)

0. Normal appetite
1. Reduced appetite, eat special food
2. Reduced appetite independently of the food
3. No appetite

Social performance

Aggression with co-mates (0-3)

0. Friendly
1. Moves away
2. Biting and aggressive when approached by other pigs
3. No aggression because immobility

Isolation (co-mates) (0-1)

0. Look actively for playing
1. Not interested in playing

Agitation at human approaching (0-3)

0. Curious, interactive, may vocalize
1. Moves away when approached
2. Biting and aggressive when approached
3. Stay immobile and disinterested

Wound palpation: gentle palpation at approximately 3 cm from the suture line. (0-3)

0. No reaction
1. Mild inconstant reaction
2. Moves away
3. Biting

Total score: Range from 0 to 20

GRAFT EVALUATION:

Grade 0: no difference between graft skin and native skin
Grade 1: mild erythema
Grade 2: moderate erythema with the beginning of scaling and scabbing
Grade 3: severe erythema and scabbing with areas of epidermolysis
Grade 4: full-thickness graft epidermolysis with areas of necrosis.

INTERVENTIONS

No intervention if the score is ≤ 4/20

Until day POD 5

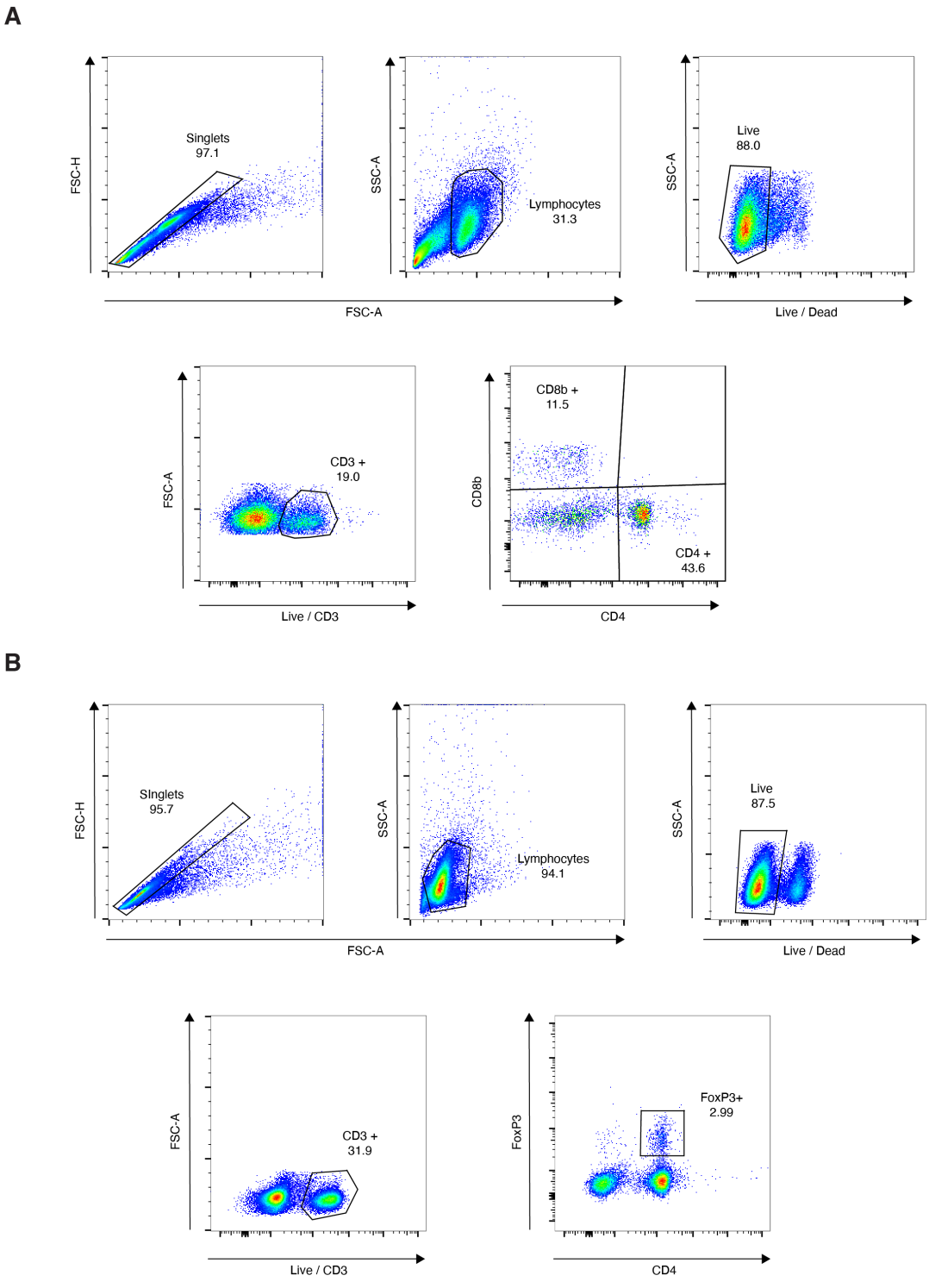
- With a score of 5-8: administer 30 mg/kg metamizole SLOWLY IV and repeat the score after 2 hours. If the score is not diminished after 2 hours, exclude confounding factors. If no confounding factors, inject buprenorphine 0,04 mg/kg IV. Repeat the score after two hours. If no changes, contact the veterinarian.
- With a score of 9-14: inject buprenorphine 0,04 mg/kg IV and metamizole 30 mg/kg SLOWLY IV. Repeat the score after two hours. If no changes, administer methadone 0,3 mg/kg IV and contact a veterinarian. If no changes after 1 hour, proceed with euthanasia. If after analgesia the score is reduced to 5-8, plan metamizole 2 times daily.
- With a score ≥15: contact immediately a veterinarian. Inject methadone (0.3 mg/kg) and metamizole 30 mg/kg SLOWLY IV. Repeat the score after 1 hour. If no changes, proceed with euthanasia. If after analgesia the score is less than 9, plan methadone every 4 hours.

After POD 6

- With a score of 5-8: administer flunixin meglumine 2.2 mg/kg and repeat the score after 2 hours. If the score is not diminished after 2 hours, exclude confounding factors. If no confounding factors, inject buprenorphine 0,04 mg/kg IV. Repeat the score after two hours. If no changes, contact the veterinarian.
- With a score of 9-14: inject buprenorphine 0,04 mg/kg IV and flunixin meglumine 2.2 mg/kg. Repeat the score after two hours. If no changes, administer methadone 0,3 mg/kg IV and contact a veterinarian. If no changes after 1 hour, proceed with euthanasia. If after analgesia the score is reduced to 5-8, plan metamizole 4 times daily.
- With a score of 15-17: contact immediately a veterinarian. Inject methadone (0.3 mg/kg) and flunixin meglumine 2.2 mg/kg IV. Repeat the score after 1 hour. If no changes, proceed with euthanasia. If after analgesia the score is less than 9, plan methadone every 4 hours.
- With a score sheet of 18-20: proceed with euthanasia.

Weight

- Weight POD 0
- Weight POD 2
- Weight POD 5
- Weight POD 7
- Weight POD 10
- Weight POD 14
- Weight POD 25
- Weight POD 45
- Weight POD 60
- Weight POD 90
- Weight POD 120
- Weight POD 180



Supplementary Figure 1: ***Gating Strategy for Flow Cytometry of T-cells. (A)****Gating strategy for CD4+ and CD8+ T-cells.****(B)****Gating strategy for FoxP3+ Tregs.*

References

1. Hopkins J. Plastic and Reconstructive Surgery. Published online 2014:16.
2. Iske J, Nian Y, Maenosono R, Maurer M, Sauer IM, Tullius SG. Composite tissue allotransplantation: opportunities and challenges. *Cell Mol Immunol*. 2019;16(4):343-349. doi:10.1038/s41423-019-0215-3
3. Petruzzo P, Sardu C, Lanzetta M, Dubernard JM. Report (2017) of the International Registry on Hand and Composite Tissue Allotransplantation (IRHCTT). *Curr Transplant Rep*. 2017;4(4):294-303. doi:10.1007/s40472-017-0168-3
4. Kueckelhaus M, Fischer S, Seyda M, et al. Vascularized composite allotransplantation: current standards and novel approaches to prevent acute rejection and chronic allograft deterioration. *Transpl Int*. 2016;29(6):655-662. doi:10.1111/tri.12652
5. Dubernard JM, Owen E, Herzberg G, et al. Human hand allograft: report on first 6 months. *The Lancet*. 1999;353(9161):1315-1320. doi:10.1016/S0140-6736(99)02062-0
6. Kaufman CL, Marvin MR, Chilton PM, et al. Immunobiology in VCA. *Transpl Int*. 2016;29(6):644-654. doi:10.1111/tri.12764
7. Morelon E, Petruzzo P. Vascularized composite allotransplantation still remains an emerging field after 17 years. *Curr Opin Organ Transplant*. 2015;20(6):593-595. doi:10.1097/MOT.0000000000000254
8. Uluer MC, Brazio PS, Woodall JD, Nam AJ, Bartlett ST, Barth RN. Vascularized Composite Allotransplantation: Medical Complications. *Curr Transplant Rep*. 2016;3(4):395-403. doi:10.1007/s40472-016-0113-x
9. Leonard DA, Kurtz JM, Cetrulo CL. Vascularized composite allotransplantation: towards tolerance and the importance of skin-specific immunobiology. *Curr Opin Organ Transplant*. 2013;18(6):645-651. doi:10.1097/MOT.0000000000000022
10. Taddeo A, Tsai C, Vögelin E, Rieben R. Novel targeted drug delivery systems to minimize systemic immunosuppression in vascularized composite allotransplantation. *Curr Opin Organ Transplant*. 2018;23(5):568-576. doi:10.1097/MOT.0000000000000564
11. Dzhonova DV, Olariu R, Leckenby J, et al. Local Injections of Tacrolimus-loaded Hydrogel Reduce Systemic Immunosuppression-related Toxicity in Vascularized Composite Allotransplantation. *Transplantation*. 2018;102(10):1684-1694.

doi:10.1097/TP.0000000000002283

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

13. Sarhane KA. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. *Front Immunol*.:7.

14. Abou-Daya KI, Oberbarnscheidt MH. Innate allorecognition in transplantation. *J Heart Lung Transplant*. 2021;40(7):557-561. doi:10.1016/j.healun.2021.03.018

15. Scozzi D, Ibrahim M, Menna C, Krupnick AS, Kreisel D, Gelman AE. The Role of Neutrophils in Transplanted Organs. *Am J Transplant*. 2017;17(2):328-335. doi:10.1111/ajt.13940

16. Zhao D, Abou-Daya KI, Dai H, Oberbarnscheidt MH, Li XC, Lakkis FG. Innate Allorecognition and Memory in Transplantation. *Front Immunol*. 2020;11:918. doi:10.3389/fimmu.2020.00918

17. Pasparakis M. Regulation of tissue homeostasis by NF-κB signalling: implications for inflammatory diseases. *Nat Rev Immunol*. 2009;9(11):778-788. doi:10.1038/nri2655

18. LaRosa DF, Rahman AH, Turka LA. The Innate Immune System in Allograft Rejection and Tolerance. *J Immunol*. 2007;178(12):7503-7509. doi:10.4049/jimmunol.178.12.7503

19. Mitchell S, Vargas J, Hoffmann A. Signaling via the NFκB system. *WIREs Syst Biol Med*. 2016;8(3):227-241. doi:10.1002/wsbm.1331

20. Zhu P, Bailey SR, Lei B, Paulos CM, Atkinson C, Tomlinson S. Targeted Complement Inhibition Protects Vascularized Composite Allografts From Acute Graft Injury and Prolongs Graft Survival When Combined With Subtherapeutic Cyclosporine A Therapy. 2017;101(4):11.

21. Grafals M, Thurman JM. The Role of Complement in Organ Transplantation. *Front Immunol*. 2019;10:2380. doi:10.3389/fimmu.2019.02380

22. Jordan SC, Toyoda M. Medical Management of the Thracic Surgery Patent - 41 - Transplant Immunology. :8. doi:10.1016/B978-1-4160-3993-8.00041-6

23. Siu JHY, Surendrakumar V, Richards JA, Pettigrew GJ. T cell Allorecognition Pathways

in Solid Organ Transplantation. *Front Immunol*. 2018;9:2548. doi:10.3389/fimmu.2018.02548

24. Abbas AK, Lichtmann AE, Pillai S. *Cellular and Molecular Immunology (Cellular and Molecular Immunology, Abbas)*. 8th ed. Elsevier Saunders; 2014.

25. Zhu X, Zhu J. CD4 T Helper Cell Subsets and Related Human Immunological Disorders. *Int J Mol Sci*. 2020;21(21):8011. doi:10.3390/ijms21218011

26. Anggelia MR, Cheng HY, Chuang WY, et al. Unraveling the Crucial Roles of FoxP3+ Regulatory T Cells in Vascularized Composite Allograft Tolerance Induction and Maintenance. *Transplantation*. 2021;105(6):1238-1249. doi:10.1097/TP.0000000000003509

27. Ma L, Zhang H, Hu K, et al. The imbalance between Tregs, Th17 cells and inflammatory cytokines among renal transplant recipients. *BMC Immunol*. 2015;16(1):56. doi:10.1186/s12865-015-0118-8

28. Schmitz R, Fitch ZW, Schroder PM, et al. B cells in transplant tolerance and rejection: friends or foes? *Transpl Int*. 2020;33(1):30-40. doi:10.1111/tri.13549

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

30. Beauvillain C, Cunin P, Doni A, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. 2011;117(4):1196-1204. doi:10.1182/blood-2009-11-254490

31. Costa S, Bevilacqua D, Cassatella MA, Scapini P. Recent advances on the crosstalk between neutrophils and B or T lymphocytes. Published online 2018:11.

32. Dunsmore G, Rosero EP, Shahbaz S, et al. Neutrophils promote T-cell activation through the regulated release of CD44-bound Galectin-9 from the cell surface during HIV infection. Rowland-Jones SL, ed. *PLOS Biol*. 2021;19(8):e3001387. doi:10.1371/journal.pbio.3001387

33. Jorch SK. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med*. 2017;23(3):9.

34. Torres-Ruiz J, Villca-Gonzales R, Gómez-Martín D, et al. A potential role of neutrophil extracellular traps (NETs) in kidney acute antibody mediated rejection. *Transpl Immunol*. 2020;60:101286. doi:10.1016/j.trim.2020.101286

35. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. 2018;18(2):134-147. doi:10.1038/nri.2017.105

36. Scozzi D, Wang X, Liao F, et al. Neutrophil extracellular trap fragments stimulate innate immune responses that prevent lung transplant tolerance. :14.

37. Safi AF, Kauke M, Nelms L, et al. Local immunosuppression in vascularized composite allotransplantation (VCA): A systematic review. *J Plast Reconstr Aesthet Surg*. 2021;74(2):327-335. doi:10.1016/j.bjps.2020.10.003

38. Ponticelli C, Reggiani F, Moroni G. Old and New Calcineurin Inhibitors in Lupus Nephritis. *J Clin Med*. 2021;10(21):4832. doi:10.3390/jcm10214832

39. Sutter D, Dzhonova DV, Prost JC, et al. Delivery of Rapamycin Using In Situ Forming Implants Promotes Immunoregulation and Vascularized Composite Allograft Survival. *Sci Rep*. 2019;9(1):9269. doi:10.1038/s41598-019-45759-y

40. Gajanayake T, Olariu R, Leclère FM, et al. A single localized dose of enzyme-responsive hydrogel improves long-term survival of a vascularized composite allograft. *Sci Transl Med*. 2014;6(249). doi:10.1126/scitranslmed.3008778

41. Etra JW, Grzelak MJ, Fidder SAJ, et al. A Skin Rejection Grading System for Vascularized Composite Allotransplantation in a Preclinical Large Animal Model. *Transplantation*. 2019;103(7):1385-1391. doi:10.1097/TP.0000000000002695

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

43. Muldur S, Vadysirisack DD, Ragunathan S, et al. Human Neutrophils Respond to Complement Activation and Inhibition in Microfluidic Devices. *Front Immunol*. 2021;12:777932. doi:10.3389/fimmu.2021.777932

44. Loeschenberger B, Niess L, Würzner R, et al. Calcineurin inhibitor-induced complement system activation via ERK1/2 signalling is inhibited by SOCS-3 in human renal tubule cells. *Eur J Immunol*. 2018;48(2):330-343. doi:10.1002/eji.201747135

45. Benichou G, Gonzalez B, Marino J, Ayasoufi K, Valujskikh A. Role of Memory T Cells in Allograft Rejection and Tolerance. *Front Immunol*. 2017;8. doi:10.3389/fimmu.2017.00170

Declaration of consent

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

Name/First Name: Helmer Anja Sophie

Registration Number: 20-117-065

Study program: Molecular Life Sciences

Bachelor ☐ Master ☑ Dissertation ☐

Title of the thesis: Tacrolimus inhibits neutrophils and neutrophil extracellular traps that are involved in vascularized composite allograft rejection

Supervisor: Supervisor: Prof. Dr. Robert Rieben
Co-Supervisor: Dr. Isabel Arenas Hoyos

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 5 September, 1996 is authorized to revoke the title awarded on the basis of this thesis.

For the purposes of evaluation and verification of compliance with the declaration of originality and the regulations governing plagiarism, I hereby grant the University of Bern the right to process my personal data and to perform the acts of use this requires, in particular, to reproduce the written thesis and to store it permanently in a database, and to use said database, or to make said database available, to enable comparison with future theses submitted by others.

Bern, 07.12.2022

Place/Date

Signature