Universität Bern
Departement Klinische Forschung
Direktor: Prof. Dr. Robert Rieben

Supervisor: Prof. Dr. Robert Rieben
Co-Supervisor: Dr. Adriano Taddeo

Vasculopathy and endothelial cell repair in vascularized composite allotransplantation

Masterthesis

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Jonas Stoffel (10-106-326)
von Visperterminen (VS)

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ABSTRACT

Background: Transplant vasculopathy and endothelial cell repair has not been systematically investigated in vascularized composite allotransplantation (VCA). The vascular endothelial layer represents the first contact of the immune system with the graft and is also the first barrier to be attacked. Moreover, the physiological vascular repair mechanism following transplantation is of pivotal importance for allograft survival and function. Our aim was to understand how the endothelium responds during acute rejection or immunosuppressive therapy in VCA. We hypothesized that after VCA the endothelium will respond trying to minimize vasculopathy and re-establish the endothelial cell function. This response could be greatly influenced by the allogeneic response with a strong imbalance towards endothelial cell (EC) damage during rejection and towards EC repair under immunosuppressive therapy. Moreover, we aimed at understanding whether the EC response involved the activation of endothelial progenitor cells (EPC) from recipient or donor bone marrow. **Methods:** To test this hypothesis, orthotopic hind limb transplantations were performed from Lewis to Lewis rats (group 1) and from Brown Norway to Lewis (group 2-4). After transplantation, we simulated four different clinical settings: 1) absence of immunoreaction (isograft); 2) acute cellular rejection (no treatment after transplantation) 3) immunosuppressive therapy (1mg/kg Tacrolimus, daily) and 4) 3-4 acute rejection episodes treated with immunosuppressive therapy (2 mg/kg Tacrolimus plus 2 mg/kg Dexamethasone). After transplantation, the rats were kept under these different treatment conditions for 30 days or until reaching grade 3 rejection. Blood was collected weekly in order to measure the levels of secreted inflammatory cytokines by luminex technology and to quantify EPC, circulating endothelial cells (CEC) and hematopoietic progenitor cells (HPC) by flow cytometry. Moreover, the EC response in the tissue was characterized by analyzing markers for EC damage and activation using immunohistochemistry. **Results:** Immunosuppressive therapy increased the frequency of HPCs (8.664±1.197% vs. pre-transplant 0.315±0.251%, $p<0.0001$). CECs were increased during acute rejection (0.832±0.821% vs. pre-transplant 0.008±0.004%, $p<0.05$). EPC frequency was significantly higher in acute rejection and immunosuppressive therapy than pre-transplantation (0.501±0.744%; 0.629±0.719%; 0.153±0.064% vs. pre-transplant 0.009±0.013%). Donor EPC, HPC and CEC were measurable after transplantation in the recipient blood. EC-related plasma cytokine levels (IL-1α, IL-1β, IL-10, IL-6, IP-10, MIP-1α, RANTES, sICAM-1, sRankl, sVCAM-1, TNF-α, VEGF-A) were increased during acute rejection compared to naïve. **Conclusion:** Acute rejection is characterized by an increased frequency of both CECs and EPCs. This leads to suggest the co-existence of EC damage and reparative mechanism. In contrast, immunosuppressive therapy promotes higher EPC frequency with minor EC damage. Interestingly, both donor and recipient EPC seem to be involved in vascular repair, with a minor contribution of donor-derived EPC. These findings have important clinical implications to design new therapeutic interventions aimed to maintain vascular integrity.

1. INTRODUCTION

Vascularized composite allotransplantations (VCA), such as facial or hand allotransplantations have become a promising clinical treatment for individuals, who have suffered severe tissue loss or who have severely damaged parts of the body, that cannot be repaired with conventional surgical techniques. The term “allotransplantation” is used for these transplants because the donor and recipient are genetically non-identical but belong to the same species. The first successful human hand allotransplantation was performed in 1998 in France (Dubernard et al. 1999). Since then, more than 100 upper extremity transplants, 30 face transplants and a variety of other vascularized composite allotransplantations have been successfully performed all over the world (Shores et al. 2015; Kueckelhaus et al. 2015). In contrast to solid organ transplantations, VCAs characteristically contain skin, vasculature, muscle, tendon, cartilage, bone and bone marrow, all of which exert different degrees of immunogenicity (Lee et al. 1991). In considerations to that, VCA has the potential to revolutionize reconstructive surgery - allowing to restore the body integrity, function and also the social reintegration - but it remains hindered by the lifelong commitment to potentially harmful immunosuppressive therapy (IST) and its attendant side-effects. As with solid organ transplantations, long-term systemic immunosuppressive therapy increases the risk of opportunistic infections, end-stage renal disease, and malignancies such as lymphomas in transplant recipients (Morelon et al. 2012). These complications prompted different novel strategies – with varying degrees of success - aimed at either minimizing the maintenance of immunosuppression or inducing donor-specific tolerance.

Despite immunosuppressive therapy, the major hurdle of this “life enhancing” rather than “life saving” intervention is acute rejection. Importantly, acute rejection episodes are reported in more than 80% of VCAs within the first year after transplantation (Petruzzo & Dubernard 2011). In contrast, the rejection rate in solid organ renal allotransplantation is approximately 10% (Fischer et al. 2014). This considerable disparity in rejection rates may be explained by the allogenic nature of the transplanted tissue, in particular the presence of vascularized skin and bone tissue (Issa 2016). In addition, skin transplants have traditionally been considered as most immunogenic, making it an obstacle to induce tolerance or minimize immunosuppression (Chadha et al. 2014). However, skin-containing vascularized composite allografts have the advantage of allowing for visual monitoring, earlier detection and subsequent treatment of acute rejection episodes. Chronic graft rejection has been rarely described in VCA, but with the increasing number of VCA recipients and with a longer follow-up, cases of chronic rejection have been observed in human VCA. Recent evidence shows, that the repetitive occurrence of acute rejection episodes leads to the development of chronic rejection (Kanitakis et al. 2016).

The vascular endothelial layer represents the first contact of the immune system with the graft and is also the primary target for alloimmune responses after transplantation. Therefore, it is mandatory to focus on the endothelial cell response following transplantation in more detail.

1.1. Allograft vasculopathy

Vascularized allografts are perfused through blood vessels composed of mural microvascular pericytes, macrovascular smooth muscle cells and the endothelium, which largely remain of graft origin. Endothelial cells are attached to the basal membrane followed by the media and adventitia layers, whereas at the capillary level, endothelial cells are directly attached to pericytes (Jiang et al. 2014). Because maintaining vascular integrity is essential for its function in providing nutrition and oxygen supply, damages to the microvasculature will impair oxygen supply and graft function. Only a healthy allograft vasculature can properly contribute to organ perfusion, maintenance of homeostatic functions and immune surveillance (Abrahimi et al. 2015).

During transplantation, graft endothelial cells may be activated by mediators of the innate as well as of the adaptive immunity leading to graft inflammation that contributes to ischemia-reperfusion injury (IRI) and/or graft rejection (Caterson et al. 2013). In IRI, reactive oxygen species (ROS) are initially produced by donor endothelial cells, followed by a larger burst production by neutrophils and macrophages, which leads to complement activation and production of pro-inflammatory cytokines; donor endothelial cells are thus allogeneic to the host and subsequently targets of alloimmunity (Madamanchi et al. 2004; Land 2012; Pober et al. 2009). Oxidative stress may induce endothelial cell apoptosis through NF-kB activation, whereas low concentrations of ROS were found not to cause irreversible injury but endothelial cell activation and inflammation, namely an increase of ICAM-1 and MHC class I expression on the EC surface (Aoki et al. 2001; Bradley et al. 1993).

Different forms of rejection, such as hyperacute rejection, cellular and antibody-mediated rejection or chronic rejection may affect the graft vasculature differentially. The respective role of the endothelium during hyperacute, acute and chronic rejection will be examined in the following sections.

1.1.1. Hyperacute rejection

Hyperacute rejection is characterized by thrombosis and ischemic fibrinoid arterial necrosis with subsequent destruction of the graft (Colvin 2007) (Fig. 1-1). This may occur within minutes to hours after anastomoses forming between donor and recipient microvessels and is mediated by preformed IgM reactive to nonself ABO blood group antigens expressed by EC. This immunopathological response is also driven - although less commonly - by preformed IgG antibodies reactive to nonself MHC class I and II molecules, that are present due to previous exposure to allogenic cells (from prior blood transfusions, pregnancies or transplants). Thus, donor endothelial cells, which are the target of this alloimmune response, are exposed to circulating antibodies that subsequently activate the

complement-, coagulation- and kinin-system, which in turn induces capillary thrombus formation and neutrophilic infiltrates (Abrahimi et al. 2015). In addition, IgG anti-donor antibodies may also activate recipient natural killer cells, which induce antibody-dependent cell-mediated toxicity (Colvin 2007).
Patients in need of allograft are routinely tested for ABO compatibility and the presence of anti-donor antibodies. As a consequence, the risk of hyperacute rejection can be avoided in clinical transplantation.

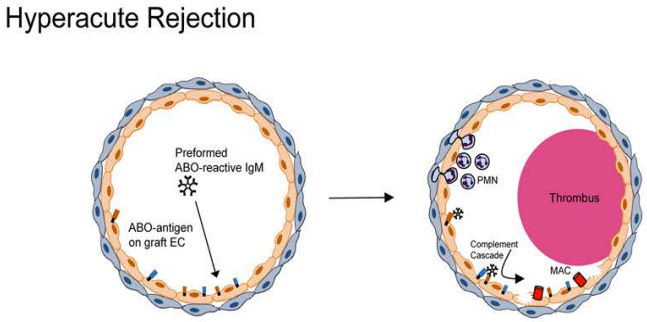

Figure 1-1: Involvement of EC in hyperacute rejection (adapted from Abrahimi et al. 2015). Hyperacute rejection is driven by preformed IgM reactive to nonself ABO blood group antigens expressed by EC. This activates the complement system, thereby inducing thrombosis and neutrophil infiltration. The resultant EC injury can lead to dysfunction and thrombosis followed by graft organ infarction and graft failure.

1.1.2. Acute rejection: cellular vs. humoral rejection

In 1980 it was first reported, that microvascular endothelium is the critical target of the immune response and that rejection manifests largely by vascular damage following vascularized skin allotransplantation (Dvorak et al. 1980). They provided further evidence that both $CD4^+$ and $CD8^+$ T cells infiltrate the perivascular cuffs and participate in the acute rejection process of skin allograft (Bhan et al. 1982).
Acute rejection is an inflammatory process of injury to the graft parenchyma and vasculature, which is orchestrated by alloreactive T cells (cellular rejection) and antibodies (humoral rejection) (Abbas Abul K. et al. 2014). Both types may typically co-exist in a graft undergoing acute rejection (Fig. 1-2). Acute rejection may usually occur a few days after allotransplantation corresponding to the time needed for T cell activation, proliferation and differentiation, whereas the immune response depends on antigen recognition, T cell activation and the signal for T cell proliferation (Ponticelli 2012). Alloantigen recognition e.g. presentation of foreign antigen to recipient T cells, can occur via three non-mutually exclusive routes, the direct, indirect and semi-direct pathway: 1) Alloreactive T cells bind directly to an intact allogeneic MHC molecule expressed on donor antigen presenting cell (APC) (Ravindra et al. 2012). 2) Allogeneic MHC molecules from graft cells are taken up, processed and expressed on recipient APCs, which are then recognized by alloreactive T (Whitelegg & Barber 2004). 3) Alloantigens get transferred from donor to recipient APCs via cell-cell contact or transfer of donor exosomes (Safinia et al. 2010).

Acute cellular rejection is mediated by the release of cytokines produced by $CD4^+$ cells and cytotoxic T lymphocytes (CTL) mediated killing of graft parenchymal cells and endothelial cells (Al-Lamki et al. 2008). Thus, cytotoxic T lymphocytes induces cell death in the target primarily through granule exocytosis of effector molecules, such as granzyme A, B and perforin or through the death receptor pathway (FAS/FASL) (Choy 2010; Russell & Ley 2002; Barry & Bleackley 2002). CTL differentiation may depend upon IL-2 release by activated effector memory $CD4^+$ T cells or IL-15 secreted by graft cells (Biedermann & Pober 1998). Moreover, activated $CD4^+$ T cells also recruit monocytes and promote differentiation of monocytes into activated macrophages. Both monocytes and host T cells may induce injury to the endothelium, which is called ‘‘endothelialitis’’ and is characterized by swelling, focal lifting and detachment of the endothelial cell layer (Abrahimi et al. 2015).

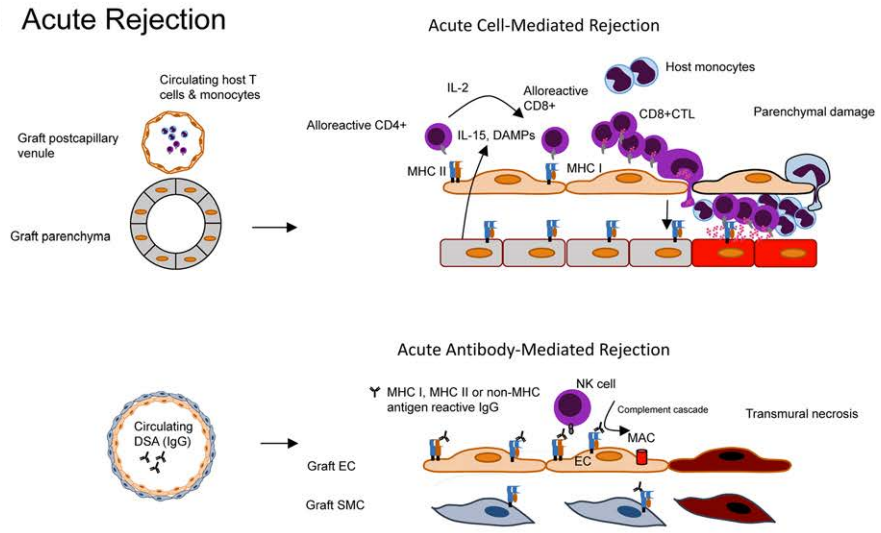

Figure 1-2: Involvement of EC in acute rejection (adapted from Abrahimi et al. 2015). Acute rejection is driven by cellular and/or humoral immunity. In acute cellular rejection, MHC molecules on EC are presented to circulating alloreactive effector memory $CD8^+$ T cells, that enter the graft, expand and eventually differentiate into CTL. This process mediates rejection by killing parenchymal cells; CTL differentiation may depend upon cytokines produced by concomitantly activated effector memory $CD4^+$ T cells or by graft cells. Activated alloreactive $CD4^+$ T cells also recruit monocytes and promote differentiation into activated macrophages. Acute antibody-mediated rejection is dominated by graft vascular injury driven by complement-activating IgG antibodies, which bind to donor-specific MHC class I and II, as well as to non-MHC alloantigens on graft EC.

Acute antibody-mediated rejection is mediated by complement activating IgG antibodies, that bind to donor-specific MHC class I and II, as well as to non-MHC alloantigens on graft endothelial cells (Colvin 2007) (Fig. 1-2). Complement activation and the formation of the membrane attack complex (MAC) thus leads to EC lysis, infiltration and activation of neutrophils followed by thrombus formation (Abbas Abul K. et al. 2014). Alloantibodies also engage natural killer cells, which then kill target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Al-Lamki et al. 2008). Acute antibody-mediated rejection tends to result in graft vascular injury rather than parenchymal cell injury, with fibrinoid necrosis of graft arteries (Colvin 2007).

In contrast, endothelial cells can acquire resistance to injury via upregulation of cytoprotective molecules and resistance to antibody-mediated cell injury via the expression of anti-apoptotic genes such as Bcl-2, A20 and Bcl-X_L (Bach et al. 1997; Tabata et al. 2003; Dorling 2012). This phenomenon is known as “accomodation” (Colvin & Smith 2005).

1.1.3. Chronic rejection

Despite the higher incidence of acute rejection episodes in VCA patients, compared to solid organ transplantations, chronic graft rejection has been rarely described in VCA and therefore the exact underlying mechanisms of chronic rejection have not been defined. Chronic rejection designates the major late terminal graft failure after a gradual process developing over the years and may manifest differently according to the type of transplanted organ (Yates & Nicholson 2006). Immunological factors that could induce chronic rejection in VCA include repeated episodes of acute rejection, both T cell and humoral rejections, HLA mismatches, the transplant relative skin and vascularized bone marrow content and infections (Fig. 1-3) (Mundinger & Drachenberg 2014). As in solid organ transplantation, transplant vasculopathy appears to be a key feature of chronic rejection in VCA (Kanitakis et al. 2016). Injury of blood vessels - primarily the endothelium - manifests with a progressive concentric narrowing of the arterial lumen due to intimal hyperplasia, which represents excessive compensatory remodeling (Kanitakis et al. 2016; Pober et al. 2014). This process may result in ischemic changes of the graft parenchyma, replacement fibrosis, dysfunction and eventually graft loss.

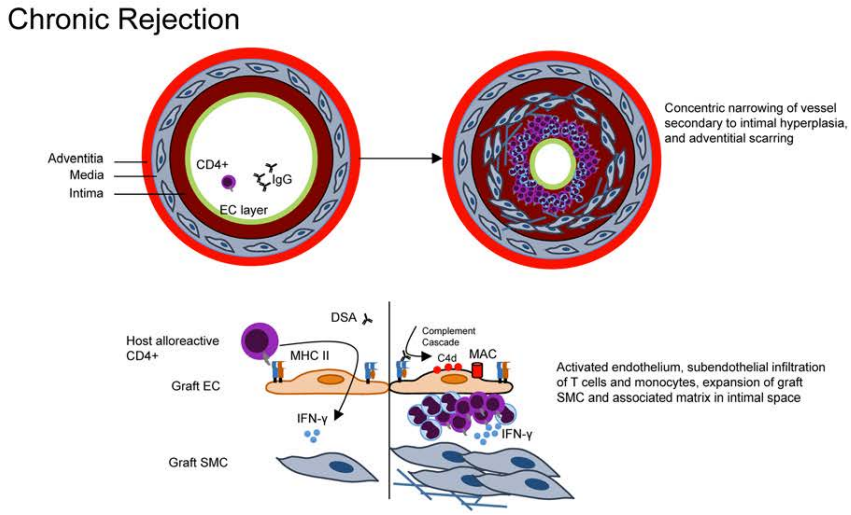

Figure 1-3: Involvement of EC in chronic rejection (adapted from Abrahimi et al. 2015). Chronic rejection may occur as progressive concentric narrowing of the graft arterial lumen (allograft vasculopathy), resulting in ischemia, fibrosis of graft parenchyma, and late graft failure. Graft vasculopathy is characterized by T cell infiltration in the sub-endothelial space of the arterial intima, which then leads to IFN-γ release, recruitment of lymphocytes and monocytes. Release of growth factors by infiltrating host cells, especially IFN-γ, results in proliferation of graft-derived SMC with associated matrix that progressively narrows the vessel lumen. Formation of donor-specific antibody increases the risk of developing vasculopathy, possibly through recruitment of IFN-γ producing natural killer cells, or by increasing the immunogenicity of EC for reactive T-cells, either directly or via MAC deposition and EC activation.

Graft vasculopathy is orchestrated by T cell infiltration in the sub-endothelial space of the arterial intima followed by IFN-γ production and lymphocytes and monocytes recruitment. It has been reported that IFN-γ has a central role in chronic rejection (Abrahimi et al. 2015; Lin et al. 2016). Release of IFN-γ and other growth factors by infiltrating host cells (T cells and macrophages), results in proliferation of graft-derived vascular smooth muscle cells and extracellular matrix which progressively narrows the vessel lumen (Abrahimi et al. 2015). Moreover, antibody-mediated rejection or the presence of donor-specific antibodies increases the risk of developing graft vasculopathy and persistent vascular inflammation through EC activation, recruitment of natural killer cells and complement-mediated pathways (Chandraker et al. 2014; Thaunat et al. 2005).

Interestingly, angiogenesis factors such as vascular endothelial growth factor (VEGF) have been reported to be overexpressed in SOT models of chronic rejection and their expression has been associated with disease progression (Ezaki et al. 2001; Ferrara 2005). Moreover, blocking of VEGF-VEGFR has been found to attenuate the progression of the chronic rejection disease process (Sho et al. 2005; Malmström et al. 2008).

We have emphasized that the endothelium is affected differentially during the varying rejection scenarios and that an intact vascular function is essential for allograft survival. Therefore, to maintain vascular integrity, physiological vascular repair following transplantation is of pivotal role.

1.2. Vascular repair in solid organ transplantation

Two different mechanisms of vascularization are known; post-natal neovascularization (“angiogenesis”), operated by sprouting and migration of endothelial cells from pre-existing vessels and embryonic neovascularization (“vasculogenesis”), namely in situ proliferation of endothelial cells to form vessels “de novo”. Vasculogenesis was believed to occur only in embryonic angiogenesis but this dogma was crushed by the discovery of endothelial progenitor cells (EPC) (Asahara et al. 1997).

During the past 20 years, endothelial progenitor cells have promoted our understanding in vasculogenesis, re-endothelialization and endothelial protection, particularly during vascular activation and injury (Lam et al. 2008; Yamada et al. 2004; Asahara et al. 1997; Asahara & Kawamoto 2004). EPC have been described as circulating progenitor cells derived from bone marrow with the capability of homing to sites of vascular injuries, where they exert their effects on endothelial cell repair and angiogenesis. It was reported that EPCs may directly integrate and incorporate into blood vessels to physically participate in the endothelial cell repair (“building block” role) (Asahara et al. 1997) or indirectly via the production and secretion of angiogenic growth factors to ischaemic tissues, thus contributing to angiogenesis via paracrine effects (Rehman et al. 2003). In addition, EPC may also release matrix metalloproteinases (MMPs) to promote a concomitant increase in matrix degradation that enables endothelial cell migration and vascular remodeling (Fadini & Avogaro 2010).

Mobilization of EPC from the bone marrow to the target tissue is a complex process regulated by a variety of growth factors and signalling cascades. EPC are located within a stem cell niche in the bone marrow and their mobilization depends on physiological factors such as peripheral tissue hypoxia, trauma, physical exercise, estrogen or age. Several studies have shown that in the presence of hypoxia, transcription factors like hypoxia-inducible transcription factor-1α (HIF-1α) are activated, leading to increased levels of VEGF and other important mediators of angiogenesis, such as stromal cell-derived factor-1 (SDF-1) and erythropoietin (EPO) (Nakamura et al. 2004; Stellos et al. 2008; Heeschen et al. 2003). VEGF, a potent angiogenic factor that binds to its receptors VEGFR-1 and VEGFR-2 (KDR), leads to EPC mobilization, proliferation and migration via activation of the Akt signaling pathway (Sen et al. 2011; Déry et al. 2005). Moreover, the adhesion molecules P-selectin and E-selectin appear to play an important role in EPC adhesion and migration, as the recruitment of EPC involves chemotaxis, tethering, adhesion, and migration of cells into the sub-endothelial tissue (Langer et al. 2006).

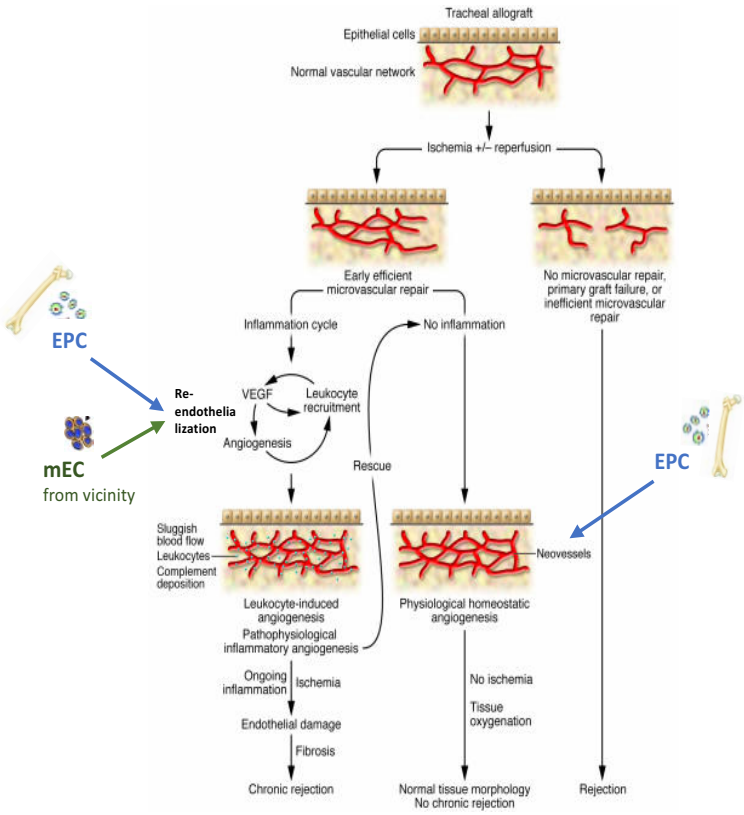

Figure 1-4: Microvascular damage and repair following transplantation (adapted and modified from Contreras & Briscoe 2007). Following orthotopic allotransplantation of trachea, re-vascularization is associated with perfusion and return of blood flow. This response involves physiological anastomoses between donor and recipient microvessels. This early repair and/or homeostatic angiogenesis is required for normal graft function. After adequate reperfusion, in absence of inflammation, physiological homeostatic vascular remodelling occurs, vascular integrity is maintained, and tissue morphology remains normal. In contrast, as leukocytes infiltrate allografts, pathophysiological inflammatory angiogenesis occurs and is only sufficient to sustain the graft function minimally. This inflammatory angiogenesis reaction likely facilitates ongoing leukocyte infiltration and endothelial damage, eventually leading to ischemia, microvascular injury, tissue fibrosis and chronic rejection.

Despite EPC activation and migration, there is a reconstitution of the endothelial cell layer that occurs via migration and proliferation of mature endothelial cells, which are resident in the vascular endothelial intima of the vicinity (Yoder 2010) (Fig. 1-4). However, differentiated endothelial cells have low proliferative potential, thus their ability to repair damaged endothelium is limited, especially when a strong inflammation response, such as in acute rejection, is triggered.

The repair of donor vessels through upregulation of endogenous repair processes in both donor and recipient may be crucial for maintaining a normal allograft. In addition, since in VCA the bone marrow is part of the graft transplant, therefore, EPC responsible for repairing the endothelial layer could originate from the graft bone marrow itself or from the recipient.

The endothelial regenerative potential of EPCs has been under intensive investigation in a variety of animal models and clinical studies. Overall, these studies implicate that circulating EPC exert important functions in endogenous repair mechanisms aiming at maintaining integrity of the endothelial monolayer by either replacing denuded parts of the injured artery and / or forming new vessels by direct incorporation and paracrine effects (Kinnaird et al. 2004; Burnham et al. 2005). However, no study so far has directly evaluated the role of transplant vasculopathy and endothelial cell repair in VCA. Due to the unique vascularization and the different cell composition with varying immunogenicity in vascularized composite allotransplantation, the concepts of vascular damage and repair in SOT cannot be adapted incidentally to VCA.

1.3. Aim and scientific question

Allograft vasculopathy and physiological vascular repair mechanisms following transplantation are of pivotal importance for allograft survival. However, no study has directly evaluated the role of transplant vasculopathy and endothelial cell repair in a VCA model. The aim of the study was to understand how the endothelium responds during acute rejection or immunosuppressive therapy in VCA. We hypothesized, that after vascularized composite allotransplantation, the endothelium will respond trying to minimize vasculopathy and re-establish the endothelial cell function. This response could be greatly influenced by the immunological response with a strong imbalance towards EC damage during rejection and towards EC repair under immunosuppressive therapy. To test this hypothesis, we simulated four different clinical settings: 1) in absence of immunoreaction, 2) under acute cellular rejection 3) under immunosuppressive therapy and 4) under 3-4 acute rejection episodes treated with immunosuppressive therapy. Moreover, we aimed at understanding whether the EC response in these conditions involved the activation of EPC from recipient or from the transplanted donor bone marrow.

2. MATERIALS AND METHODS

2.1. Overall study design

In order to understand the role of the endothelium in VCA we simulated four different clinical settings:

1. **Isograft**

   Lewis to Lewis hind limb isotransplantations were performed, essentially eliminating the immunological barrier, allowing to assess the impact of surgical trauma and ischemia reperfusion injury (IRI). This group was expected not to exhibit any signs for acute rejection.

2. **Immunosuppressive Therapy (IST)**

   For maintenance of an allograft, without acute rejection. Brown Norway to Lewis hind limb allotransplantations were performed. The animals were treated daily with a standardized immunosuppressive treatment using Tacrolimus (1 mg/kg, s.c.) daily. This group was expected not to exhibit signs for acute rejection.

3. **Acute Rejection**

   Brown Norway to Lewis hind limb allotransplantations were performed. No immunosuppressive drugs were given in order to observe an acute rejection.

4. **Acute Rejection Therapy (ART)**

   Brown Norway to Lewis hind limb allotransplantations were performed. The group was treated with a high dose of immunosuppressive treatment using Tacrolimus (2 mg/kg) and Dexamethasone (2 mg/kg, s.c.) at onset of early signs of an acute rejection episode until the signs vanish, then treatment was stopped until signs appeared again.

Since ischemia related injury (IRI) is known to cause endothelial injury, ischemia time for all animals was kept at 90 minutes.

2.2. Animals

Six to eight weeks old male Lewis (LEW) and Brown Norway (BN) rats (wild type, purchased at Charles River), weighting between 250g and 300g, were used in this study. Animals were randomly divided into four groups. The animals were maintained in a specific pathogen-free environment at the central animal facility of the University of Bern. All animals were housed under standard conditions with water and food ad libitum and all animal experiments were performed in accordance with the terms of the Swiss animal protection law and were approved by the animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland). Experimental protocols were refined according to the 3R principles and state-of-the-art anesthesia and pain management, which are used to minimize the number of animals and to reduce the exposure of the animals to stress and pain during all the experiments.

2.3. Orthotopic hind limb allotransplantation

Orthotopic hind limb transplantations were performed from BN to LEW (MHC-mismatched) and from LEW to LEW in the isograft group respectively. Both hind limbs of a BN donor (or LEW donor) were retrieved at mid-thigh level and transplanted to two LEW recipients. The surgeries were performed concomitantly by two surgeons as described previously with some modifications (Sacks et al. 2012).
The transplantations were performed under continuous inhalation anesthesia (Table 2-1). Isoflurane 5% with oxygen (1 L/min) was used for the induction of anaesthesia (2-3 min) in the induction chamber. Then, the animals were placed in maintenance anesthesia at 2-3 % Isoflurane with 0.6 L/min oxygen. All rats were maintained at normal body temperature using heating pads and received pre-emptive buprenorphine analgesia (50 µg/kg, s.c.) 30-60 minutes before operation. Furthermore, the eyes of the rats were treated with ophthalmic ointment to avoid desiccation.
After the hind limb was shaved and the circumferential incision of the thigh at the inguinal crease was made, heparin (300 IU) was given intravenously in the penile vein. The femoral nerve, artery and vein were isolated close to the inguinal ligament and the thigh muscle and the sciatic nerve were transected to expose the femur. After the transverse osteotomy using a liston forceps, the limb was put in saline-soaked gauze and kept on ice. Upon harvesting the hind limbs, the donor rats were euthanized (as described later). Meanwhile, the recipient Lewis hind limb was prepared in a similar way; only the transection of nerve and vessels was done more proximal.
Ischemia time for all animals was kept at 90 minutes. To transplant the allograft, a femoral osteosynthesis was performed using an intramedullary fixation with 18-gauge needle with blunted ends. Then, donor and recipient femoral veins were anastomosed using a “cuff technique” with a polyimide medical tube (Vention Medical® Inc, Denver, USA), the arteries were anastomosed using interrupted 10-0 Nylon sutures and the sciatic and femoral nerves were coapted in an end-to-end fashion (via neurorrhaphy with interrupted 10-0 Nylon sutures). The muscular groups were

approximated using interrupted 4-0 Vicryl and the skin was sutured in a continuous manner with 4-0 Vicryl. Prior to skin closure, Buprenorphin (50 µg/kg) was injected subcutaneously. In order to prevent hypothermia, all rats were kept on heating pads for at least one hour post-transplantation. Analgesic Buprenorphin (50 µg/kg) was administered every 12 hours until post-operative day two (POD 2) or whenever animals showed pain.

Table 2-1: List of drugs that were used in the experiments.

Drug	Generic name	Company	Concentration	Solvent	Dose	Route
Buprenorphine	Temgesic®	Reckitt Benckiser AG	0.03 mg/ml	NaCl 0.9%	50 ug/kg	s.c.
Isoflurane	Forene®	AbbVie AG	pure	N/A	**Induction:** 5% with 1L/min O_2 **Maintenance:** 1-1.5% with 0.6 L/min O_2	inhalation
Pentobarbital	Esconarkon ad us. vet. Injektionslösung	Streuli Pharma AG	300 mg/ml	Ethanol	150 mg/kg	i.p.
Heparin	N/A	Inselspital	20'000 units E/48	NaCl 0.9%	80 UI/kg	i.v.
FK-506 (Tacrolimus)	N/A	LC Laboratories	1 mg/ml	Ethanol / Kollifor 1:1	1 mg/kg	s.c.
Dexamethasone	Mephameson®-4	Mepha Pharma AG	2 mg/ml	NaCl 0.9%	2 mg/kg	s.c.

2.4. Graft monitoring and immunosuppression

All animals were examined daily for either clinical signs of rejection or transplant failure. In this study, clinical acute rejection was graded as 0 = no rejection, 1 = erythema and edema, 2 = epidermolysis and exudation, 3 = desquamation, necrosis and mummification. First occurrence of edema and erythema was considered the start of acute rejection and resolution of those symptoms was considered complete reversal of rejection. Post-operative day 30 (POD 30) or grade 3 rejection was defined as the endpoint of the experiment. All animals of the immunosuppressive therapy group (IST) were treated daily with systemic immunosuppression (Tacrolimus 1 mg/kg/day, s.c.) for the complete experimental period of 30 days whereas no immunosuppressive drugs were given in the isograft and acute rejection group. Upon signs of grade 1-2 rejection, the animals in the acute rejection therapy group (ART) received Tacrolimus (1 mg/kg/day, s.c.) and Dexamethasone (2 mg/kg/day, s.c.). Pulse treatment with Tacrolimus + Dexamethasone was continued until signs vanished and acute rejection was clinically reversed. Allografts in this group underwent multiple episodes of acute rejection; the first episodes were clinically reversible until they reached grade 3 rejection.

2.5. Euthanasia and sample collection

In order to analyze endothelial progenitor cells, circulating endothelial cells and hematopoietic progenitor cells, peripheral whole blood was collected at post-operative day (POD) 1, post-operative week (POW) 2, POW 3 and at the end of the experiments (endpoint). Therefore, the animals were placed in the induction chamber and Isoflurane 5% with oxygen (1 L/min) was used for the induction of anaesthesia (2-3 min). Then, the sublingual vein was punctured in order to collect blood into an EDTA tube (500 µl). Moreover, the blood samples were analysed using a cell counter hematology analyzer (Sysmex KX-21N™, Hyogo, Japan) to receive the actual absolute white blood cell count. In a further step, peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples in order to characterize donor-specific cells (EPCs, CECs and HPCs) with flow cytometry and EDTA plasma was isolated to quantify plasma cytokine expression.

At the end of the experiment (POD 30 or grade 3 rejection), muscle and skin tissue samples from the contralateral and transplanted site were obtained for immunohistochemistry analysis in order to examine tissue-specific allograft responses. After harvesting samples, the rats were euthanized by injecting 150 µg/kg pentobarbital intraperitoneally and death was confirmed by bilateral thoracotomies. All euthanasia procedures in our study were performed according to current Swiss Laws on Animal Protection.

2.6. Histopathology

Muscle and skin tissues from the graft were analyzed by histology to evaluate the pathological score of inflammation. Tissue samples were harvested at the end of the experiment (endpoint). Biopsies of formalin-fixed muscle and skin tissues were embedded in paraffin wax and sectioned (5 µm). Sectioned tissues were stained with hematoxylin and eosin (H&E). The tissue samples from different experimental groups were observed under a light microscope and scored blindly by a professional pathologist.

2.7. Immunofluorescence analysis

Muscle samples from the graft, retrieved at the end of the experiments, were preserved in a suitable tissue mold with Tissue-Tek O.C.T. (Sakura Finetek, 4583), an embedding compound for cryosectioning, and were rapid frozen at -80°C. Subsequently the frozen samples were placed in the Cryostat (Leica CM3000, Wetzlar, Germany) and 5 µm thick tissue sections were cut with the microtome at −20°C and picked up instantly on a glass slide. If necessary, the temperature of the cutting chamber (±5°C) was adjusted, according to the tissue under study.

The 5 µm tissue sections were dried in air for 30 minutes at room temperature. After labelling, the samples were fixed in – 20°C cold acetone or methanol for ten minutes using glass jars. Afterwards,

the slides were gently removed from the jar and left to dry for about five minutes. The tissue samples were marked around using a Dako pen (Dako, s-2002). Then the slides were rehydrated in cold 1x tris-buffered saline (TBS) in glass jars for five minutes at room temperature. After removing and gently flicking the slides from the TBS bath, the slides were placed onto the slide holder, which has been previously wetted with TBS. Prior to incubation of the sample with the primary antibodies, 150 µl 3% bovine serum albumin (BSA) + TBS was added and incubated for one hour at room temperature to block non-specific interactions. After removing the blocking solution by rinsing with 1x TBS, 150 µl of primary antibodies (diluted in TBS-1% BSA) were added and incubated overnight at 4°C (Table 2-2). HSPG, VE-Cadherin and CD31-Biotin were used to show EC preservation and damage in the graft tissue whereas E-selectin was used to show EC activation. Goat anti-rat IgG-Cy3™ and goat anti-rat IgM-R-PE were used to detect tissue immunoglobulin deposition and association with EC damage.

Table 2-2: Staining panel. Overview of the different staining with corresponding fixation, primary and secondary antibody.

Staining	Fixation	Primary Antibody 1:100	Number	Company	Secondary Antibody 1:500	Number	Company
1	Aceton	CD31-Biotin	130-105-877	Miltenyi Biotec	Streptavidin-Cy3™	S-6402	Sigma-Aldrich
2	Methanol	Heparan sulfate (HSPG)	370255-1	AMS Biotechnology	Goat anti-mouse IgM-Cy3™	115-167-020	Jackson ImmunoResearch Laboratories
3	Aceton	E-selectin	bs-1273R	Bioss Inc.	Sheep anti-rabbit IgG-Cy3™	C2306	Sigma-Aldrich
4	Aceton	VE-cadherin	sc-6458	Santa Cruz Biotechnology	Donkey anti-goat IgG - Alexa Fluor 488	A-11055	Molecular Probes
5	Aceton	Goat anti-rat IgG-Cy3™	112-166-003	Jackson ImmunoResearch Laboratories	-	-	-
6	Aceton	Goat anti-rat IgM-R-Phycoerythrin	112-116-075	Jackson ImmunoResearch Laboratories	-	-	-

Then the slides were rinsed with 1x TBS and washed three times in 1x TBS on an orbital shaker. After drying the slides as mentioned above and placed again onto the slide holder, 150 µl of secondary antibody (diluted in TBS-1% BSA) and DAPI (1 µg/ml) were added and incubated at room temperature for one hour protected from light. After another washing step, the slides were dried on a heated block set at 42°C. Prior to mount the slides using cover slips, a small drop of pre-warmed glycergel (Dako, C0563) was put onto the tissue. The slides were then visualised by the examiner, who was blinded to the sample identities, using the immunofluorescence microscope Leica DMI4000 and LAS AF Software (Wetzlar, Germany) and all images were captured with identical exposure times

and settings in each experimental group. Quantitative analysis of fluorescence intensity (integrated density) was performed by ImageJ software (http:/rsb.info.nih.gov/ij/).

2.8. Characterization of EPC, CEC and HPC using flow cytometry

Whole blood samples, retrieved at the different time points, were diluted with 1x phosphate buffered saline (PBS), an iso-osmolar buffer solution to maintain the cellular integrity, and transferred to a falcon tube. Then, the blood was stratified gently on 2 ml of pre-warmed (room temperature) Ficoll-Paque Plus (GE Healthcare, 17-1440-02) and centrifugated for 20 minutes at 2200 rpm without brake and acceleration. In another step, the ring of cells was collected and transferred to another falcon tube using a pipette. After a washing step with 1x PBS, the PBMCs were ready for the staining. Therefore, 100 µl of PBMC solution per vial was transferred to FACS tubes. In order to detect all nucleated cells, Hoechst dye was added and incubated for 20 minutes at 4°C. After washing with 2 ml of PBS-1% BSA and centrifugating five minutes at 1500 rpm, the supernatants were discarded. Then the antibodies were added and incubated for 20 minutes at 4°C (Table 2-3). $CD34^+$ and $CD34^+RT1Ac^+$ was used to detect hematopoietic progenitor cells (HPC) which derived from recipient bone marrow or donor bone marrow, respectively. $CD34^+KDR^+CD45^{low}$ were used to detect circulating endothelial progenitor cells (EPC) and $CD31^+CD45^-$ were used to select mature circulating endothelial cells (CEC). Donor cells were identified as $RT1Ac^+$ cells.

Table 2-3: List of antibodies used to detect (donor-) circulating EPCs, mature circulating ECs, hematopoietic progenitor cells.

Antibody	Fluorophore	Number	Company
Hoechst 33342	Hoechst 33342	5117	Tocris Bioscience
CD45	APC-Vio770	130-107-792	Miltenyi Biotec
CD34 Streptavidin	Biotin BV650	NBP2-33076B 405231	Novus Biologicals BioLegend
KDR	PerCP	NB200-208PCP	Novus Biologicals
CD31	PE-Cy7	25-0311-82	eBioscence
RT1Ac	PE	MCA156PE	AbD Serotec
CD3	FITC	130-102-678	Miltenyi Biotec
CD45R (B220)	VioBright FITC	130-106-778	Miltenyi Biotec
CD11b/c	FITC	130-105-273	Miltenyi Biotec
CD133	DyLight650	NB120-16518C	Novus Biologicals

After another washing and centrifugation step, the supernatants were again discarded. Then Streptavidin BV650 was added and incubated for 20 minutes at 4°C, so that it conjugated with the biotinylated CD34 antibody. After the final washing step and centrifugation, the supernatants were again discarded. After resuspending the samples with FACS-flow buffer (BD Biosciences, San Jose, USA), the tubes were acquired using the flow cytometer LSR II Special Order System H274 (BD Biosciences, San Jose, USA) and the FACSDiva Software (BD Biosciences, San Jose, USA). Analysis of the flow cytometry data was performed using Flow-Jo software (Tri-Star, Ashland, USA) in order to display the frequencies of (donor-) hematopoietic progenitor cells, endothelial progenitor cells and circulating endothelial cells.

Although the following gating strategy was pursued (Fig. 2-1): PBMCs were gated by using forward scatter (FSC) and side scatter (SSC) to eliminate any cell debris, dead cells, clumps or double cells. In the second plot, only nucleated cells (Hoechst$^+$ cells) were selected. Within this population, in the one hand all CD34$^+$ cells were selected to receive the amount of hematopoietic progenitor cells. In the other hand, circulating EPCs were determined out of this population by the expression of surface markers CD34$^+$, KDR$^+$ and CD45^{low}. Moreover, all fluorescence activated CD31$^+$ and CD45$^-$ cells were selected out of the nucleated Hoechst$^+$ cell population in order to obtain mature circulating ECs. We further selected all RT1Ac$^+$ cells – a BN-specific MHC class II molecule – to differentiate the donor-derived cells from the LEW recipient-derived cells.

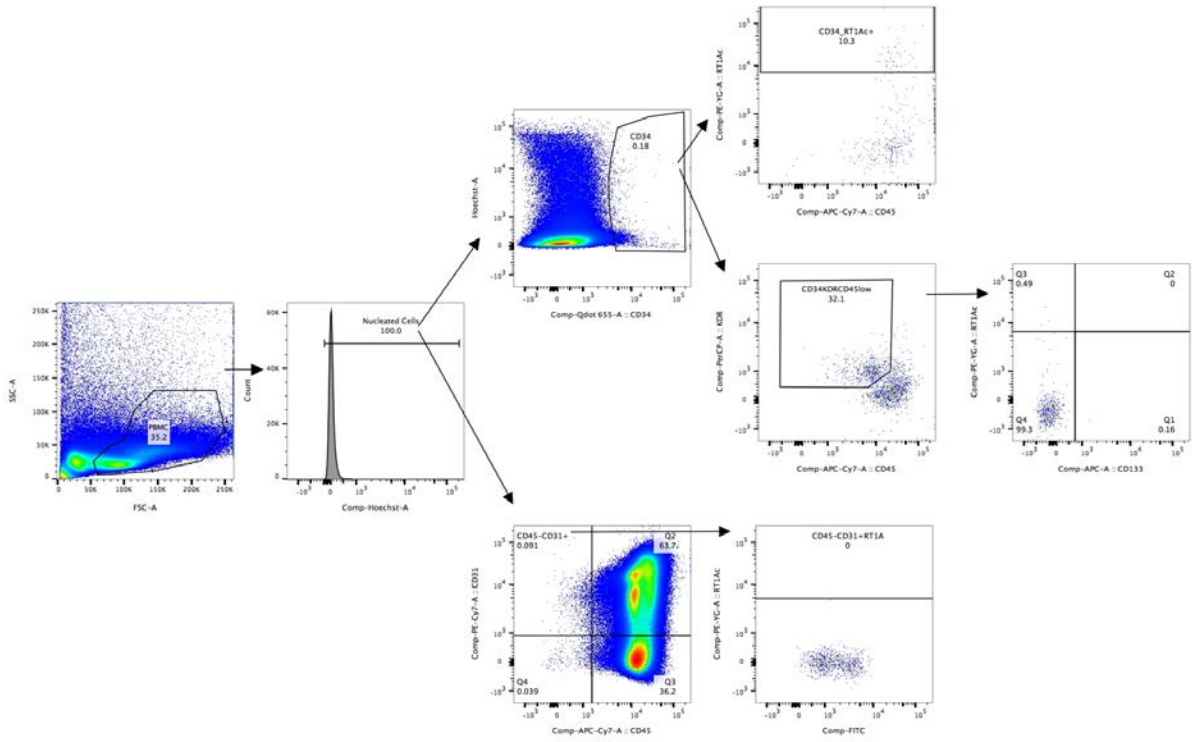

Figure 2-1: Gating strategy. Frequency of hematopoietic progenitor cells (CD34$^+$), circulating EPCs (CD34$^+$KDR$^+$CD45^{low}) and mature circulating ECs (CD31$^+$CD45$^-$) were analyzed by flow-cytometry after exclusion of debris, double cells and selection of nucleated cells (Hoechst$^+$ cells). Donor EPCs, CECs and progenitor cells were identified as RT1Ac$^+$ cells.

2.9. Plasma cytokine analysis

Plasma cytokine expression was quantified using a ProcartaPlex® Mix&Match Rat 13-plex Immunoassay (Affymetrix, Santa Clara, USA) for each of the following groups: naïve, Isograft, acute rejection, immunosuppressive therapy and acute rejection therapy. Levels of secreted inflammatory and EC-related cytokines (IL-1α, IL-1β, IL-10, IL-17A, IL-6, IP-10, MIP-1α, RANTES, sICAM-1, sRankl, sVCAM-1, TNF-α, VEGF-A) in the plasma collected at POD1, POW1, POW2, POW3 and day of rejection, were analyzed according to the manufacturer's instructions (Affymetrix, Santa Clara, USA). In short, plasma samples, containing unknown quantities of the cytokines, were incubated together with magnetic beads conjugated with the respective antibodies for 60-120 min in a micro plate well. After a washing step, the magnetic beads were incubated with the detection antibody mix (25 µl) for 30 min. After another washing step, the magnetic beads were incubated with streptavidin-PE (50 µl) for another 30 min. After the final washing step, beads were resuspended via adding reading buffer (120 µl) and then the concentrations were measured using the Bio-Plex 3D® suspension array system (Bio-Rad, Hercules, USA)

2.10. Statistical analysis

Prism software version 7 (GraphPad Software, La Jolla, CA) was used for statistical analysis. The data are presented as means ± SD unless otherwise indicated. Differences between groups were assessed by using one-way ANOVA with Tukey's and Dunnett's multiple comparisons test. Graft survival was compared between the different groups using log-rank (Mantel-Cox) test and represented as Kaplan-Meier curve. A value of p less than 0.05 was considered statistically significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3. RESULTS

3.1. Allograft survival

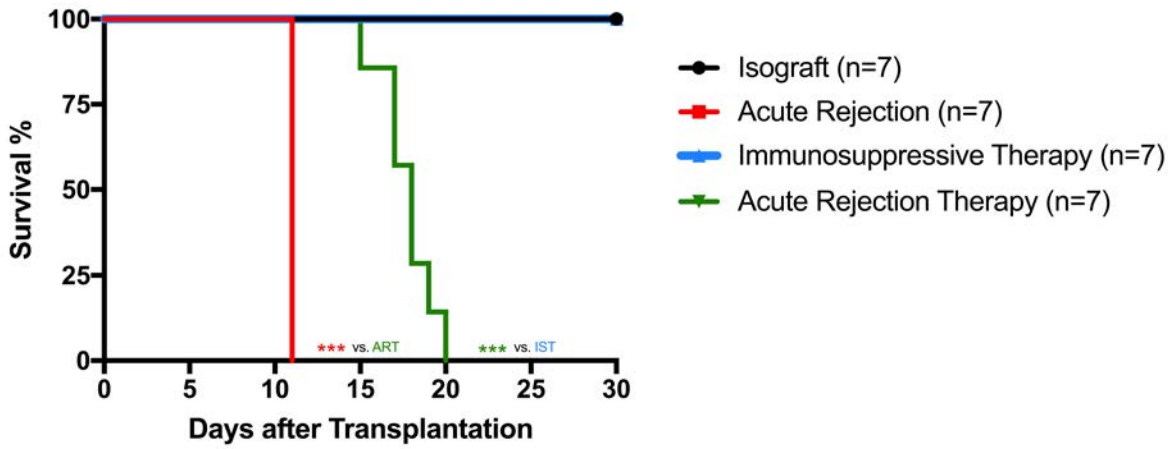

Figure 3-1: Vascular composite allograft survival curve. Graft survival was compared between the different experimental groups as indicative of four different clinical outcomes. Data are represented as Kaplan-Meier curve. n=7 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by log-rank (Mantel-Cox) test.

Syngeneic and allogeneic hind limb transplantations from Lewis-to-Lewis or Brown Norway-to-Lewis rats were performed, respectively. Graft rejection was evaluated macroscopically and graded from 0 = no rejection to 3 = desquamation, necrosis and mummification (see chapter 2.4). No animal of the Isograft group, which underwent syngeneic transplantation without treatment, exhibited signs of rejection or complications. All animals reached the final endpoint of the experiment and were euthanized at day 30 post operation (POD 30).

The median survival time of the acute rejection group was 11 days (POD 11±0, n=7, Fig. 3-1). The first signs of rejection (grade 1) appeared at POD 6 (2 out of 7 animals), whereas all animals showed grade 2 rejection at POD 8. All recipient in this untreated acute rejection group, showed grade 3 rejection of the graft with erythema and edema formation and necrosis. Therefore, all animals were euthanized at day 11 post operation.

All animals of the immunosuppressive therapy group, which were treated daily with tacrolimus (1 mg/kg/day, s.c.), showed no clinical signs of rejection. All animals in this group reached the endpoint of the experiment and were sacrificed at day 30 (POD 30).

Treatment of acute rejection (ART) resulted in a median survival time of 18 days, which is significantly longer with respect to the group that did not receive any treatment (POD 18±4 vs. POD 11±0, $p \leq 0.001$, n=7). The last group of animals received Tacrolimus (1 mg/kg/day, s.c.) and Dexamethasone (2 mg/kg/day, s.c.) upon the appearance of grade 1-2 rejection to revert the acute rejection episode.

Pulse treatment with Tacrolimus + Dexamethasone was continued until signs vanished. Then, the treatment was stopped until signs appeared again. The first signs of rejection (grade 1) appeared at POD 4 (3 out of 7 animals). Due to the systemic immunosuppressive pulse treatment, acute rejection episodes were fully reversed in 5 out of 7 animals (grade 0); in two animals, the signs were only partially reversed to grade 1. Overall, allografts in the ART group underwent 3 to 4 multiple episodes of acute rejection; the first episodes were reversible, then the therapy was not able to revert the rejection process anymore. Though, the animals reached grade 3 rejection with erythema, edema formation, epidermolysis and necrosis and were euthanized at POD 18±4.

3.2. Histopathological evaluation of the groups

In order to evaluate vasculopathology and the pathological score of inflammation, muscle and skin tissue samples from the graft were stained with hematoxylin & eosin and analyzed by histology (Fig. 3-2). The histopathological evaluation was done for the group 1, 2 and 4. The acute rejection group is currently under evaluation.

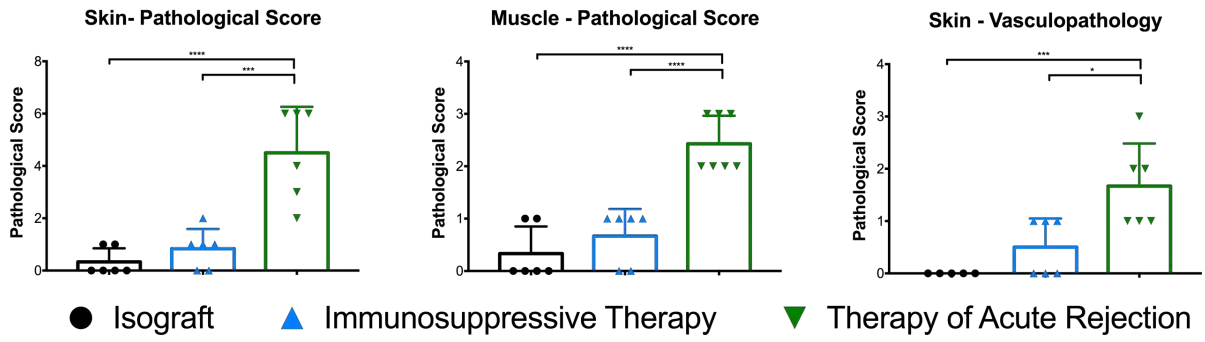

Figure 3-2: Histopathological evaluation of skin and muscle samples from Isograft, immunosuppressive therapy and acute rejection therapy group. Necrosis and cell infiltration was evaluated in skin and muscle. Vasculopathology (swelling and EC damage) was evaluated in skin. Data are presented as mean + SD. n=5-7 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test.

In the Isograft group, no animal showed any signs of skin vasculopathology (mean histopathological grade: 0±0, n=5). In contrast, the vasculopathological score was significantly higher in the ART group than in the IST and Isograft group (mean histopathological grade: 1.67±0.82 vs. 0.5±0.55 and 0±0, $p \leq 0.05$ and $p \leq 0.001$, n=5-6). Namely, swelling and EC damage was found in the skin of the acute rejection therapy group, but not in Isograft. A mild vasculopathy was observed in the immunosuppressive therapy, but did not reach statistical significance. Moreover, a significant increase of the pathological score in muscle was found in ART compared to IST and Isograft (mean histopathological grade: 2.43±0.53 vs. 0.67±0.52 and 0.33±0.52, $p \leq 0.0001$, n=6-7). Similarly to the muscle, the skin of the ART showed a significantly higher pathological score than in IST and Isograft (mean histopathological grade: 4.5±1.76 vs. 0.83±0.75 and 0.33±0.52, $p \leq 0.001$ and $p \leq 0.0001$, n=6).

Though, necrosis and mononuclear cell infiltration were observed both in skin and muscle of the acute rejection therapy but not in Isograft and immunosuppressive therapy, where the rejection process was avoided by daily tacrolimus administration.

3.3. Tissue characterization of the EC response

To determine, whether the endothelium in the different clinical settings is activated, preserved, damaged or reveal immunoglobulin deposition, we stained and analyzed muscle tissue from transplanted and contralateral site (Fig. 3-3).

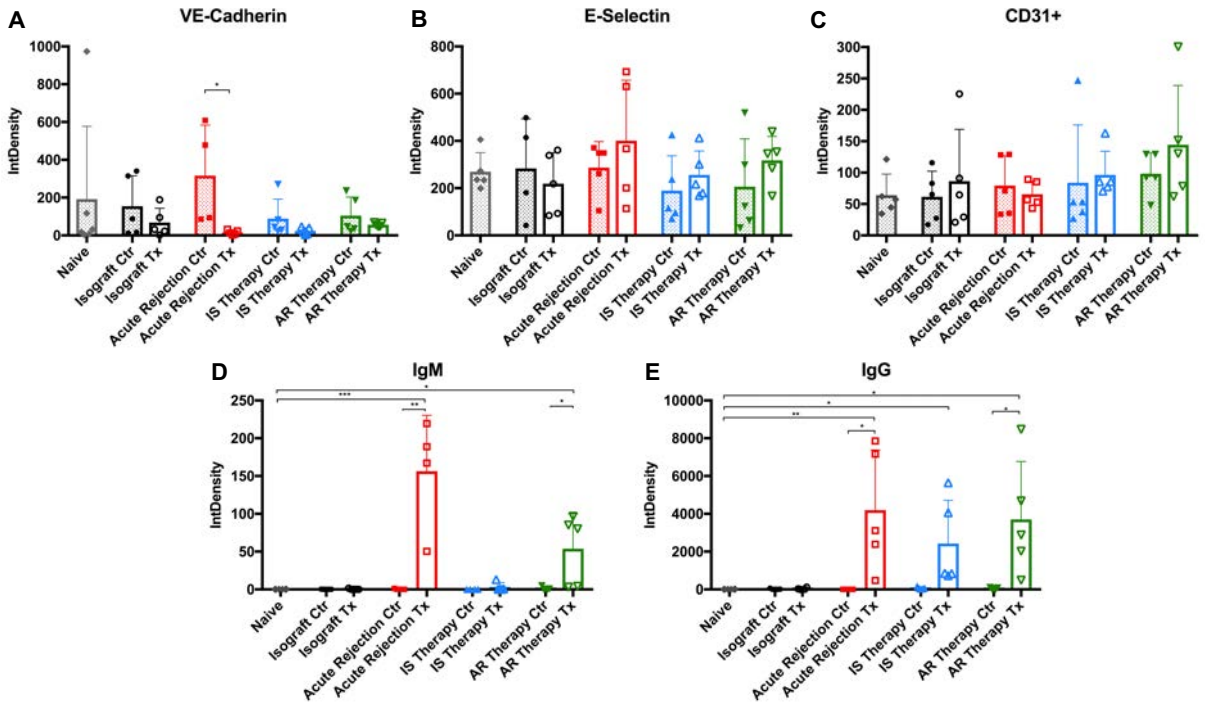

Figure 3-3: Tissue characterization of the endothelial cell response in transplanted (Tx), contralateral (Ctr) and naïve muscle tissue. Allografts and contralateral sites were immunostained for VE-Cadherin (A), E-Selectin (B), $CD31^+$ (C) and deposition of IgM and IgG (D and E). Quantitative analysis of fluorescence intensity (integrated density) was performed by ImageJ software. Data are presented as mean + SD. n=4-6 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by unpaired T test. IntDenisity, integrated density; IS Therapy, immunosuppressive therapy; ART, acute rejection therapy.

To asses EC preservation and damage, staining for VE-Cadherin and $CD31^+$ expression was carried out. VE-Cadherin expression was significantly decreased during acute rejection in the transplanted muscle, than in the contralateral muscle (AR Tx 15.52±12.13 vs. Ctr 316.6±267.3, $p \leq 0.05$). A similar VE-Cadherin expression pattern with a slight decrease of VE-Cadherin in the transplanted site compared to contralateral site was also visible in the Isograft, IST and ART group, but did not reach statistical significance (Fig. 3-3 A). All experimental groups showed similar $CD31^+$ expression in the contralateral hind limb when compared to naïve (healthy control) and no statistical differences in $CD31^+$ were detected between the experimental groups (Fig. 3-3 C).

To assess EC activation, we performed tissue staining for the adhesion molecule E-Selectin (Fig. 3-3 B). A slight increase of E-Selectin expression was observed in transplanted muscle tissue; E-Selectin expression was higher in the transplanted limb of acute rejection, ART and IST group, than in the contralateral hind limbs, but did not reach statistical significance.

To determine whether immunoglobulin deposition is present during acute rejection or immunosuppressive therapy, we examined expression of IgM and IgG in the experimental groups. IgM deposition was significantly higher both in transplanted limbs of acute rejection and acute rejection therapy group than in the contralateral limb (AR Tx 156.4±73.9 vs. Ctr 0.34±0.53, $p \leq 0.01$; ART Tx 53.77±46.23 vs. Ctr 1.08±2.04, $p \leq 0.05$). In addition, IgG deposition was found to be significantly increased in transplanted tissue of the acute rejection as well as of the ART group compared to contralateral (AR Tx 4197±3182 vs. Ctr 4.65±2.93, $p \leq 0.05$; ART Tx 3707±3063 vs. Ctr 41.52±42.64, $p \leq 0.05$). Interestingly, IgG deposition was significantly higher in the transplanted hind limb during immunosuppressive therapy, than in the naïve hind limb (IST Tx 2426±2289 vs. Naïve 3.16±4.88, $p \leq 0.05$). In the IST group (IST Tx), there was a significant increase in IgG but not in IgM deposition.

3.4. Plasma cytokine response

To determine, whether the endothelium expresses EC-related cytokines during acute rejection or immunosuppressive therapy, plasma cytokine expression was quantified using rat 13-plex immunoassay. Thus, plasma levels of IL-1α, IL-1β, IL-10, IL-17A, IL-6, IP-10, MIP-1α, RANTES, sICAM-1, sRankL, sVCAM-1, TNF-α, VEGF-A were measured in the experimental groups at different time points (Fig. 3-5). We focused our analysis on the fluorescence intensity due to some low abundant analytes (concentrations not shown). As suggested in literature; using fluorescence analysis instead of concentration based analysis allows the analysis of low abundant analytes (Breen et al. 2016).

Our analysis showed, that one week after transplantation, the cytokine levels of IL-1α, IL-1β, IL-10, IL-6, MIP-1α, IP-10, RANTES, sICAM-1, sRankL, sVCAM-1, TNF-α, VEGF-A were significantly elevated in the acute rejection group (POW1 vs. naïve), that did not receive any treatment. When the rats were treated daily with systemic immunosuppressive therapy, one week after transplantation, the cytokines IL-6, IL-17, IL-10, RANTES, sICAM-1 and VEGF-A were significantly elevated, whereas when the rats were treated with the acute rejection therapy (Tac + Dex) at onset of rejection signs, only IP-10 was significantly elevated at POW1 (ART 669.6±511.4% vs. naïve 233.3±131.5%, $p \leq 0.05$). One week after transplantation, the cytokine levels of IL-1β, IL-10, IL-6, TNF-α, MIP-1α, IP-10, RANTES, sICAM-1, sRankL, VEGF-A were significantly higher in Isograft than in healthy naïve.

Three weeks after transplantation, the cytokine levels of IL-6, IL-1β, TNF-α, MIP-1α, IP-10, RANTES, sRankL, sICAM-1 and sVCAM-1 were significantly elevated in the Isograft group (POW3 vs. naïve), whereas in the immunosuppressive therapy group only IL-1β, TNF-α, sRankL and sICAM-1 were significantly elevated at POW3 (Fig. 3-5).

During acute rejection, no cytokine was observed to be significantly elevated in the ART group, whereas the cytokine levels of IL-6, IL-10, IP-10, RANTES, sICAM-1 and VEGF-A were significantly increased in the acute rejection group (AR vs. naïve).

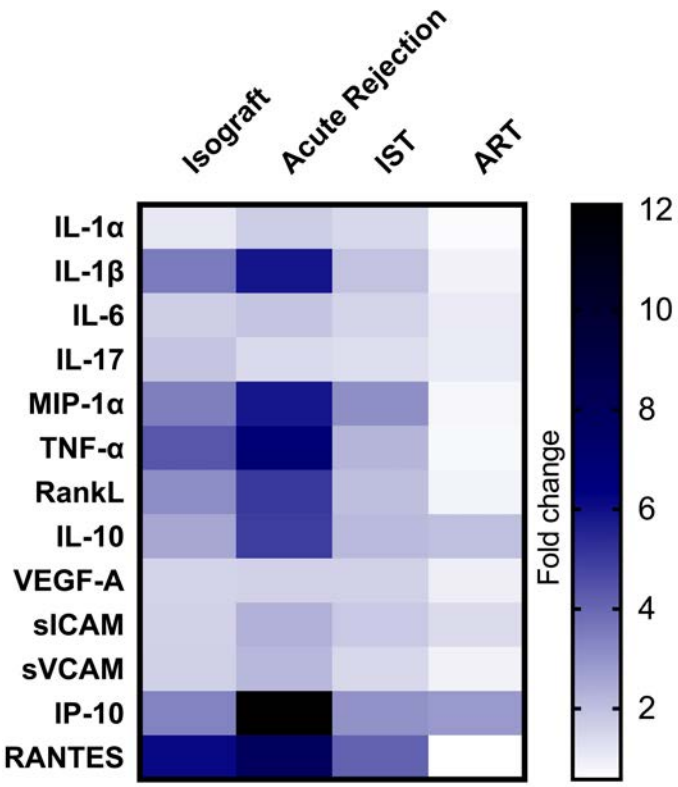

Figure 3-4: Heat map of 13 inflammatory and EC-related cytokine level at POW 1. Expression levels of IL-1α, IL-1β, IL-10, IL-17A, IL-6, IP-10, MIP-1α, RANTES, sICAM-1, sRankL, sVCAM-1, TNF-α, VEGF-A were quantified in the experimental groups at week 1 post-operation using rat 13-plex immunoassay. Data are presented as mean, fold change to naïve (pre-transplantation). n=6-10 per group. IST, immuno-suppressive therapy; ART, acute rejection therapy; POW; post-operative week.

Vascular cell adhesion molecule-1 (sVCAM-1) was significantly increased only in the Isograft (POW3) and in the acute rejection group (POW1) (2629±1067%; 3021±993.4% vs. naïve 1395±831.9%, $p \leq 0.05$ and $p \leq 0.001$). Interleukin-17 and Interleukin-1α expression was found to be elevated only in IST (POW1) or acute rejection (POW1), respectively. IL-1β, sRankL and TNF-α showed identical cytokine expression pattern; a significant increase in Isograft (POW1 and POW3), acute rejection (POW1) and IST (POW3). VEGF-A, a prominent EC-related proangiogenic cytokine, was significantly elevated during acute rejection, immunosuppressive therapy and Isograft but not during acute rejection therapy (POW1 vs. naïve) (Fig. 3-4). Furthermore, IL-6 and RANTES showed identical cytokine expression pattern; a significant enhancement in Isograft (POW1 and POW3), acute rejection (POW1 and Rejection) and IST (POW1). In contrast, the expression levels during acute rejection therapy resembled those of naïve and they were not seen to increase with time, except of IP-10. The acute rejection group was seen to express the highest cytokine level (except IL-17) among all groups (Fig. 3-4). Moreover, we observed that the expression of EC-related cytokines accompanied inflammation pattern during acute rejection.

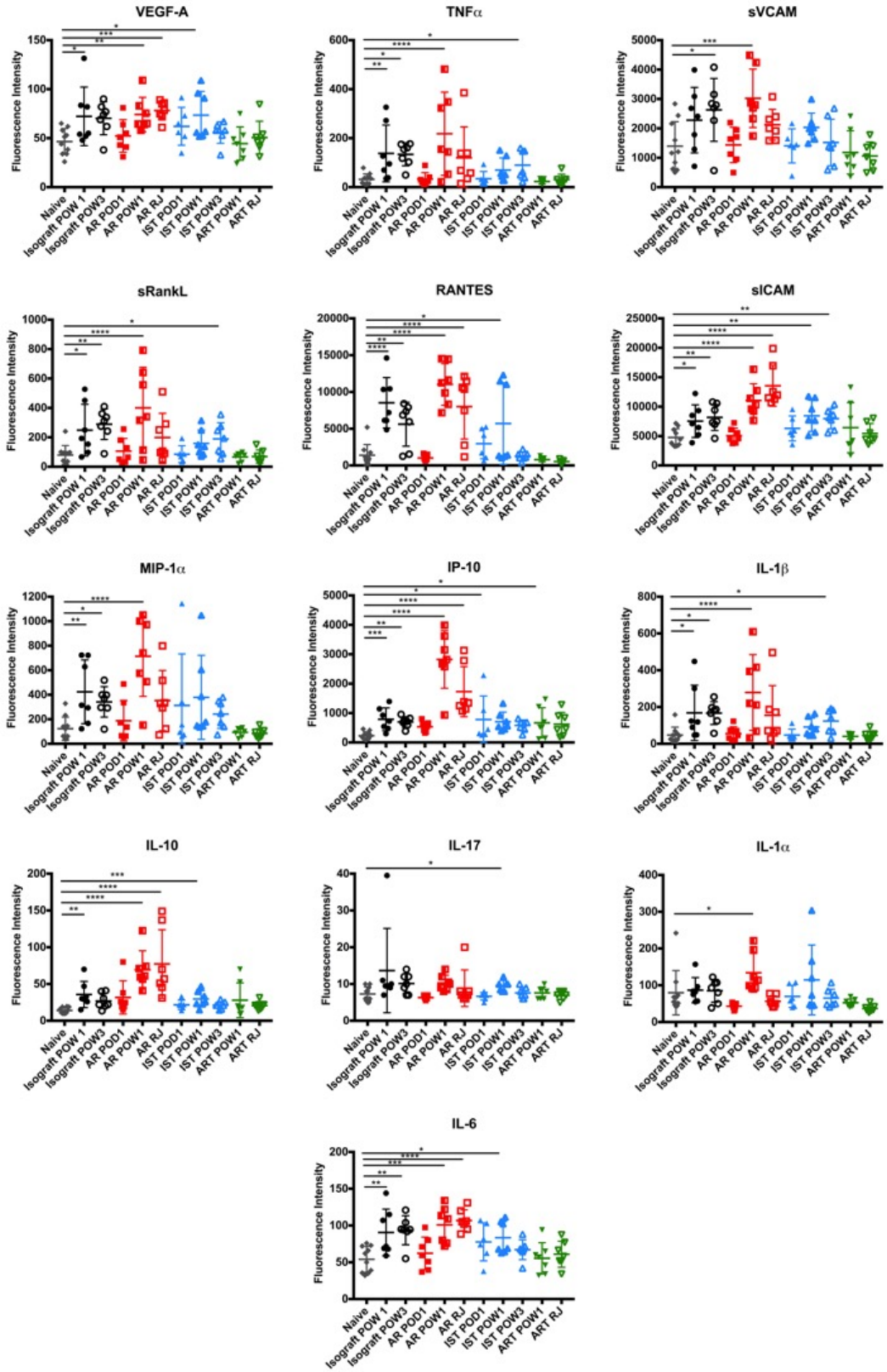

Figure 3-5: Plasma cytokine expression was quantified using rat 13-plex immunoassay. Levels of IL-1α, IL-1β, IL-10, IL-17A, IL-6, IP-10, MIP-1α, RANTES, sICAM-1, sRankL, sVCAM-1, TNF-α, VEGF-A were measured in the experimental groups at different time points. Data are presented as mean + SD. n=6-10 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Dunnett's multiple comparisons test. AR, acute rejection; IST, immuno-suppressive therapy; ART, acute rejection therapy; RJ, rejection; POD, post-operative day, POW; post-operative week.

3.5. Characterization of circulating endothelial cell response and hematopoietic progenitor cells

In order to characterize the response of donor and recipient endothelial cell to the different treatment, we analyzed the number of circulating endothelial cells with an either mature or progenitor phenotype by flow cytometry. Therefore, peripheral blood mononuclear cells (PBMC) were collected at the indicated time points and stained with the respective markers. Hematopoietic progenitor cells (HPC) were defined as $CD34^{+}$, circulating endothelial progenitor cells (EPC) were defined as $CD34^{+}KDR^{+}CD45^{low}$ and mature circulating endothelial cells (CEC) were defined as $CD31^{+}CD45^{-}$. Moreover, donor cells were identified as $RT1Ac^{+}$ cells. Even if we focused the analysis on frequencies, the same differences were seen in the absolute numbers of the IST and ART group (data not shown). Hence, an increase of the frequency is a real increase of cell numbers.

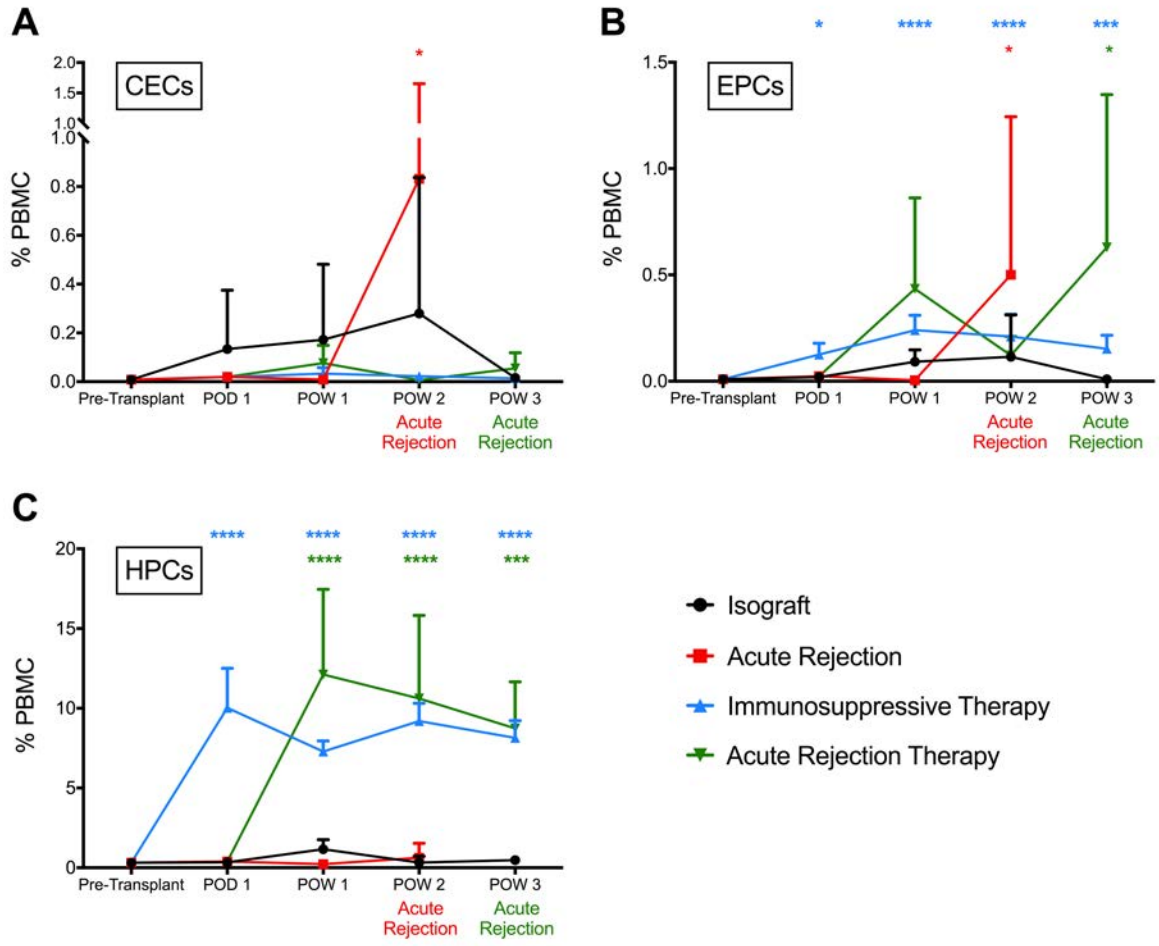

Figure 3-6: Frequency of circulating endothelial cells (A), endothelial progenitor cells (B) and hematopoietic progenitor cells (C) in the circulating blood of recipient rats. PBMCs from the respective experimental groups were collected at the indicated time points and analyzed using flow cytometry. Data are presented as mean + SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Dunnett's multiple comparisons test vs. pre-transplant values. CEC, circulating endothelial cells; EPC, endothelial progenitor cells; HPC, hematopoietic progenitor cells; PBMC, peripheral blood mononuclear cells; POD, post-operative day; POW, post-operative week.

3.5.1. Circulating endothelial cells are increased during acute rejection

Frequency of circulating endothelial cells (CEC) was significantly higher only during acute rejection, than pre-transplantation (AR 0.832±0.821% vs. pre-transplant 0.008±0.004%, $p \leq 0.05$). A slight increase in CEC was also visible in the Isograft group, but did not reach statistical significance (Fig. 3-6 A). Moreover, no considerable changes in CEC were observed during immunosuppressive therapy and acute rejection therapy.

The significant increase of CEC during acute rejection was of recipient origin (AR 0.019±0.022% donor-CEC among all CEC) (Fig. 3-7). In addition, donor-derived CEC frequency in the acute rejection group was significantly decreased during acute rejection compared to post-operative week 1 (AR 0.019±0.022% vs. POW1 8.886±7.286%, $p \leq 0.05$). In acute rejection therapy, the frequency of donor-CEC remained unchanged at ±15% over the experimental period, whereas in immuno-suppressive therapy, the frequency of donor-CEC first slightly increased up to 25.7% (at POW1) and then decreased to 6.6% (at POW3).

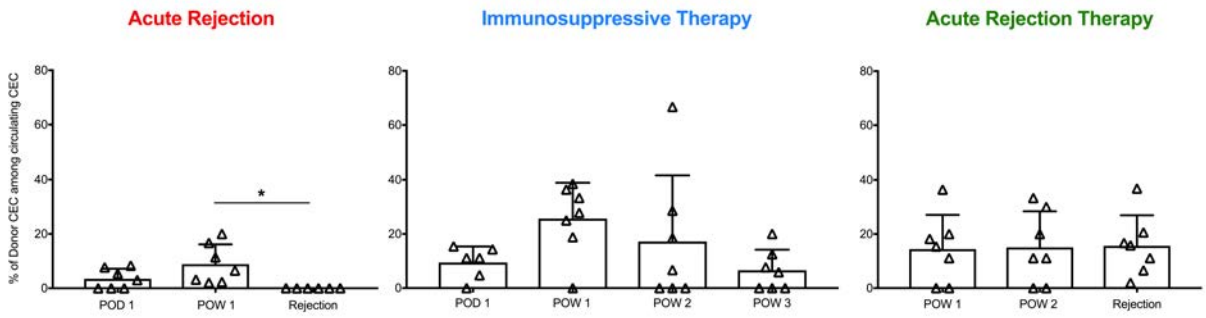

Figure 3-7: Analysis of donor specific circulating endothelial cells at different time points by flow cytometry. Donor specific CEC among all circulating CEC at day 1, week 1, 2 and 3 post operation. Data are presented as mean + SD. n=6-7 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. CEC, circulating endothelial cells; POD, post-operative day; POW, post-operative week.

3.5.2. EPC frequency is higher during acute rejection and immunosuppressive therapy

EPC frequency was significantly higher in acute rejection and immunosuppressive therapy, than pre-transplantation (AR 0.501±0.744%; ART 0.629±0.719%; IST 0.153±0.064% vs. pre-transplant 0.009±0.013%) (Fig. 3-6 B). We measured a continuous increase of EPC during immunosuppressive therapy, starting at post-operative day 1 till post-operative week 1, then at post-operative week 3, the EPC frequency decreased slightly (POD1 0.126±0.053%; POW1 0.240±0.070%; POW2 0.210±0.104%; POW3 0.153±0.064%). In addition, a mild increase of EPC was noted in the Isograft group, but did not reach statistical significance. A correlation-test between EPC frequency and VEGF-A cytokine level was performed, but no correlation could be established.

We detected both donor and recipient-derived EPC in the peripheral blood during acute rejection, immunosuppressive therapy and acute rejection therapy (Fig. 3-8). During immunosuppressive therapy, the frequency of donor-EPC remained unchanged at ±1% over the complete experimental period (POD1 1.52±1.50%; POW1 0.30±0.64%; POW2 0.25±0.44%; POW3 0.78±0.85%). The significant increase of EPC frequency during acute rejection was composed of only 0.116% donor-derived EPC (mean frequency: 0.116±0.187%), whereas the increase of EPC in the acute rejection therapy was composed of 0.9% donor-derived EPC (mean frequency: 0.907±1.266%). Thus, the majority of detected EPC derived from recipient.

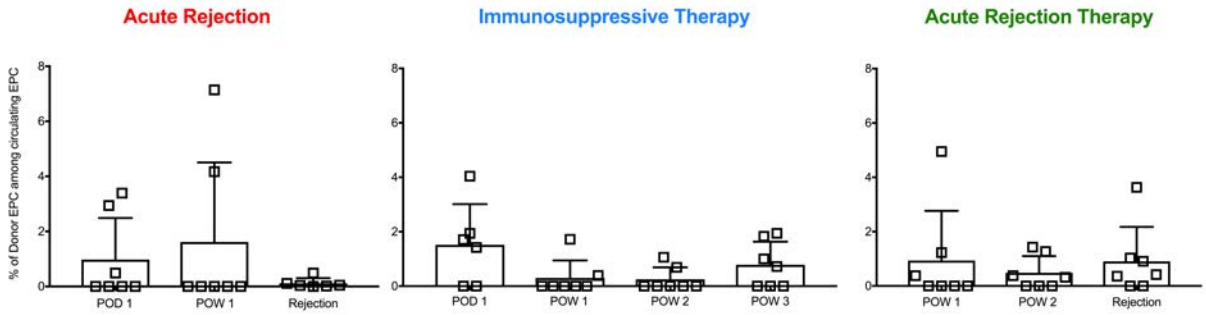

Figure 3-8: Analysis of donor specific endothelial progenitor cells at different time points by flow cytometry. Donor specific EPC among all circulating EPC at day 1, week 1, 2 and 3 post operation. Data are presented as mean + SD. n=6-7 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. EPC, endothelial progenitor cells; POD, post-operative day; POW, post-operative week.

3.5.3. Immunosuppressive therapy induces hematopoietic progenitor cells

Frequency of hematopoietic progenitor cells (HPC) was significantly higher during immuno-suppressive therapy and acute rejection therapy, than pre-transplantation (IST POW1 7.289±0.666; IST POW2 9.196±1.112; ART POW1 12.113±5.343; ART POW2 10.616±5.214 vs. pre-transplant 0.315±0.251, $p \leq 0.0001$). Interestingly, the acute rejection therapy group, which were treated with Tacrolimus + Dexamethasone, showed a delayed increase of HPC, compared to the immuno-suppressive therapy, which were treated only with Tacrolimus (Fig. 3-6 C). Additionally, the observed

significant increase in HPC in the IST and ART group remained increased at POD1 for the IST and at POW1 for the ART. Moreover, no considerable changes in HPC frequencies were observed in the Isograft and acute rejection group.

Donor hematopoietic progenitor cells were measurable in recipient blood (Fig. 3-9). Whereas the increase of HPC in the IST remained unchanged, the frequency of donor-derived HPC decreased significantly over the experimental period (POD1 6.22±2.60%; POW1 1.03±0.66%; POW2 2.71±1.34%; POW3 2.86±1.43%). Moreover, donor-derived HPC frequency in the ART group was significantly lower during acute rejection, than one week after transplantation (Rejection 1.009±0.682% vs. POW1 0.307±0.204%, $p \leq 0.05$).

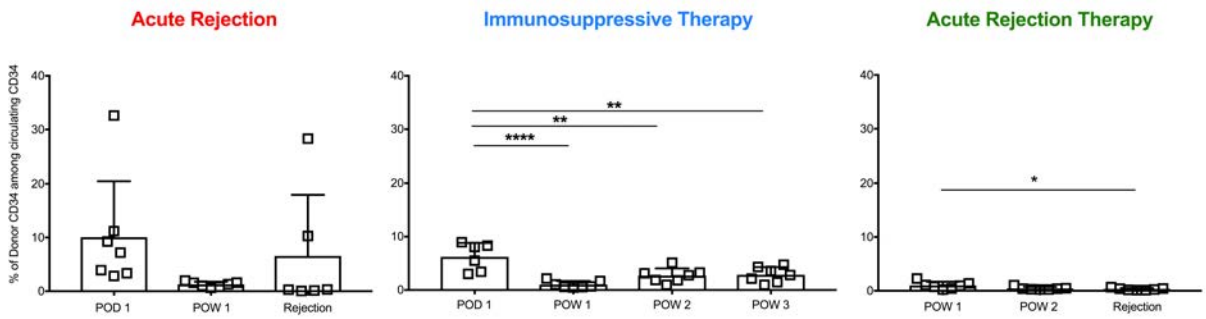

Figure 3-9: Analysis of donor specific hematopoietic progenitor cells at different time points by flow cytometry. Donor specific HPC among all circulating PBMC at different time points. Data are presented as mean + SD. n=6-7 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. HPC, hematopoietic progenitor cells; POD, post-operative day; POW, post-operative week.

4. DISCUSSION

This is the first study that directly evaluates the role of transplant vasculopathy and endothelial cell repair in a VCA model. The aim of the study was to understand, how the endothelium respond during acute rejection or immunosuppressive therapy in VCA.

We show that in VCA, similarly to SOT, endothelial cell damage is a main characteristic in acute rejection. This is paralleled by an increase of both pro-inflammatory and pro-angiogenic cytokines as well as by the release of CEC and EPC. Firstly, we observed a massive immunoglobulin deposition (IgG and IgM) and a significant decrease in VE-Cadherin. These results imply that the endothelial cells were affected and damaged during acute rejection. Secondly, as expected, the acute rejection group was seen to express the highest cytokine level among all experimental groups. We found increased expression levels of prominent pro-inflammatory cytokines (e.g. TNF-α, IL-1α, IL-1β, IL-6) during acute rejection. In the other hand, EC-related cytokines (e.g. VEGF-A, sVCAM-1, sICAM-1, IP-10, RANTES) were also found to be increased during acute rejection. These results provide evidence to suggest that EC-related cytokines accompanies inflammation pattern during acute rejection. To maintain endothelial homeostasis and vascular integrity, endothelial cell damage should be balanced by endothelial cell repair mechanisms. Thus, after endothelial cell lifting and detachment induced by prolonged EC activation or immunologic injury – what may occur during acute rejection – as a consequence, circulating EC can be detected in the peripheral blood (Woywodt et al. 2002). In response to inflammatory and angiogenic signals, EPC are mobilized into circulation to the damaged tissue (Asahara et al. 1997; Basile & Yoder 2014). Finally, we demonstrated that during acute rejection, CEC as well as EPC were significantly increased compare to pre-transplantation. These results suggest the co-existence of EC damage and reparative mechanism.

We show that the use of immunosuppressive therapy targeting T cell activation is able to mitigate EC damage and inflammatory response promoting EC repair through the release of EPC. Firstly, we observed a slight change in E-Selectin and VE-Cadherin but these differences did not reach statistical significance. In addition, a mild vasculopathy and a slight increase in the histopathological score of muscle and skin was found during immunosuppressive therapy. These results imply that EC damage is mitigated by the immunosuppressive therapy in comparison to no treatment (acute rejection). Interestingly, we observed an increase in IgG deposition but not in IgM. In the literature it is shown, that this could be due to B cell activation and humoral rejection (Colvin 2007; Mathieux et al. 2014). But we did not investigate further whether the antibodies were donor-specific or not. Secondly, evidence that the immunosuppressive therapy mitigates the inflammatory response is that one week after transplantation only the cytokine levels of IL-6, IL-17, IL-10, RANTES, sICAM-1 and VEGF-A were significantly elevated and that the cytokine expression was

less pronounced compared to acute rejection. Finally, no considerable changes in CEC were observed during immunosuppressive therapy whereas we found a significantly continuous increase of EPC over the experimental period compare to pre-transplantation. These results suggest that immunosuppressive therapy promotes higher EPC frequencies with minor EC damage.

We show that the use of a strong anti-inflammatory therapy to treat acute rejection episodes blocks the inflammatory response as well as the EC response but this do not prevent damage but only repair increasing vasculopathy and EC necrosis. Firstly, we observed a significantly high histopathological and vasculopathological score in the acute rejection therapy group. This is in line with data by Unadkat et al. 2010 that vasculopathy occurred after treatment of multiple acute rejection episodes. In addition, we found a massive immunoglobulin deposition (IgG and IgM) and a slight increase of E-Selectin (not significant) compared to healthy naïve. These results suggest that necrosis, swelling and EC damage is present in the ART group whereas endothelial cells are not highly activated. Secondly, in contrast to the other experimental groups, pro-inflammatory and EC-related cytokine levels during acute rejection therapy resembled those of naïve and they were not seen to increase with time (except of IP-10). Based on these results, we suggest that the strong immunosuppressive therapy blocks both the inflammatory and the EC response. Finally, no considerable changes in CEC were observed in the ART group, whereas a significant increase of EPC was observed compare to pre-transplantation. These observations are surprising and contrarily to what we observed in acute rejection and brings up the question whether we simply failed to detect CEC in the circulation or whether there is another explanation. We demonstrated that the endothelium, especially EC seemed not to be strongly activated in the ART group but from a histopathological point of view there was strong EC damage and vasculopathy. Moreover, we found massive immunoglobulin deposition that may trigger the complement system and initiate cell death. In the literature it is already reported that allospecific antibodies were elevated in a Brown Norway-to-Lewis rat transplantation (Unadkat et al. 2009; Gajanayake et al. 2014). Therefore, we think that there is a strong innate response leading subsequently to apoptosis and cell lysis. Moreover, EPCs in healthy individuals may be part of a homeostatic mechanism whereby they are attracted to sites of vascular injury and repair denuded parts, but slight imbalances in the process may cause dysfunction (Hill et al. 2003). Allograft rejection - as demonstrated in SOT - may represent pathological EPC repair in response to continuous and persistent damage to the endothelium, eventually leading to vasculopathy (Sathya et al. 2010). Meaning, that EPC may contribute to vasculopathy during acute rejection therapy.

It is widely accepted that tissue trauma and surgical interventions are able to mobilize EPC, depending on the extent of the intervention (Schillaci et al. 2009; Foresta et al. 2011; Condon et al. 2004; Laing et al. 2007). We observed a slight increase of CEC and EPC in the Isograft group, which did not reach statistical significance and a mild expression of pro-inflammatory and EC-related cytokines. That

leads to suggest, that there is physiological repair present in the Isograft group. These results go along with those of previous studies (Condon et al. 2004; Schillaci et al. 2009).

4.1. Donor vs. recipient EC response

The repair of transplanted vessels through an upregulation of endogenous repair processes in both donor and recipient may be crucial for maintaining a normal allograft. Evidence in heart transplantations suggests, that recipient-derived cells occupy allograft endothelium (Hillebrands et al. 2001; Simper et al. 2003). Hu et al. 2003 demonstrated in a murine model, that allograft cells were replaced with recipient-derived cells with ongoing allograft age. In addition, as in VCA bone marrow is part of the graft transplant, thus EPC responsible for repairing the endothelial layer could originate from the graft bone marrow itself or from the recipient. Indeed, we found that EPC from both donor and recipient may participate in vascular repair but with a minor contribution of donor derived EPC. To our knowledge their respective roles in VCA has not been examined.

4.2. Effect of immunosuppressive therapy on EC damage & repair

It is now widely accepted, that many of the immunosuppressive drugs used to prevent rejection can cause EC damage and dysfunction (Tepperman et al. 2010; Trapp & Weis 2005). One study showed, that patients with kidney transplants treated with cyclosporine A, a calcineurin inhibitor such as tacrolimus, had impaired NO production compared to patients treated with azathioprine and healthy controls (Morris et al. 2000). Additionally, cyclosporine A led to microvascular endothelial dysfunction in patients with heart transplantation but another study showed impaired endothelial wound repair by methylprednisone treatment but not by cyclosporine A (Fyfe et al. 1995).

Despite prevention of acute cellular rejection, tacrolimus also causes glomerular injury through induction of EC dysfunction by direct upregulation of the NADPH oxidase activity and ROS production following kidney transplantation (Kidokoro et al. 2012). Furthermore, the endothelium is the key regulator of microvascular thrombosis; tacrolimus treatment has been shown to enhance thrombus formation via a profound increase in the vasoconstrictor endothelin-1 (ET-1) production (Püschel et al. 2012). Moreover it was shown, that tacrolimus induces endothelial dysfunction through attenuation of Akt and ERK1/2 (Eguchi et al. 2013) and modulates TGF-β signaling to induce epithelial-mesenchymal transition in epithelial cells (Bennett et al. 2016).

We found significantly higher EPC during immunosuppressive therapy compare to pre-transplantation. Our results are in line with those of previous studies showing higher EPC numbers and function in patients suffering acute rejection after solid organ transplantation (Di Marco et al. 2011; Sathya et al. 2010). Di Marco et al. 2011 were demonstrating that kidney transplantation and its associated use of Tacrolimus increased the number of circulating EPCs. In contrast, Plischke et al.

2015 showed no favourable effect on EPC level after conversion to Tacrolimus in kidney transplantation. In addition, we demonstrated in our study that hematopoietic progenitor cells (HPC) were significantly higher during immunosuppressive therapy and acute rejection therapy, than pre-transplantation. Therefore, we think that Tacrolimus may directly induce EPC and / or HPC. This may explain the significant increase in EPC in the acute rejection therapy group in absent of EC activation and plasma cytokine expression. However, we could not finally determine whether the increase of EPC is due to a direct effect of Tacrolimus or due to an indirect effect through the control of inflammation. The exact role of Tacrolimus treatment on EPC and CEC counts is still controversial. Moreover, the precise definition of EPC was not consistent in these studies. Most of the studies used varying methods to identify EPC. Firstly, there is no specific unique marker for EPC at present. Thus, to evaluate the exact mechanism of EPC, an exact terminology is crucial. Secondly, techniques of EPC isolation are not standardized, preventing direct comparison between different studies. Overall, these studies still demonstrated that commonly used immunosuppressive drugs, such as Tacrolimus may clearly have an impact on EC dysfunction and repair.

We observed 3-4 acute rejection episodes in the acute rejection therapy group; the first episodes were reversible, then the pulse treatment with Tacrolimus and Dexamethasone was not able to revert the rejection process anymore. Though, the animals reached grade 3 rejection with erythema, edema formation, epidermolysis and necrosis. This is in contrast to the study of Unadkat et al. 2010. They treated the acute rejection episodes with Cyclosporin A (10 mg/kg/day i.p.) and Dexamethasone (2 mg/kg/day i.p.) at onset of signs; all of them were clinically reversible. However, we observed similar histopathological signs (vasculopathy, EC damage) in the acute rejection therapy group to those found in the study of Unadkat et al. 2010. We demonstrated in our study, that the acute rejection therapy shut off the inflammatory response as well as the EC response. We think – in consideration of our results – that the acute rejection therapy could be too strong. This leads to suggest to either reduce the dose of the acute rejection therapy or to replace Dexamethasone with a drug that blocks innate immunity.

4.3. Clinical application

VCAs have the advantage of direct observation of the graft but split rejections in VCA are demonstrating, that the skin can be rejected while the muscle and bone remain intact (Sinha & Pomahac 2013). This has implications on flap monitoring, as it leads to suggest, that skin biopsies alone may not be sufficient in graft monitoring. However, there are no assays available for graft rejection, similar to those used in solid organ transplantation for graft monitoring (e.g. creatinine levels in kidney transplants). Our study shows, that during acute rejection both circulating EC and EPC are increased and can be detected in the peripheral blood. Other studies performed in SOT support our findings, saying that the degree of injury and microvascular EC loss early times after transplantation can be predictive of long-term graft survival (Fine & Norman 2008; Steegh et al.

2011). Singh et al. 2012 already introduced CEC as a potential predictive marker for cardiac allograft vasculopathy and allograft survival. Despite all reported beneficial characteristics of EPC, Feng et al. 2009 showed in a murine model of transplant vasculopathy, that increased number of EPC and enhanced EPC function attenuated the progression of the disease. With this in mind, EPC and CEC may be used as clinical markers for allograft status independent of the macroscopic evaluation of the graft. Additionally, pro-inflammatory and EC-related cytokine level may also provide a tool for graft evaluation.

4.4. Further research

In order to observe signs of chronic rejection and its effect on EC damage and repair, it is essential to perform a longer follow up. There are studies showing that acute rejection episodes could be reverted (Unadkat et al. 2010). Further approaches, such as EPC isolation, characterization and administration may represent novel treatments for EC damage and vasculopathy after transplantation in the future. It has been already shown in a porcine lung transplantation model, that autologous EPC administration improved allograft survival and attenuated lung injury (Yen et al. 2016). Overall, the potential of EPC for further clinical applications remains promising. While a great deal remains to be understood.

4.5. Conclusion

In summary, our study show that endothelial cell damage is a main characteristic in acute rejection. This is paralleled by an increase of both pro-inflammatory and pro-angiogenic cytokines as well as by the release of CEC and EPC. This leads to suggest the co-existence of EC damage and reparative mechanism. In contrast, we provide evidence that the use of immunosuppressive therapy targeting T cell activation is able to mitigate EC damage and inflammatory response promoting EC repair through the release of EPC. Interestingly, both donor and recipient EPC seem to be involved in vascular repair, with a minor contribution of donor-derived EPC. These results revealed that the characterization of endothelial cell damage and repair mechanism during acute rejection or immunosuppressive therapy in VCA has important clinical implications to design new therapeutic interventions aimed to maintain vascular integrity and, therefore, VCA function.

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DECLARATION OF AUTHORSHIP

I herewith confirm that I wrote this thesis without external help and that I did not use any resources other than those indicated. I have clearly acknowledged all parts of the text where material from other sources has been used, either verbatim or paraphrased. I am aware that non-compliance with the above statement may lead to withdrawal of the academic title granted on the basis of this master's thesis by the Senate, according to the law governing the University of Bern.

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