HABILITATIONSSCHRIFT

AUS DER KLINIK FÜR HERZCHIRURGIE

DIREKTOR: PROF. DR. MED. CHRISTIAN HAGL

Lymphendothel in terminaler Herzinsuffizienz und nach Herz- und Lungentransplantation

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2017

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ABBREVIATIONS

BOS	bronchiolitis obliterans syndrome
CMV	cytomegalovirus
CsA	Cyclosporine A
DA	Dark Agouti
DAMP	danger associated molecular pattern
DC	dendritic cell
FEV1	forced expiratory pressure in one second
HAS	hyaluronic acid synthase
HMBG1	high-mobility box group 1
ICAM-1	intracellular adhesion molecule-1
IFN- γ	γ -interferon
IL	interleukin
IP-10	IFN-γ -inducible protein 10
IRI	ischemia-reperfusion injury
LEC	lymphatic endothelial cell
MHC	major histocompatibility complex
NF-κB	nuclear factor-κB
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGD	primary graft dysfunction
PRR	pattern recognition receptor
PROX-1	prospero homeobox protein 1
RECA-1	rat endothelial cell antigen 1
TGF-β	transforming growth factor β
TLR	Toll-like receptor
TNF- α	tumor necrosis factor α
TnT	troponin T
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WF	Wistar Furth

INTRODUCTION

Transplantation is a well-established and often the final therapeutic option for the patients with end-stage heart and lung failure. The various advances in immunosuppression and human leucocyte antigen matching techniques have significantly reduced the incidence of acute allograft rejection, whereas the chronic allograft rejection is still difficult to treat and causes the absolute majority of terminal graft failure. The lymphatic endothelium is important in the pathogenesis of various cardiac and pulmonary diseases. However, the exact knowledge about the functions and role of lymphatic endothelium in heart and lung failure as well as after transplantation is still lacking. Therefore, the study focused on the investigation of the changes in lymphatic endothelial phenotype in patients with terminal heart failure and after heart and lung transplantation. Further, the mechanisms of lymphatic endothelial activation in the ischemia-reperfusion injury were evaluated in experimental rat and mouse cardiac transplantation. Finally, novel clinically feasible strategies of lymphatic endothelial activation inhibition were tested in experimental models.

REVIEW OF THE LITERATURE

1 Clinical heart transplantation

Christian Barnard, assisted by Rodney Hewitson, was the first surgeon worldwide to successfully transplant a human heart on the 3rd December of 1967. The operation took place in Cape Town, at the Groote Schuur Hospital (Barnard 1967). Louis Washkansky, the first human recipient of a heart transplant, died 18 days after the transplantation on pneumonia. Nevertheless, the era of clinically feasible heart transplantation had begun, significantly promoted by Drs. Shumway and Lower (who previously built up the theoretical platform and developed the surgical techniques of heart transplantation in Stanford), as well as Drs. Demikhov, Ross, Cooley, Kantrowitz and other dedicated surgeons. Further improvement of operative techniques, postoperative care, and especially the wide introduction of immunosuppressant Cyclosporine A (CsA) (Borel et al. 1976) has made the procedure clinically relevant. Currently, heart transplantation is a valid therapeutic option for end-stage heart failure, and approximately 4000 procedures are reported annually (Stehlik et al. 2012).

1.1 Indications

Nowadays, non-ischemic cardiomyopathy is the leading diagnosis in adult patients undergoing heart transplant (54%), followed by ischemic cardiomyopathy (37%). The remaining indications for heart transplantation are congenital heart failure (3%), valvular heart disease (3%) or repeated transplantation (3%). The majority of transplanted patients is 40 to 60 years of age and recipients older than 60 years of age are being transplanted with increasing frequency, which results from a combination of changing demographics of the general population, as well as the willingness of clinicians to transplant higher risk patients (Stehlik et al. 2012).

1.2 Survival

Currently, the median survival for the entire cohort of adult and pediatric heart recipients who completed more than one year of follow-up is almost ten years. Patients who live past the first year post-transplant have a 63% likelihood of being alive ten years post-transplant and a 27% chance of being alive 20 years post-transplant. Further, the survival rates have continuously been improving over the last three decades. Most of this survival improvement is related to mortality reduction during the first post-transplant year. The patient survival stratified by different era is represented in **Figure 1**. It is likely that interventions resulting in a reduction of events leading to long-term mortality will be needed to achieve further improvements in survival after heart transplant (Stehlik et al. 2012).

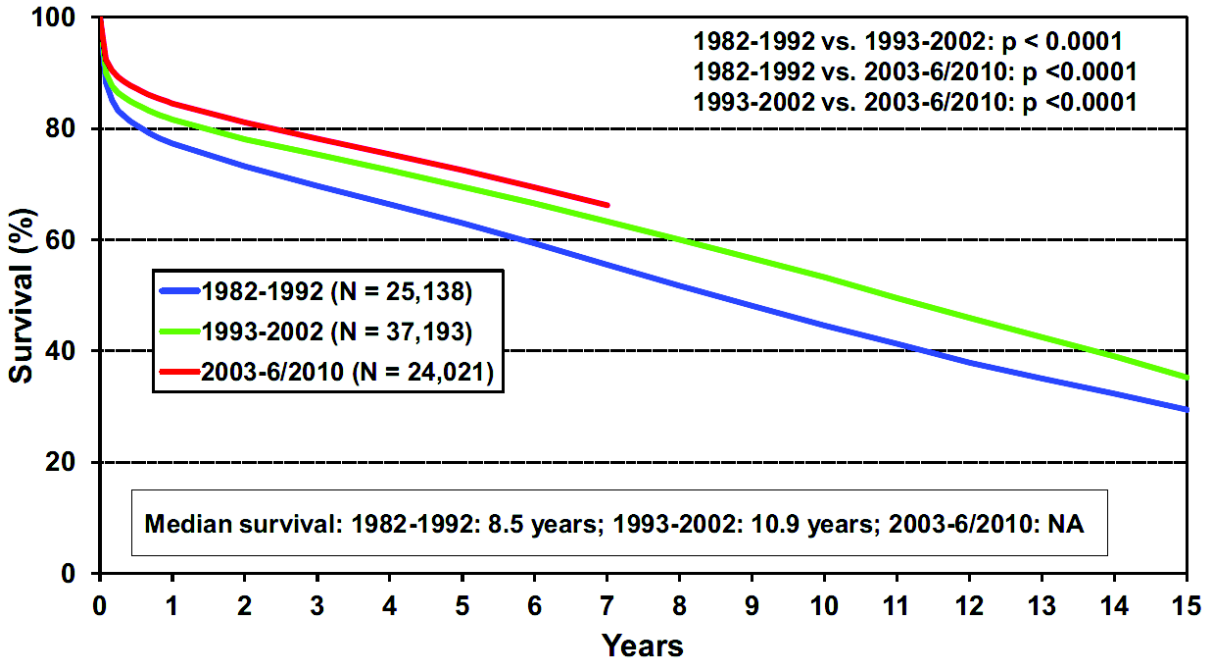

Figure 1. Kaplan-Meier survival depending on the era for adult heart transplantation. Data from the ISHLT Registry (Stehlik et al. 2012).

1.3 Complications and comorbidity

The leading causes of death after transplant are acute rejection, graft failure, cardiac allograft vasculopathy (CAV), infection, renal failure and malignancy. The relative contribution of these etiologies to post-transplant mortality changes with time elapsed since transplant (summarized in **Table 1)**. Graft failure and infection are currently the leading causes of death in patients dying during the first three years after heart transplantation. Beyond approximately three years after transplant, malignancy and cardiac allograft vasculopathy become the major contributors to mortality. Interestingly, acute rejection has been directly responsible for only a minority of deaths; however, it is likely that consequences of acute rejection, as well as chronic immune injury are responsible for deaths denoted as "graft failure" mortality (Stehlik et al. 2012).

Table 1. Contribution of death causes to mortality by time since heart transplantation

Cause of death	Time after heart transplantation				
	0-30 days	31 days – 1 year	>1-3 years	>3-5 years	>10 years
Acute rejection	4%	9%	12%	5%	1%
CAV	1%	3%	10%	10%	13%
Graft failure	34%	17%	27%	25%	16%
Renal failure	0%	1%	2%	4%	9%
Infections (Non-CMV)	13%	29%	14%	11%	11%
Malignancy	0%	3%	11%	20%	23%

2 Clinical Lung transplantation

James Hardy performed the first lung transplantation in a human in 1963 in University of Mississippi Medical Center (Hardy et al. 1963). It has needed a lot of effort to give the procedure a worldwide clinical acceptance as a valid therapeutic tool for end-stage lung failure. As well as in the case of heart transplantation, first the invention and clinical use of CsA allowed the wide and clinically relevant application of the procedure. So, over 3,5 thousand lung transplantations were performed in 2010. The constantly growing number of procedures was largely the result of growing numbers of bilateral lung transplants while the numbers of single-lung transplantations mainly remained on the same level (Christie et al. 2012).

2.1 Indications

Lung transplantation is a valid therapeutic tool for patients with severe end-stage lung failure. Following lung diseases are mainly underlying the end-stage lung failure: chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary arterial hypertension and idiopathic interstitial pneumonia (Arcasoy and Kotloff 1999). Most procedures performed between January 1995 and June 2011 were indicated in 34% for chronic obstructive pulmonary disease, in 23% for idiopathic pulmonary fibrosis, in 17% for cystic fibrosis and 6% for alpha-1-antitrypsin-deficiency emphysema. Bilateral transplantation accounted for the largest proportion of transplant procedures (74%) across all age groups and diagnoses in 2010 (Christie et al. 2012).

2.2 Survival

According to current reports (Christie et al. 2012), median overall survival is 5.5 years. The unadjusted survival rate at three months is currently 88%, at one year – 79%, at three years – 64%, at five years – 53% and at ten years – around 30% (**Figure 2**).

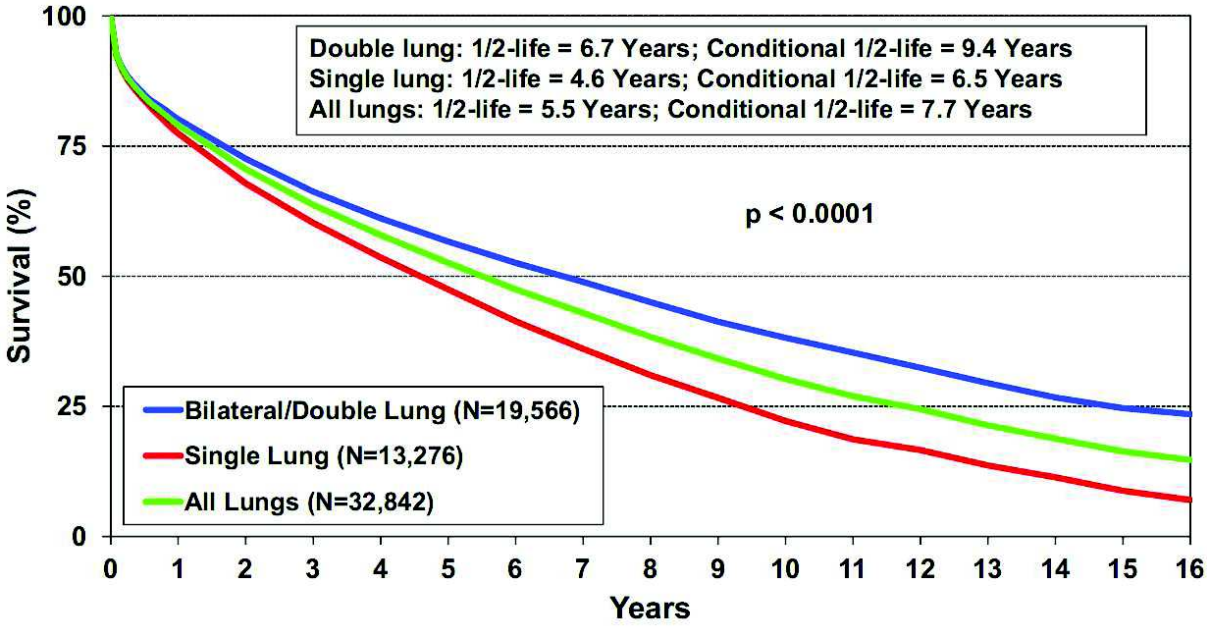

Figure 2. Kaplan-Meier survival depending on the type of procedure in adult lung transplants. Data from the ISHLT Registry (Christie et al. 2012).

2.3 Complications and comorbidity

Graft dysfunction and infections are the major causes of death within the first month and during the first year after lung transplantation. The most common causes of death after the first year were bronchiolitis obliterans and non-CMV infections. Late deaths were reported as "graft failure" and are generally difficult to classify. Most likely, the graft failure might be associated with graft rejection or BOS. Further, the rate of reported deaths due to malignant tumors permanently increases with the time after the transplantation (Christie et al. 2012).

The immunosuppressive medication is mainly causing the common comorbidities such as hypertension, renal dysfunction, diabetes, and hyperlipidaemia. The incidence of severe renal dysfunction among survivors is substantial and reaches a reported incidence of 14.4% at ten years.

BOS remains a common long-term complication. It is reported in every second recipient at five years after transplantation, increasing up to 76% at ten years. Further, malignancies are common after lung transplantation. At least one case of malignancy is reported in 14% of surviving recipients at five years and almost 30% at ten years. As seen in prior years, skin and other cancers become more common than lymphoma as the interval from transplantation increases (Christie et al. 2012).

3 Immunobiology of thoracic transplantation

The various advances in immunosuppression and MHC matching techniques have significantly reduced the incidence of acute allograft rejection, whereas the chronic allograft injury remains a serious problem and causes the absolute majority of terminal graft failure (Christie et al. 2012; Stehlik et al. 2012). Thus, it is important that the development of chronic injury can take its origin even before the transplantation as a consequence of donor brain death and allograft ischemia.

Furthermore, implantation and reperfusion of the allograft induces injury-related stress responses, which in turn initiate the alloimmune response (Wood and Goto 2012). In other words, local allograft damage and ischemia-reperfusion injury activate the non-specific innate immunity system. This marks the allograft as a site of damage and inflammation and leads to the activation of the more specific adaptive immunity, finally setting the scene for allograft rejection.

3.1 Innate immunity

Innate immunity is a rapid immunological response with limited specificity and no memory. Innate immunity includes cellular (neutrophils, macrophages, dendritic cells and natural killer cells) and molecular components (toll-like receptors, complement proteins, chemokines, cytokines and others) (Cristofaro and Opal 2006; Murphy et al. 2011; Trouw and Daha 2011). Local tissue damage and ischemia-reperfusion injury lead to secretion of potential damage-associated molecular patterns (DAMPS). Following DAMPs are relevant in transplantation: reactive oxygen species, heat shock proteins, heparin sulphate, high mobility group box-1 and fibrinogen. DAMPs can bind to pathogen-associated pattern recognition receptors (PRRs), which are expressed on the surface of inflammatory cells (Wood and Goto 2012). This binding reaction further induces the production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), interferons and chemokines (Carvalho-Gaspar et al. 2005; Lo et al. 2011). The following inflammation of the allograft modifies the vascular permeability and the endothelial cells viability. Further release of complement, acute phase proteins and graft antigens stimulate the exit of antigen-presenting cells (APCs) from the allograft towards recipient's secondary lymphoid organs (Larsen et al. 1989; van Kooten et al. 2011), initiating the consequent allorecognition and adaptive immune responses.

3.2 Adaptive Immunity

Adaptive immunity comprises allorecognition, T- and B-cell activation and differentiation and effector destruction mechanisms. Adaptive immunity is slow but has a high specificity and long-term memory.

3.2.1 Allorecognition and T-cell activation

The recognition of an alloantigen by T-cells (defined as allorecognition) is the beginning of the immune response to the allograft.

T-cell activation occurs upon binding to their specific MHC antigens, which are usually expressed on antigen-presenting cells. Appropriate costimulatory molecules on the surface of APC, such as CD40, CD80 or CD86, are required. (Sayegh and Turka 1998; Clarkson and Sayegh 2005).

Transplantation represents a very special immunological situation, where priming of recipient T cells can occur by three different pathways (Afzali et al. 2008): direct, indirect and semidirect allorecognition. The interaction of recipient´s T-cell receptor with allogeneic MHC complexes, which

are presented by donor APCs, is defined as direct allorecognition. The same process with the presentation of degraded donor MHC peptides by recipient APCs is defined as indirect allorecognition. And the uptake and presentation of donor antigens by recipient APCs is defined as semi-direct allorecognition.

In the case of MHC-mismatched transplantation, the direct allorecognition is mainly involved in the initiation of the adaptive immune responses. However, due to the limited number of donor APCs transferred from an allograft, the direct allorecognition is only relevant for the initial phase of allograft rejection. The indirect pathway, on the other hand, remains maintained for the whole lifetime of the transplanted organ (Wood and Goto 2012).

T-cell activation that is not accompanied by costimulatory signals can lead to further deletion of these T-cells. Alternatively, these T-cells can differentiate into protective regulatory cells (Tregs), which are capable of inhibiting cellular immune responses and are believed to be essential for prevention of autoimmune disease (Long and Buckner 2011; Heeger and Dinavahi 2012)

3.2.2 T-cell differentiation

Successful T-cell activation initiates a cascade of intracellular signaling pathways that lead to secretion of interleukin 2 (IL-2) and further T-cell proliferation and differentiation to effector cells.

One important subset of effector T-cells is the CD4+ T-cells with the helper function (Th). Several subgroups of Th cells exist and are referred to as Th1, Th2, Th17, Th9, and Tfh (follicular helper) populations.

Second important subset of effector T-cells is CD8+ T cells with cytotoxic activity (Tc). They are divided into Tc1 and Tc2 (Burrell and Bishop 2010; Wood and Goto 2012).

The activated effector T-cells acquire the ability to travel towards the sites of inflammation, directed by chemoattractant chemokines. Here, the specific target cells antigens can be recognized, which launces the effector mechanisms (release of proinflammatory cytokines and cytolytic killing). As a result, the antigen-expressing cells are destructed (Heeger and Dinavahi 2012).

3.2.3 B-cell Activation

B cells are multifunctional, as they mainly secrete antibodies but can also present antigens by expressing MHC and even secrete costimulatory molecules (Tarlinton et al. 2008). B cells can facilitate antigen presentation and so regulate the adaptive immunity (Carroll 2004). And serving as APC, B-cells are capable of communication with T-cells. Furthermore, B-cells require T-cell help for their activation and antibody production (Cyster 2010).

The importance of antibody-mediated rejection is increasingly becoming evident nowadays (Montgomery et al. 2011). Experimental B-cell deficiency or disturbed production of donor-specific antibodies are beneficial for cardiac allograft survival (Brändle et al. 1998; Wasowska et al. 2001). In

clinical setting, the diagnosis of antibody-mediated cardiac allograft rejection is defined as the allograft dysfunction combined with the histological evidence of C4d complement deposits on the capillary endothelium and macrophages in the capillary lumen in endomyocardial biopsies, together with the presence of donor-specific antibodies (Behr et al. 1999). Although very little is currently known about the influence of antibody-mediated rejection on the outcome after heart transplantation in adults, it seems to be significantly associated with reduced allograft survival after paediatric heart transplantation (Everitt et al. 2012).

3.2.4 Lymphatic endothelium linking innate and adaptive immunity

In the perspective of the current study, it is important to underline, that the development of alloimmune response after organ transplantation requires the migration of antigen presenting cells from allografts to secondary lymphoid organs (Larsen et al. 1990; Lakkis et al. 2000). Both, donor and recipient-derived APCs, travel towards secondary lymphoid organs via the afferent lymphatics of the allograft, which they use as an exit from the allograft. Thus, allograft lymphatics are specifically important in a setting of transplantation, linking the innate and adaptive immunity (**Figure 3)**.

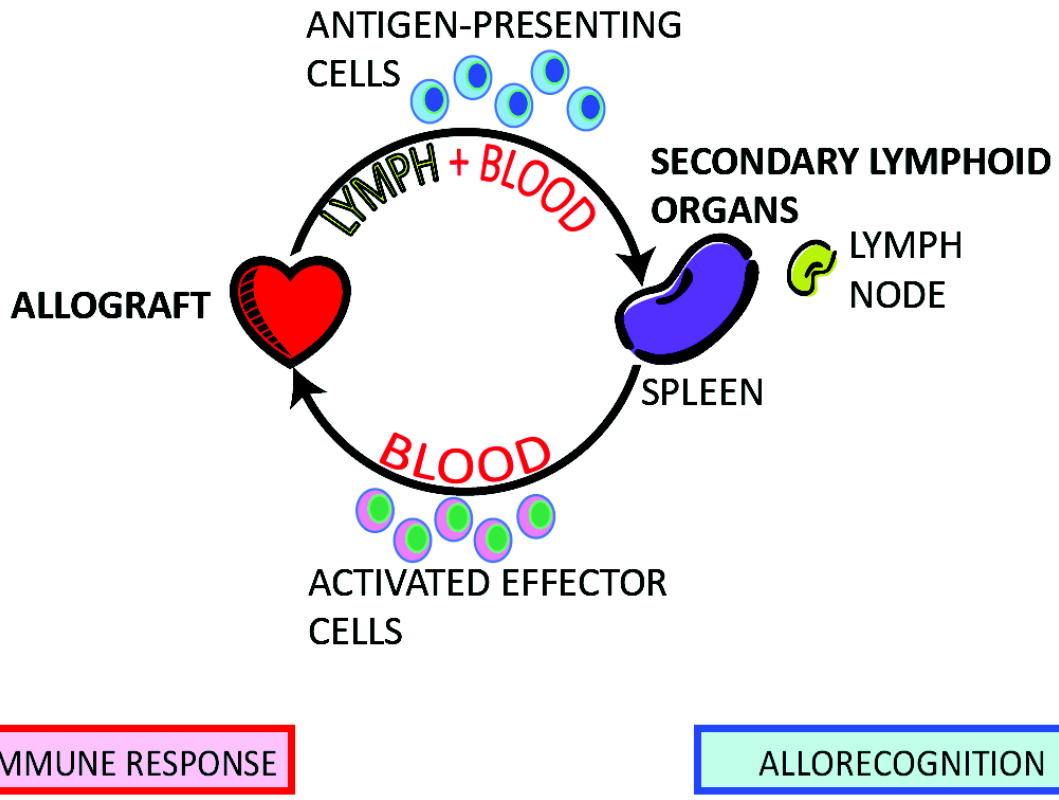

Figure 3. Schematic overview of the alloimmunity initiation in the setting of cardiac transplantation. The migration of antigen presenting cells from vascularized allografts to secondary lymphoid organs is the first step in the initiation of alloimmune response after organ transplantation. Antigen-presenting cells enter the afferent lymphatics of the allograft and use them as an exit from the transplanted graft. The allorecognition takes place in secondary lymphoid organs. The activated effector cells migrate to the allograft and lead here to alloimmune response. Figure authored by Alexey Dashkevich, copyright by John Wiley and Sons; reprinted with permission.

This phenomenon is very well described for the various inflammatory states. So, in contact hypersensitivity, inflamed lymphatic endothelium of the skin promotes the exit of leukocytes from tissue to afferent lymph through the newly induced expression of the adhesion molecules ICAM-1 and VCAM-1 (Johnson et al. 2006). Inflammation-induced lymphangiogenesis is considered to represent an endogenous anti-inflammatory mechanism aimed at limiting edema formation and accumulation of inflammatory cells (Huggenberger et al. 2010). For example, lymphatic activation is beneficial in case of acute skin inflammation (Huggenberger et al. 2011) and stimulation of lymphangiogenesis inhibits chronic skin inflammation (Huggenberger et al. 2010). Similarly, promotion of lymphangiogenesis benefits the treatment of asthma and other inflammatory airway diseases (Baluk et al. 2005). However, the functions and biology of lymphatic endothelium in the setting of terminal heart and lung failure and after transplantation are barely known. Thus, it was the main focus of this study.

4 VEGF family

4.1 Overview of the VEGF family members

VEGF-A was discovered as a vascular permeability inducing tumour-secreted factor and was first named vascular permeability factor (Senger et al. 1983). Currently, five ligands belonging to the VEGF family are known: placental growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D (Jeltsch et al. 1997; Carmeliet and Jain 2011; A. Alitalo and Detmar 2011 Dec 19; Koch and Claesson-Welsh 2012). Following VEGF receptors are known nowadays: VEGFR-1, VEGFR-2, VEGFR-3 and neuropilins (NRP-1 and NRP-2). The general properties of VEGF ligands and the principal effects of VEGF receptors are combined in **Figure 4**. Simplified, the preferential expression of VEGFR-1 and -2 is restricted to blood vascular endothelial cells (EC) and of VEGFR-3 – to lymphatic EC.

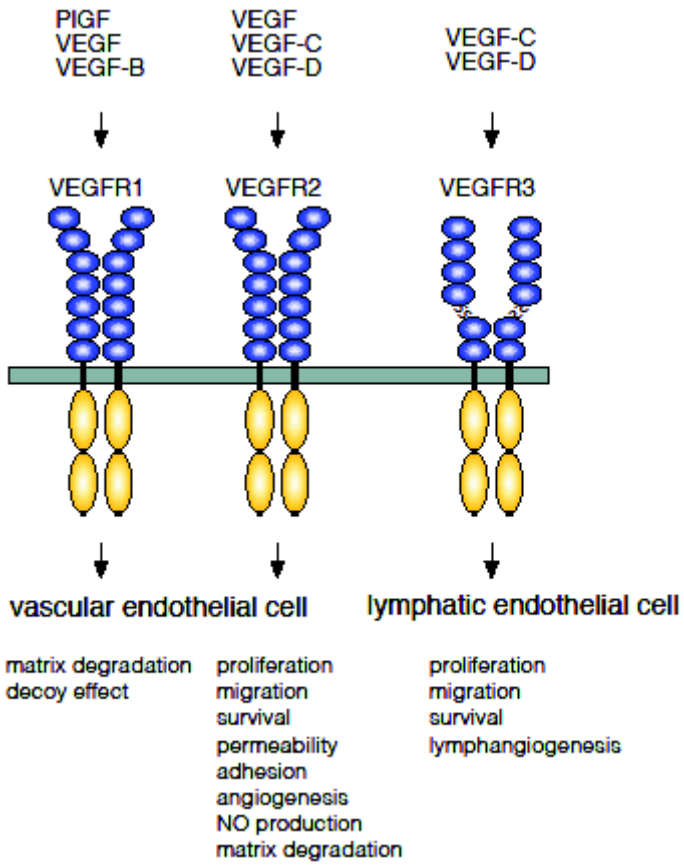

Figure 4. VEGF ligands and receptors. VEGF ligands are binding to specific VEGF receptors. VEGFR-1 is expressed on endothelial cells and attracts monocytes and smooth muscle cells. VEGFR-2 is highly relevant for mitogenic effects on vascular endothelial cells. VEGFR-3 mainly controls the development and functionality of lymphatic endothelial cells and migration of antigen-presenting cells. PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. Figure authored by Alexey Dashkevich, copyright by John Wiley and Sons; reprinted with permission.

4.1.1 Angiogenic VEGF ligands and receptors

The core receptors of VEGF-A are VEGFR-1, VEGFR-2, NRP-1 and NRP-2. Vascular and inflammatory cells in a variety of adult tissues are a rich source of VEGF-A. Its secretion is induced by hypoxia, inflammation and different growth factors (Tomizawa et al. 1984; Ferrara et al. 1990; Berse et al. 1992; Freeman et al. 1995; McCourt et al. 1999; Melter et al. 2000). VEGF-A signalling leads to angiogenesis, which is a result of endothelial cell migration, proliferation and sprouting. The angiogenic function of VEGF-A is pivotal for vascular development during embryogenesis (Carmeliet et al. 1996; Ferrara et al. 1996).

Several directions for clinical use of VEGF-related therapeutic targets are of special interest. For instance, anti-VEGF targeting for the treatment of malignancies and eye disease have been suggested (Carmeliet and Jain 2011). Although this filed appears to be promising, the clinical use could not be established because of the lacking proof of evidence (Giacca and Zacchigna 2012).

The core receptors of VEGF-B are VEGFR-1 and NRP-1. The main expression sites of VEGF-B are skeletal muscles and myocardium (Olofsson et al. 1996; Carmeliet and Jain 2011; Koch 2012). VEGF-B

is not critical for vascular development, as lack of VEGF-B doesn´t result in vascular malformation, but can lead to conducting defects and cardiac size reduction. (Bellomo et al. 2000; Aase et al. 2001; A. Alitalo and Detmar 2011 Dec 19). Importantly, VEGF-B is an important player in cardiac arteriogenesis (Bry 2010; Koch and Claesson-Welsh 2012) and hypertrophy (Kärpänen et al. 2008; Koch and Claesson-Welsh 2012).

The core receptors of Placental growth factor (PlGF) are VEGFR-1 and NRP-1 (Maglione et al. 1991; Koch 2012). PlGF itself plays no crucial role in vascular formation during the embryogenesis (Gigante et al. 2003; Carmeliet and Jain 2011) but it is an important costimulator of VEGF effects (Park et al. 1994; Carmeliet et al. 2001). Under certain circumstances, PlGF can promote cardiac hypertrophy (Accornero et al. 2011).

VEGFR-1 (alternatively defined as fms-like tyrosine kinase, Flt-1) is a predominant receptor for VEGF-A, VEGF-B and PlGF (Carmeliet and Jain 2011; Koch 2012). Two forms of VEFGR-1 exist: the membrane-based VEGFR-1 and a soluble form (sVEGFR-1) (Kendall and Thomas 1993; Ambati et al. 2006; Giacca and Zacchigna 2012). Multiple cell types express VEGFR-1: endothelial cells, smooth muscle cells, monocytes, macrophages, and haematopoietic stem cells (Maglione et al. 1991; Couper et al. 1997; Gigante et al. 2003; Carmeliet and Jain 2011; Koch 2012). Depending on the site of VEGFR-1 expression, it can regulate angio- and arteriogenesis, myelomonocyte cell recruitment or even lipid metabolism (Luttun et al. 2002; Pipp et al. 2003; Kärpänen et al. 2008; Hagberg et al. 2010; Hagberg et al. 2012 Sep 25). Deletion of VEGFR-1 leads to global vascular malformations and embryonic death (Fong et al. 1995; Fong et al. 1999). Important for the interplay of the receptors, VEGFR-1 has a high binding capacity to VEGF-A but a very low activity of tyrosine kinase, thus functioning as a negative regulator of VEGFR-2 in endothelial cells (Hiratsuka et al. 1998; Accornero et al. 2011).

VEGFR-2 (alternatively defined as kinase-insert domain receptor, KDR or Flk-1) has a predominant binding affinity to VEGF-A, and to a lesser extent to VEGF-C and VEGF-D. Embryonic haematopoiesis and vasculogenesis are highly dependent on VEGFR-2 expression, whereas it is usually silent in healthy adults (Kaipainen et al. 1993; Shalaby et al. 1995). Vascular endothelial cells are the main expression site of VEGFR-2, which is thus crucial for the VEGF-A-related endothelial effects (Carmeliet and Jain 2011; Koch and Claesson-Welsh 2012). The sites of active angiogenesis during wound healing, myocardial infarction or malignancies are actively expressing VEGFR-2 (Shibuya 1994; Li et al. 1996).

The simplified schematic overview suggests straight differentiation of VEGF ligands and receptors into either mainly angiogenic or mainly lymphangiogenic. However, various cross-reactions are possible (Nagy et al. 2002).

4.1.2 Lymphangiogenic VEGF ligands and receptors

VEGF-C is the main ligand involved in the development of lymphatic vascular system. VEGF-C demonstrates the highest binding affinity to its core receptor VEGFR-3, although also being affine to VEGFR-2 (Jeltsch et al. 1997; Oh et al. 1997; K. Alitalo 2011; Kim et al. 2012). Accordingly, the predominant lymphatic effects of VEGF-C can be combined with angiogenic actions determined by VEGFR-2 binding (Saaristo et al. 2002; Tammela et al. 2011; Benedito et al. 2012 Mar 18). The VEGF-C precursor protein requires proteolytic processing to transform to the active ligand form (Joukov et al. 1996; Kukk et al. 1996). Proinflammatory cytokines TNF-α, IL-1 α and IL-β induce the VEGF-C gene expression (Ristimäki et al. 1998). Further, PlGF, epidermal growth factor and transforming growth factor-b have the ability to induce the VEGF-C secretion (Enholm et al. 1997). Importantly, inflammatory cells, antigen presenting cells and some subsets of T-lymphocytes are a rich source of VEGF-C (Schoppmann et al. 2002; Hamrah et al. 2003; Cursiefen et al. 2004; Baluk et al. 2005; Nykanen et al. 2010).

VEGF-C is crucial for the development during the embryogenesis, thus, loss of VEGF-C activity leads to embryonic death (Karkkainen et al. 2004). Therapeutic inhibition of VEGF-C-VEGFR-3 axis leads to suppression and degradation of existing lymphatics and ends in lymphedema (Makinen et al. 2001). The VEGF-C overexpression, on the other hand, leads to lymphatic hyperplasia (Jeltsch et al. 1997; Oh et al. 1997). Due to the crucial role of the lymphatic vascular system in the processes of inflammation, VEGF-C is induced in many malignancies and is very likely to play a role in lymphatic tumor metastasis (Mandriota et al. 2001; Skobe, Hawighorst, et al. 2001).

VEGF-C is not only directly involved in the process of lymphangiogenesis, but also affects the phenotype of the lymphatic endothelial cell depending on the physiological or pathological condition. So, VEGF-C can turn lymphatics more attractive for tumor cells or antigen-presenting cells by secreting a chemokine CCL-21 (Issa et al. 2008; Nykanen et al. 2010). And as the core receptor of VEGF-C, VEGFR-3, can be expressed on the macrophages and dendritic cells, direct cellular effects of VEGF-C are described (Chen et al. 2004; Hajrasouliha et al. 2012). The divertive stimulating affinity of VEGF-C to both, lymphatic endothelial cells and inflammatory cells, that travel towards lymphatics, explains the important function of VEGF-C-VEGFR-3 interaction in the regulation of various immune response reactions (Skobe et al. 2001; Chen et al. 2004; Nykanen et al. 2010; Hajrasouliha et al. 2012). Thus, several processes can be orchestrated by lymphangiogenic factors: transportation of antigen-presenting cells, the initiation of immune responses, induced lymphatic drainage of the inflamed sites and the resolution of inflammation (Huggenberger et al. 2010; Huggenberger et al. 2011; Zhou et al. 2011).

The second known lymphangiogenic ligand is VEGF-D, which also binds to VEGFR-2 and VEGFR-3 (Baldwin et al. 2001). Importantly, VEGF-D is not crucial for the initial lymphangiogenesis (Baldwin et

al. 2005). VEGF-D repeats in main functions and physiological roles the key roles of VEGF-C (Stacker et al. 2001; Byzova et al. 2002; Rissanen et al. 2003).

VEGFR-3 is the main lymphangiogenic receptor with the predominant binding affinity to its primary ligands VEGF-C and VEGF-D (Jeltsch et al. 1997; Karkkainen et al. 2004). VEGFR-3 is a selective director of the lymphatic growth and lymphatic vessel maintenance, mainly being expressed on the lymphatic endothelial cells (Makinen et al. 2001). However, VEGFR-3 is also expressed in various inflammatory cells, mainly described for macrophages and dendritic cells (Hamrah et al. 2003; Chen et al. 2004; Maruyama et al. 2005). The angiogenic effects of VEGFR-3 have also been well described and can be explained by VEGFR-3 expression in blood endothelial cells (Tammela et al. 2011; Benedito et al. 2012 Mar 18). Also, VEFG-C binding to VEGFR-3 positive macrophages can lead to VEGF-A upregulation with consequent angiogenic cascade development (Chung et al. 2009).

4.2 Lymphatic specific cardiac VEGF pathways

4.2.1 in embryogenesis and in healthy adult heart

The development of the vascular system and the heart, in particular is a complex process of coordinated signalling pathways, where the vascular endothelial growth factors are central. The lymphatic system is taking its origin from the venous endothelial cells in the early embryogenesis, which transform to lymphatic endothelial cells upon binding with VEGF-C (Wigle and Oliver 1999; Karkkainen et al. 2004). These very initial LECs already express the whole spectrum of classical lymphatic receptors, such as PROX-1, VEGFR-3 and Podoplanin. Exposed to further VEGF-C signals, the processes of sprouting and migration of LECs result in the creation of lymphatic sacs (Karkkainen et al. 2004). The final formation of lymphatic capillaries requires the involvement of Notch signaling pathways, the VEGF-C co-receptor NRP-2 and ligand ephrin B2 (Mäkinen et al. 2005; Xu et al. 2010; Niessen et al. 2011).

Lymphatic specific receptor VEGFR-3 is inevitable during the early vascular development and becomes restricted to the regulation of lymphatic endothelial biology during the first 12 weeks (Dumont et al. 1998; Partanen et al. 1999). During the whole period of gestation, the lymphatic capillaries can only be detected in the human fetal epicardium and besides VEGFR-3 positivity, these vessels demonstrate strong VEGFR-2 expression (Partanen et al. 1999).

Even less is currently known about the phenotype of adult cardiac lymphatics. The available reports on cardiac lymphatics in adult rats describe the predominant distribution of VEGFR-3 expressing capillaries mainly in the epicardium, whereas the myocardial area has fewer lymphatics (Nykanen et al. 2010).

4.2.2 in heart failure

The lymphatic endothelial biology in patients with terminal heart failure has never been studied

sufficiently, although the details of endothelial changes in myocardial ischemia and infarction have been very well highlighted. However, the impact of the lymphatic system in cardiac failure cannot be exaggerated, as the microvascular environment, hemodynamical conditions, fluid and metabolic balance are roughly impaired. Our current knowledge is only based on the spare reports of lymphatic signaling involvement in ischemic and dilative cardyomyopathy. For instance, VEGF-C encoding is severely induced in patients with any aetiology of terminal heart failure (Aharinejad et al. 2001). Further, various vascular endothelial growth factors have a differential expression pattern in different forms of heart failure (Dashkevich et al. 2010).

Obviously, further evidence of the lymphatic role in heart failure is required to elaborate on potential therapeutic targets in this field.

4.2.3 in heart transplantation (allorecognition and rejection)

The development of alloimmune response after cardiac transplantation begins with the migration of antigen presenting cells from cardiac allograft to secondary lymphoid organs (Larsen et al. 1990; Lakkis et al. 2000). The afferent lymphatics provide the initial route for the APCs ´travel. Thus, the allograft lymphatics are important in the initiation of allorecognition.

Recently, a detailed description of cardiac lymphatic changes in myocardial rejection has been provided (Nykanen et al. 2010). Interestingly, experimental acute and chronic rejection episodes lead to controversial changes of lymphatic vessel density, which significantly drops in acute rejection and is doubled in chronic rejection. These changes of lymphatic density seem to represent their secondary reaction on various inflammatory processes associated with allograft rejection. Importantly, the role of cardiac lymphatics in the setting of transplantation is much wider, as they seem to be crucial in the initiation of the alloimmune cascade.

So, experimental therapeutic VEGFR-3 blocking in cardiac allograft recipients has reduced allograft inflammation and the development of chronic allograft vasculopathy (Nykanen et al. 2010).

The early involvement of lymphatic endothelial cells and lymphangiogenesis in the initial processes of allograft rejection seemed of high relevance to me and have therefore been chosen as the central focus of the experimental part of the presented work.

The members of the VEGF family seem to be intimately involved in the normal healthy development of cardiac vascular and lymphatic systems. They further represent important functional units in the pathogenesis of various cardiac diseases. As many of them are beyond the scope of this review, a short overview of lymphatic specific VEGF expression and signaling in cardiac disease are presented in **Figure 5.**

It was the principle aim of the performed work to broaden the understanding of lymphatic signaling mechanisms in failing hearts and after heart transplantation and to possibly highlight potential therapeutic approaches.

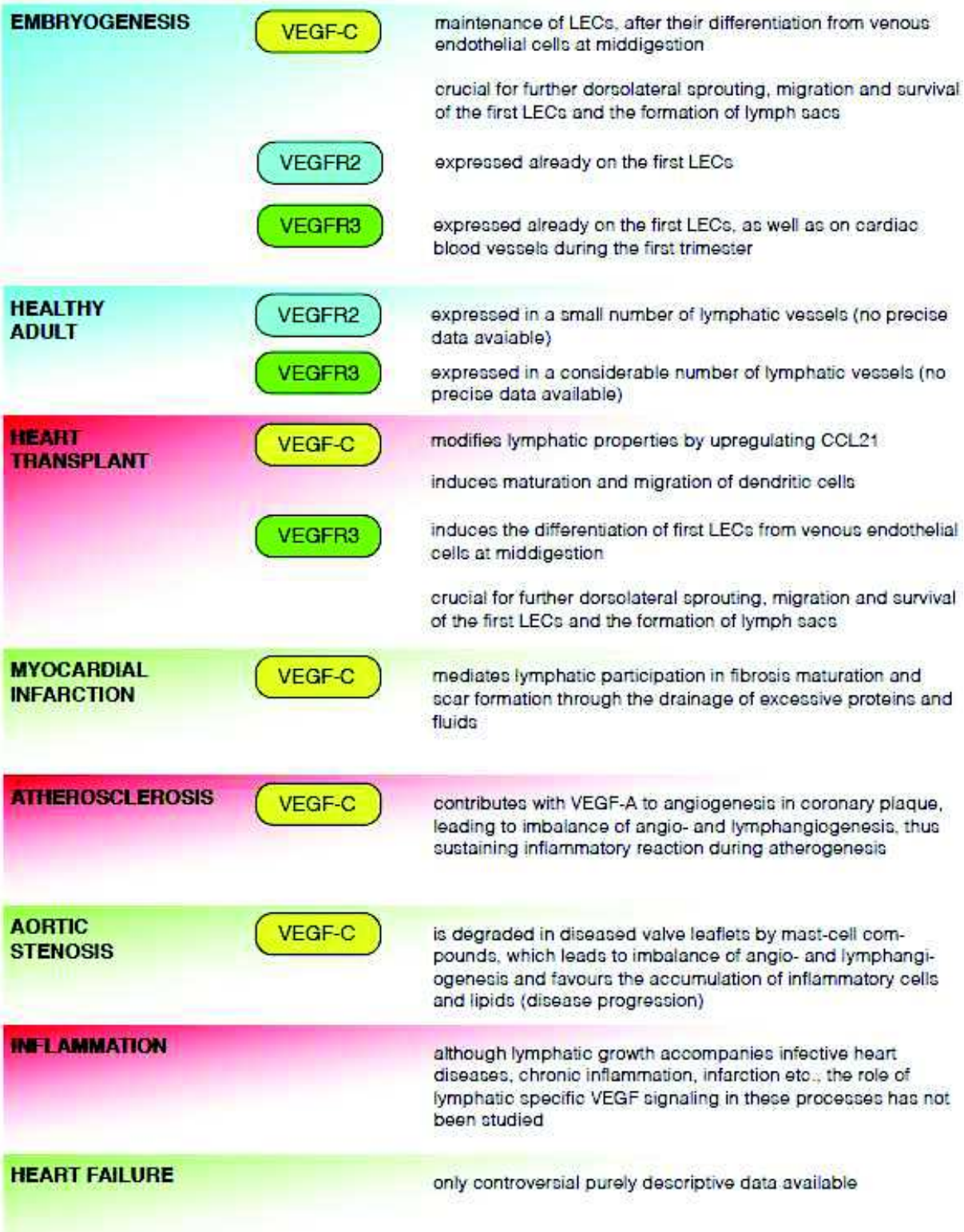

Figure 5. Brief overview of cardiac lymphatic VEGF expression and signaling in healthy and diseased heart. Figure authored by Alexey Dashkevich, copyright by John Wiley and Sons; reprinted with permission.

4.3 Lymphatic specific VEGF pathways in the lung

4.3.1 in embryogenesis and in healthy adult lungs

During the pulmonary growth, vascular endothelial growth factors are actively participating in vasculo- and lymphangiogenesis. VEGF-C and its receptor VEGFR-3 are in particular also expressed here during embryogenesis (Kukk 1996). In mouse lungs, VEGF-D cannot be found during the early phase but is later on expressed in the mesenchymal tissue of the lungs, disappearing again after the birth (Greenberg et al. 2002).

The classical angiogenic growth factor VEGF-A has also been shown to overtake a certain role in lymphangiogenesis of developing lungs, as its overstimulation leads to increased lymphatic density in a foetus. This lymphangiogenic switch of VEGF-A might be explained by it cross-reaction with VEGFR-3 (Mallory et al. 2006). Further, VEGF-A is important for maturation and alveolarization of the developing lung (Lassus et al. 1999).

The detailed role of lymphatic endothelium in healthy lungs is still not clearly understood, with only fragmental pieces of evidence found in the literature. For instance, VEGF-C is detectable in tracheal fluid samples of the new-born, being present in the bronchial epithelium. Reduced level of the lymphangiogenic ligand is associated with underdevelopment of pulmonary lymphatic system and subsequent lung edema, as described in prematurely born. Prenatal injection of corticosteroids increases VEGF-C levels, which may promote the maturation process (Janér et al. 2006).

4.3.2 in lung transplantation

Lung transplantation is very similar to cardiac transplantation regarding the transplantation immunology. Thus, the lymphatic system might also play an important role in the initiation of alloimmune responses after clinical lung transplantation. However, even fewer data than for cardiac transplantation are currently available for the lungs.

The inevitable cold preservation of the lung allograft leads to pulmonary edema, which is associated with increased levels of VEGF-a and VEGF-C (Abraham et al. 2002). These changes may be the initiation point of later BOS development. In the pathogenesis of BOS, VEGF-A is also likely to show lymphangiogenic cross-reaction, stimulating lymphatic growth, thus inducing the alloimmune responses (Krebs et al. 2005).

In human lung transplant recipients, the pathogenetic relevance of vascular endothelial growth factors has been proven to be evident, in particular for the development of BOS (Luckraz et al. 2004; Belperio et al. 2005; Dutly et al. 2005; Langenbach et al. 2005). However, there are are currently almost no data on the pathogenetic role of lymphangiogenesis and lymphangiogenic factors in lung transplantation, although they appear relevant. For instance, total body lymphoid irradiation is beneficial for the prevention of primary lung dysfunction in lung allograft recipients (Fisher et al.

2005).

In summary, lymphangiogenic and angiogenic growth factors are evidently relevant for the development of healthy heart and lung, as well as for pathogenesis of various cardiac and pulmonary diseases. And although the current body of evidence appears to be very limited, available data can give the direction for the elaboration of potentially valid therapeutic approaches in the future. Apparently, this field still warrants further research, as various questions have not been answered yet.

METHODS

In the study, the functional parameters and biopsies from the human patients who underwent heart or lung transplantation were analysed. Further, experimental studies using heterotopic abdominal heart transplantation model in rat and mouse were applied.

1 Patients with end-stage heart failure

All procedures were fully approved by the ethics committee of the medical faculty of the University of Cologne and followed the Declaration of Helsinki guidelines. Transmural left ventricular endomyocardial biopsies taken from recipient hearts group failing heart) immediately after explantation of the organ, and from donor hearts (control) taken during organ harvest, immediately after aortic cross-clamp, were investigated. The biopsies of the patients were analysed in the retrospective manner. The demographic data of the included patients are represented in the **Table 2**.

Table 2. Demographic characteristics of the study cohort used.

	Terminal heart failure	Control (donor hearts)
n	7 Dilative cardiomyopathy (n=5) Ischemic cardiomyopathy (n=1) Vasculitis (n=1)	8
sex	7 males	7 males, 1 female
age	57,1 ± 3,4	38,1 ± 11,0

2 Heart transplant patients

All heart transplantations were performed in the department of cardiothoracic surgery, University of Cologne, Germany. All procedures were approved by the University of Cologne Medical Faculty Ethics Committee and followed the Declaration of Helsinki guidelines.

The biopsies of the patients were analysed in the prospective manner. The demographic data of the included patients are represented in the **Table 3**. The indication for HTX was terminal heart failure due to dilative cardiomyopathy in eight patients, ischemic cardiomyopathy in 17 patients, and congenital heart disease in one patient. All transplantations were performed with bicaval anastomosis.

Table 3. Demographic characteristics of the study cohort used.

	Heart transplant recipients	Heart donors
n	26	26
sex	21 males	17 males
age	55.8 ± 9 years (20—68 years)	35.8 ± 12.4 years (17—59 years)

Myocardial biopsies

In order to investigate a possible link between histologically verified rejection and lymphatic endothelial marker density, the biopsies from patients with no rejection episode during the first 12 months after HTX (n = 17) were compared to biopsies from patients with at least one rejection of ISHLT grade IIIa or higher (n = 9).

Right ventricular endomyocardial biopsies from 26 heart transplant recipients, taken for routine rejection monitoring during the first 12 months after HTX, were used for analysis. For the study, the following time points were investigated: 0.5, 1, 1.5, 6, and 12 months after HTX.

3 Lung transplant patients

All lung transplantations were performed in the departments of cardiovascular and thoracic surgery of Freiburg University Medical Centre, Freiburg, Germany. All procedures were approved by the Ethics Committee of Freiburg University Medical Centre and followed the Declaration of Helsinki guidelines. Written informed consent was obtained from all patients. 23 lung transplant recipients underwent routine rejection monitoring at 14 days and at 90 days after LTX by transbronchial biopsy. The indication for LTX was cystic fibrosis in 12 patients, chronic obstructive pulmonary disease in 7 patients, emphysema in 2 patients, bronchoalveolar carcinoma in 1 patient, and bronchiolitis obliterans in 1 patient. The demographic data of the included patients are represented in the **Table 4**. In 19 patients with double LTX, surgery was performed as sequential single LTX; 4 patients received a single lung.

Table 4. Demographic characteristics of the study cohort used.

	Lung transplant recipients	Lung donors
n	23	23
sex	14 males	11 males
age	47 ± 15 years (17 – 65 years)	32.0 ± 13.2 years (16—67 years)

Primary Graft Dysfunction

Primary graft dysfunction (PGD) was defined as a severe radiological infiltrate in association with a PaO2:FiO2 ratio less than 200 during the first 48 hours after transplantation.

Spirometric Analysis

Spirometry was performed for routine clinical monitoring in every patient with an 830-L whole-body plethysmograph (Masterlab Jaeger, Wurzburg, Germany). The results of spirometric measurements 14 and 90 days after LTX (the same day as transbronchial biopsies) were recorded. The following lung function indices were obtained: inspiratory vital capacity (IVC [L]), forced expiratory volume in 1 second (FEV1 [L]), and forced midexpiratory flow rate at 25% to 75% of forced vital capacity (FEF75/25 [L/s]). The FEF75/25 at chosen time-points was set in relation with the measurements

during the first year after LTX. A reduction of FEF75/25 compared with the best postoperative value was considered to represent the clinical manifestation of BOS. Spirometric analysis in every patient allowed the strict differentiation between BOS and acute allograft rejection in the examined patients.

Lung Biopsies

Transbronchial lung biopsies, taken during routine rejection monitoring, were used for analysis. For the study, the time points 14 and 90 days after LTX were investigated. Samples from patients with histologically verified pneumonia were excluded from further analysis.

4 Heart transplantation model in rat and mouse

The State Provincial Office of Southern Finland approved all animal experiments. The animals received care in compliance with the Guide for the Care and Use of Laboratory Animals as outlined by the National Academy of Sciences (ISBN 0-309-05377, revised 1996). For anaesthesia and perioperative analgesia, the recipient rats and mice inhaled isoflurane (Isofluran, Baxter, Deerfield, IL) and received s.c. buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ). Each group had 5 to 10 animals.

The heterotopic cardiac transplantations were performed in full-mismatched major histocompatibilty rat and mouse cardiac allograft models. The donor hearts were perfused with ice-cold heparinized phosphate-buffered saline (PBS), and excised. The donor heart was preserved at +4°C PBS for 4 h (Tuuminen et al. 2011). The operation time (time of warm ischemia) was standardized to 1 h.

Heterotopic heart transplantation in rat

After preservation, heterotopic cardiac transplantations were performed between fully MHC-mismatched, pathogen-free, inbred 8- to 12-week-old male DA donor and Wistar Furth (WF, RT^{1u}) recipient rats (Harlan Laboratories, Boxmeer, The Netherlands). Syngeneic heart transplantations were performed between DA rats.

After reperfusion, the recipients were sacrificed at 6 h to analyse lymphatic endothelial activation with immunofluorescence, inflammatory cell influx with immunohistochemistry, phenotype and proportions of antigen-presenting cells by flow cytometry, myocardial injury with serum troponin T (TnT) analysis, and innate immune activation with real-time quantitative reverse-transcription PCR.

At 10 d after reperfusion, adaptive immune response activation was analysed with real-time quantitative reverse-transcription PCR and the inflammatory cell influx with immunohistochemistry. At 8 weeks, the degree of cardiac fibrosis and allograft vasculopathy was assessed with histological stainings.

To determine the effect of donor treatment with VEGF-C/D trap on allograft survival, recipients received low dose immunosuppression of cyclosporine A 1 mg/kg/d s.c. until the deterioration of graft function, assessed by palpation.

Heterotopic heart transplantation in mouse

Inbred male BALB/c (B/c, H-2d) and C57BL/6J (B6, H-2b) mice (Harlan Laboratories, Boxmeer, The Netherlands) 2-3 months of age were used for heterotopic heart transplantation model in mouse. In the mouse model, allografts were harvested at 5 days after reperfusion to analyse the inflammatory cell influx and the activation of alloimmune response. To determine the effect of donor treatment with VEGFR-3 antibody on allograft survival, recipients received low dose immunosuppression of FK506 (Astellas, Tokyo, Japan) 1.5 mg/kg/d s.c. until the deterioration of graft function, assessed by palpation.

Medication

The coronary arteries of the donor rat hearts (Dark Agouti; DA, $RT1^{av1}$) were perfused with 200 µl of mutant VEGF-C (1 µg/ml, in PBS), VEGF-C/D trap (10 µg/ml, in PBS) or with PBS.

Allograft donors (BALB/c) were treated intraperitoneally with 800 µg rat anti-mouse VEGFR-3 neutralizing antibody (mF4-31C1; ImClone, New York, NY) or 800 µg rat IgG (Sigma-Aldrich, St. Louise, MO).

In the rat heart transplantation model, to prevent irreversible episodes of acute allograft rejection, to achieve long-term allograft survival, and to enable the development of chronic rejection, the recipients in the 10-d and 8-week groups received s.c. Cyclosporine A (Novartis, Basel, Switzerland) 2 mg/kg/day for the first 7 days and 1 mg/kg/day thereafter.

VEGF-C/D trap

VEGF-C was inhibited using a protein chimera of VEGFR-3 (soluble VEGFR-3(D1-3)-Fc fusion protein), which was produced in a baculovirus *Drosophila* S2 system. The protein is capable to bind full-length, processed and mature forms of VEGF-C and D in human, rat and mouse. The soluble VEGFR-3(D1-3)-Fc fusion protein was produced and purified as previously described (Anisimov et al. 2009).

$Vegfr3^{i\Delta LEC}$ mice

To dissect whether LECs are mechanistically responsible for the beneficial effects of VEGFR3 inhibition on allograft rejection, we constructed C57BL/6J mice with conditionally knocked out VEGFR3 in LECs (*$Vegfr3^{i\Delta LEC}$*), and used them as cardiac allograft donors in acute allograft survival study. *$Prox1iCreER^{T2}$;$Vegfr3^{flox/flox}$ ($Vegfr3^{i\Delta LEC}$)* and *$Vegfr3^{flox/flox}$* control mice were treated with intraperitoneal injections of tamoxifen for 3 consecutive days. Ten days after the last injection the mice were anesthetized and their hearts were used for transplantation.

5 Immunohistochemistry and immunofluorecence stainings

Cryostat sections were stained for subsets of inflammatory cells and blood and lymphatic microvascular vessels using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories) and the reaction was developed with 3-amino-9-ethylcarbazole (AEC, Vectastain). Immunofluorescent stainings were performed using Alexa 564 red and Alexa 488 green (Promega, Madison, WI) secondary antibodies. We used the following antibodies and dilutions: VEGFR-3 (10

µg/ml, AF743, R&D Systems, Minneapolis, MN), VCAM-1 (10 µg/ml, MMS-141P, Covance, Princeton, NJ); ICAM-1 (10 µg/ml, 1A29, Seikagaku, Tokyo, Japan), rabbit anti-human LYVE-1 (1 µg/ml, Molecular Cancer Biology Laboratory, Helsinki), RECA-1 for rat endothelium corresponding to human CD31 (50 µg/ml, MCA97, AbD Serotec, Dusseldorf, Germany); CD4 for T cells (5 µg/ml; anti-rat 22021D and anti-mouse 553043, BD Pharmingen, San Diego, CA), CD8 for T cells (5 µg/ml; anti-rat 22071D and anti-mouse 553027, BD Pharmingen), ED1 for macrophages (5 µg/ml, 22451D, BD Pharmingen, San Diego, CA); CD11b (553308) and CD11c (553799) for mononuclear cells in mouse (BD Pharmingen, San Diego, CA); MPO for neutrophils (20 µg/ml, ab9535, Abcam, Cambridge, UK); OX62 for dendritic cells (10 µg/ml, MCA 1029G, Serotec, Oxford, UK); OX76 for DA-specific MHC Class II RT1Ba/c molecule (10 µg/ml, MCA826, AbD Serotec, Düsseldorf, Germany). The immunoreactivity was quantified in a blinded manner using 400x magnification.

6 Acute myocardial injury

Myocardial troponin T (TnT) levels in serum samples, derived at 6 h after reperfusion, determined acute myocardial injury. We analysed TnT with the fifth generation TnT test (Troponin T STAT, Roche Diagnostics, Mannheim, Germany), which shows cross-reactivity of 0.001 % with TnT originating in skeletal muscle at a concentration of 2.000 ng/ml. The functional sensitivity is 0.05 µg/l and the lower detection limit 0.01 µg/l. The TnT was measured by electrochemiluminescence immunoassay (ECLIA) with the Elecsys 2010 immunoassay analyser (Roche Diagnostics).

7 Flow Cytometry

Isolation and preparation of myocyte-depleted cardiac cells

Cardiomyocyte-depleted mononuclear cell suspensions were prepared as previously described (Pfister et al. 2005) with modifications. Briefly, the cardiac tissue was minced and digested in the enzymatic solution, consisting of 10mg/mL collagenase IV (Gibco BrL, New York, NY), 2.4 U/mL dispase II (Roche Molecular Biochemicals, Mannheim, Germany), 1mg/mL DNase I (Sigma-Aldrich, St. Louise, MO) and 2.5 mmol/L CaCl2 at 37°C for 60 minutes. The cell suspensions were then filtered through 70µm mash, and washed with Hanks' balanced salt solution (HBSS) buffer.

Cell surface antigen staining was performed using fluorochrome-conjugated monoclonal mouse anti-rat antibodies CD45-V450 (BD Pharmingen, San Diego, CA), CD103-PE (OX62, eBioscience, San Diego, CA), CD68-Alexa700 (ED1), OX76-FITC, CCR7-PE/Cy7 and CD86-Alexa647 (all AbD Serotec, Düsseldorf, Germany) and VEGFR3-Alexa488 (Bioss, Woburn, MA) at 4°C for 15 minutes. Respective isotype controls were used as negative controls.

FACS analysis

FACS was performed using BD FACSAria Iiu (BD Biosciences, San Diego, CA) equipped with three lasers (blue 488 nm, red 633 nm and violet 407 nm). Acquired data were analysed using BD

FACSDiva™ software 6.1 (BD Biosciences, San Diego, CA).

8 RNA isolation and quantitative RT-PCR

We isolated total RNA from tissue samples, dissected from the myocardial apex of the allografts, using RNeasy kit according to the Manufacturer's instructions (Qiagen, Hilden, Germany) and reverse transcribed the RNA by using the High-RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). We performed quantitative real-time PCR on a RotorGene-6000 (Corbett Research, Doncaster, Australia) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland) and measured the mRNA quantities of the following factors from each group: lymphangiogenic factors VEGF-C, VEGFR-3, PROX-1, chemokines CXCL-2, CXCL-5, CCL-20, CCL-21, CINC-1, innate immune receptors TLR2 and TLR4, their ligands HAS1-3, HMGB1, transcription factor NF-kappa B subunits p50, p52, p65, dendritic cell maturation markers CD40, CD80, CD86, CD83, CCR7 and CCR8, as well as inflammatory cytokines interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12p35, IL-17A, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IP-10, transforming growth factor (TGF)-β. The number of mRNA copies of the gene of interest was calculated from a corresponding standard curve using the RotorGene software. Of the tested housekeeping genes (18SRNA, GAPDH, b-actin and TBP), 18SRNA was most stably expressed (data not shown) and therefore all RT-PCR data were normalized against 18SRNA.

9 Histology

We used paraformaldehyde-fixed paraffin mid-cardiac cross-sections for histological stainings. We determined the degree of cardiac fibrosis from cross sections subjected to Masson's trichrome staining and computer-assisted image processing (Zeiss Axiovision 4.4, Munich, Germany) by measuring the average proportional area stained for fibrosis from photographs captured with 100x magnification. We evaluated cardiac allograft vasculopathy from cross sections stained with hematoxylin-eosin, and Resorcin-Fuchsin for internal elastic lamina, by measuring the area between the internal elastic lamina and vessel lumen. The ratio of neointimal area to internal elastic lamina determined the arterial occlusion percentage.

10 Statistics

Data are presented by Kaplan-Meier survival plot or by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box. The data are analysed by Student t-Test or Mann-Whitney *U* test using PASW Statistics 19.0 (SPSS Inc., Chicago, IL). Log-rank analysis was used to evaluate allograft survival. $P<0.05$ was considered statistically significant.

RESULTS

1 The phenotype of lymphatic endothelium is modified in terminal heart failure

End-stage cardiac insufficiency is often associated with chronic myocardial edema. Increased myocardial lymph flow can be of specific pathophysiological importance in this situation. Thus, the aim of the study was to analyse the morphological and quantitative characteristics of the initial myocardial lymphatics in patients with terminal heart failure.

The density and the ratio of lumen presenting VEGFR-3+, LYVE+ and PROX-1+ vessels were determined in failing and control hearts. **Figure 6** represents typical microscopic morphology of the open and collapsed lymphatic vessels.

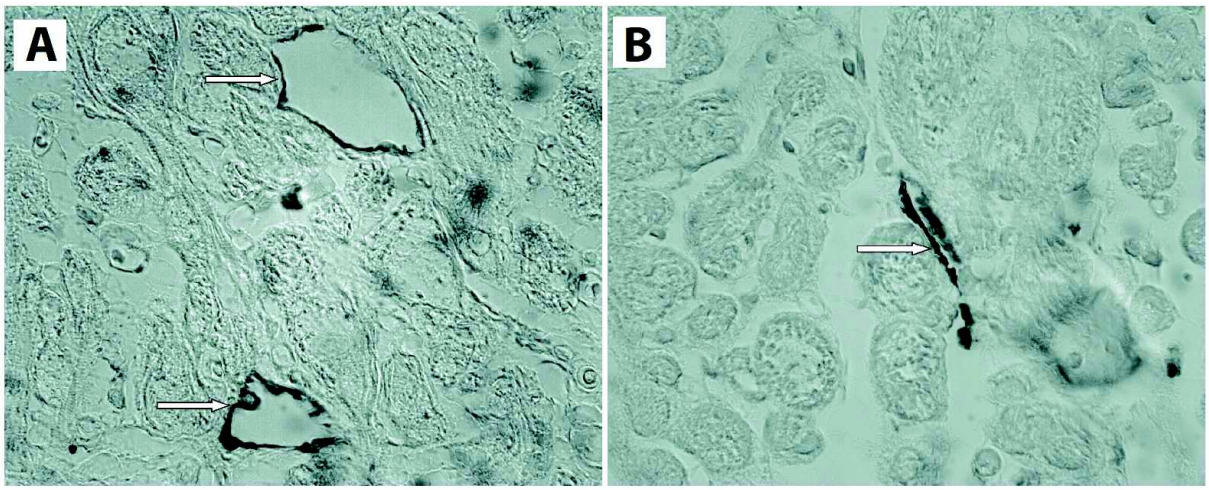

Figure 6. VEGFR3 positive lymphatics in left ventricular myocardium with typical morphology for an "open" (**A**) and collapsed (**B**) lymphatic vessel.

A significantly higher ratio of LYVE-1+ open vessels was revealed in failing hearts, indicating possibly the modified functionality of the challenged draining system of oedematous cardiac tissue. In summary, the initial myocardial lymphatics undergo significant changes in patients with terminal heart failure.

2 The phenotype of myocardial lymphatic endothelium is modified after heart transplantation in humans

In solid organ transplantation, the lymphatics have been shown to participate in the transfer of alloantigens to secondary lymphoid organs, initiating alloimmune responses (Larsen et al. 1990; Lakkis et al. 2000). Thus, our initial goal was to analyse the myocardial lymphatic endothelial cell phenotype during the first year after heart transplantation in humans. The further goal was to find a possible association of the lymphatic phenotype with rejection episodes.

Our data demonstrated a significant decrease in the density of myocardial lymphatics (LYVE-1+ and PROX-1+ vessels) during the first year after cardiac transplantation. VEGFR-3+ vessels remained

unchanged in their density **(Figure 7)**. Further, multiple fluorescent stainings for VEGFR-3, LYVE-1, and PROX-1 demonstrated the accidence of VEGFR-3+ vessels that were negative for LYVE-1 and PROX-1 and were thus interpreted to be blood capillaries.

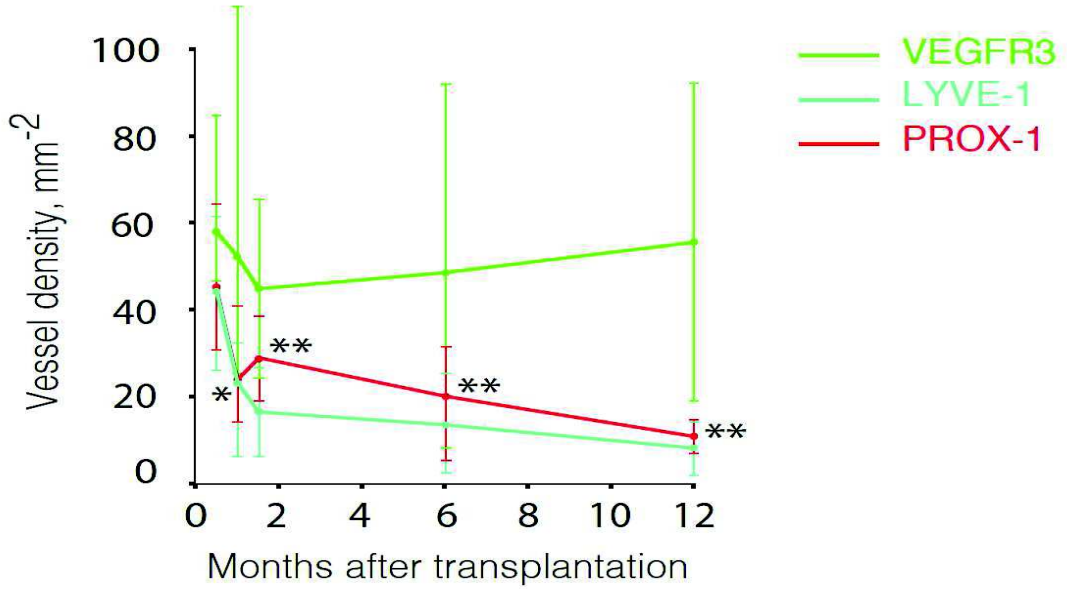

Figure 7. Densities of VEGFR-3+, LYVE-1+, and PROX-1+ capillaries during the first year after cardiac transplantation. The densities of positively marked capillaries were manually evaluated in myocardial cross-sections by immunohistochemistry. *p < 0.05 and **p < 0.01 using t-Test.

Patients with no significant rejection episodes during the first postoperative year showed a significantly higher density of VEGFR-3+ vessels two weeks after transplantation compared to the patients with at least one episode of significant acute rejection during the same period. These results for the first time described the change of lymphatic endothelial phenotype over time after heart transplantation in human patients and demonstrated a significant link between the episodes of acute allograft rejection and change in lymphatic phenotype.

3 Acute allograft rejection after lung transplantation in humans is associated with upregulation of prolymphangiogenic factor PROX-1.

The relation between the verified acute rejection episodes and lymphatic endothelial cell phenotype early after lung transplantation was analyzed in humans. The density of PROX-1+ vessels was 22.7 ± 14.9 vessels mm^{-2} 14 days after LTX, and that of VEGFR-1 and -2 were 14.5 ± 11.5 and 12.9 ± 7.1, respectively. No significant change of the marker densities between two weeks and three months after LTX was found. Further, biopsies from patients with significant acute rejection episodes showed a significantly higher density of PROX-1+ lymphatics, compared with patients who had no rejection, p < 0,001. For VEGFR-1 and VEGFR-2, no similar trend could be found (**Table 6**).

Here, for the first time a link between a verified acute allograft rejection and lymphatic density after lung transplantation in human patients was demonstrated.

Table 6. Vessel densities in patients with histologically evident acute rejection and patients with no rejection

	Acute rejection (n=27)	No rejection (n=19)	p-value
PROX-1	12.8 - 10.43	35.0 - 16.8	0.001
VEGFR-1	17.2 - 10.6	13.0 - 8.3	0.78
VEGFR-2	13.8 - 7.7	15.0 - 8.1	1.0

4 VEGF-C-VEGFR-3 signaling in IRI is linked to acute lymphatic endothelial activation.

The allograft harvesting and organ transportation time lead in clinical setting to inevitable tissue damage due to cold ischemic preservation and further ischemia-reperfusion injury in the acute phase after the transplantation. Importantly, these processes are taking place in the allogeneic environment. It is unclear how this damage-associated cascade affects the lymphatic activation and consequent leukocyte trafficking and antigen presentation. Therefore, the further goal was to investigate the impact of organ preservation and IRI on the lymphatic vessel activation and the initiation of alloimmunity in rat cardiac allografts. According to our results, IRI induced the activation of lymphatic endothelial cells in rat cardiac allografts and had direct consequences for the development of alloimmune responses.

We demonstrated that not the cold ischemic preservation per se, but reperfusion injury induced acute lymphatic vessel activation in rat cardiac allografts subjected to 4 h cold ischemic preservation. We found that 4 h cold ischemic preservation significantly increased the level of VEGFR-3, ICAM-1 and VCAM-1 expression in LYVE-1 positive lymphatics during IRI when compared to allografts subjected to 0 h ischemia. Real-time reverse-transcription polymerase chain reaction analysis showed that 4 h cold ischemia significantly increased VEGFR-3 and VEGF-C, as well as CXCL-2 mRNA levels during reperfusion.

On the molecular level, we showed the importance of VEGF-C – VEGFR-3 signaling for IRI-related lymphatic activation. Even without 4 h cold ischemic preservation intracoronary *ex-vivo* perfusion of the allograft with mutant VEGF-C induced lymphatic vessel activation and increased mRNA expression of CCL-20, CCL-21 and CXCL-2.

In summary, our results could show that specific VEGFR-3 stimulation induced lymphatic vessel activation in the allograft without prolonged cold ischemia, which is similar to the lymphatic activation in the setting of 4h cold graft ischemia. However, mutant VEGF-C treatment did not enhance lymphatic vessel activation nor mRNA levels of VEGFR-3 or any lymphatic related cytokines in cardiac allografts, subjected to 4h of cold ischemia.

5 VEGF-C/D inhibition prolonged allograft survival and prevented chronic rejection by regulating dendritic cell and lymphatic endothelial cell activation

Our next goal was to prove the role of VEGF-C in the lymphatic vessel activation, which was associated with IRI. For this purpose, we used a synthetic chimera of VEGFR-3 as a trap of autologous VEGF-C, given in the one single dose intracoronarily immediately after allograft harvesting. We found that VEGFR3-trap significantly reduced the expression of VEGFR-3, ICAM-1 and VCAM-1 expression in LYVE-1+ lymphatics of rat cardiac allografts subjected to 4 h cold ischemia at 6h after transplantation, when compared to nontreated controls. However, the analysis by immunohistochemistry and flow cytometry demonstrated a significantly higher density of OX62+ dendritic cells in the VEGF-C/D trap treatment group, when compared to nontreated cardiac allografts.

Moreover, treatment with VEGFR3 trap significantly reduced the level of CCR7 expression on OX-62+ dendritic cells in the allograft. Thus, our results have demonstrated a central role for VEGF-C signaling in lymphatic vessel activation and dendritic cell maturation in cardiac allografts during ischemia-reperfusion injury.

In chronically rejecting rat cardiac allografts, treatment with VEGFR3 trap was also linked with prolonged graft survival and decreased cardiac fibrosis and allograft vasculopathy (**Figure 8**).

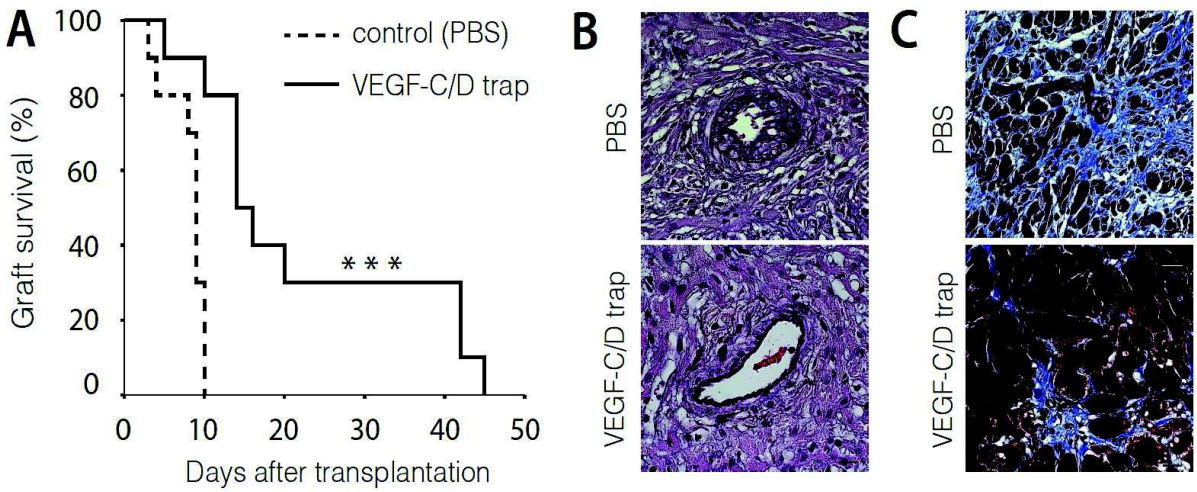

Figure 8. Ex vivo intracoronary treatment with a single dose of VEGF-C/D trap prolongs cardiac allograft survival and prevents the development of cardiac fibrosis and allograft vasculopathy in rat cardiac allografts. The coronaries of DA donor hearts were ex vivo perfused with a one single dose of VEGF-C/D trap, or PBS. The allografts were then subjected to 4-h cold ischemia, and transplanted to WF rats. (A) Effect of single-dose VEGF-C/D trap on rat cardiac allograft survival. (B) Representative images for the degree of luminal arterial occlusion in Hematoxylin-eosin and Resorcin-Fuchsin stained myocardial cross-sections. (D) Representative images for the extent of fibrotic area in Masson's trichrome stained myocardial cross-sections. Survival data are given by Kaplan-Meier survival plot, ***P<0.001 by the Mann-Whitney U test.

In mice cardiac allografts, donor treatment with VEGFR3 blocking monoclonal antibody confirmed survival benefit of cardiac allografts.

Our results suggest single-dose *ex-vivo* intracoronary treatment with VEGF-C/D inhibitor at the time of organ procurement as an alternative clinically feasible dendritic cell and lymphatic vessel targeted immunomodulatory approach.

6 Conditional deletion of VEGFR3 in lymphatic endothelial cells of mouse cardiac allografts improves graft survival

Our previous results indicated two possible targets of VEGFR3 inhibition: lymphatic endothelial cells and dendritic cells, both expressing VEGFR3. Thus, our next aim was to determine which of the VEGF-C signaling arms is primarily responsible for the beneficial results of VEGFR3 inhibition on the development of alloimmune response. For this, a line of C57BL/6J mice with a possibility for the conditional knock out of VEGFR3 in LECs (*Vegfr3*$^{i\Delta LEC}$) was used. For knock out, the *Vegfr3*$^{i\Delta LEC}$ mice and *Vegfr3*$^{flox/flox}$ control mice were treated with i.p. injections of tamoxifen for three consecutive days. Ten days after the last injection, the hearts were harvested and used as cardiac allografts for the heterotopic transplantation into BALB/c recipients. A standard setting of 4-h cold and 1-h warm ischemia before transplantation was used. Conditional knock out of VEGFR3 selectively in lymphatic endothelial cells of cardiac allografts significantly prolonged graft survival, compared with littermate controls (**Figure 9**).

Thus, conditional deletion of *VEGFR3* specifically in lymphatic endothelial cells in cardiac allograft donors proved that VEGF-C/VEGFR3 pathway in lymphatic endothelium, and not in myelomonocytic cells, is central in the bridging of innate and adaptive immune responses in the cardiac allografts.

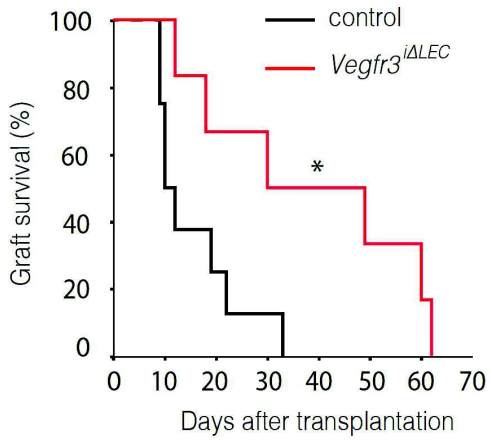

Figure 9. Conditional deletion of Vegfr3 in lymphatic endothelial cell in the donor hearts improves cardiac allograft survival. Vegfr3 knock-out donor hearts were transplanted to balb/c mice after 4-h cold ischemic preservation. Effect of lymphatic specific Vegfr3 knock-out in donors on mouse allograft survival. Survival data are given by Kaplan-Meier survival plot, *P<0.05 by the Mann-Whitney U test.

DISCUSSION

1 The evidence of a link between lymphatic endothelial phenotype and alloimmunity in human thoracic transplantation

Solid organ transplantation results in complete mechanical disruption of all lymphatic connections of the allograft. The establishment of new connections to secondary lymphoid organs requires, at least, one to two weeks after the transplantation, as has been demonstrated in experimental animal trials (Málek and Vrubel 1968). This process is believed to require lymphatic neoangiogenesis, finally providing the pathway for donor antigen presentation to secondary lymphatic organs. Thus, the mechanisms of lymphatic reconnection between the transplanted organ and the recipient lymphatic system may be of special importance for understanding the process of donor antigen presentation and the development of alloimmune response. And although lymphatic biology appears to be crucial in terminally insufficient or transplanted solid organs, concrete changes have not been investigated before.

Thus, the initial purpose of our research was to investigate the modification of cardiac lymphatic endothelial cell phenotype after heart transplantation and to analyse a possible association of with changes with allograft rejection.

The data demonstrated that myocardial lymphatic endothelium showed a significant change of phenotype after transplantation, expressed in a modification of lymphatic receptor expression pattern. Further, lymphatic vessel activation correlated strongly with acute allograft rejection, as patients in acute allograft rejection after heart transplantation had a significantly lower density of VEGFR3+ lymphatics (Geissler et al. 2006). This finding can be explained by the acute destruction of initial lymphatics in terms of the general necrotic reaction of the acutely rejecting myocardium.

Further, we demonstrated that cardiac lymphatics in patients with terminal heart failure undergo significant morphological changes compared to healthy hearts (Dashkevich et al. 2009).

In the following paper, the endotracheal biopsies of human lung transplant recipients were analysed for lymphatic angiogenesis and allograft rejection. These data were correlated with diverse clinical examinations during the postoperative follow-up. Here, activated lymphangiogenesis after lung transplantation was associated with histologically evident allograft rejection (Dashkevich, et al. 2010).

The findings in heart and lung transplant patients are opposite. Thus, it appears that allograft rejection can be associated with both: elevated and increased densities of lymphatic vessels. This controversy might be explained by differences between acute and chronic allograft rejection. Thus, as recently demonstrated for rejecting rat cardiac allografts (Nykanen et al. 2010), acute rejection decreases the epicardial lymphatic vessel density (mainly due to the tissue necrosis), and chronic rejection doubles the myocardial lymphatic vessel density (as a sequence of chronic inflammation).

For this reason, it appears to be critical to differentiate between the two modalities of allograft rejection. Further, these opposite findings raised the question whether the quantitative and qualitative changes of lymphatic endothelium after heart and lung transplantation are not just a secondary reaction, but might also reflect their regulatory role in the initiation of alloimmune reactions.

These observations of the changes in microangiogenesis after heart and lung transplantation and in patients with terminal heart failure in human patients were the first of their kind. The core message of these studies can be summarized as follows: acute allograft rejection after heart and lung transplantation in human patients is associated with significant changes in the phenotype of lymphatic endothelium.

As far as the studies on human patients could not reveal the exact mechanistic role of lymphangiogenesis in allograft rejection or clarify the relation between rejection and lymphatic endothelial phenotype, the experimental studies involving heterotopic heart transplantation in rat and mouse were conducted.

2 The role of lymphatic endothelial cell activation in the initiation of alloimmune responses in rat cardiac transplants.

The initial aim of our further research was to clarify the impact of the tissue damage during organ preservation and ischemia-reperfusion injury on the lymphatic endothelium.

Our results confirmed the hypothesis and demonstrated that cardiac lymphatic endothelium responded to prolonged cold graft preservation by rapid overexpression of endothelial adhesion molecules ICAM-1 and VCAM-1, and VEFGR-3 already 6h after graft reperfusion. These results go in hand with similar processes in contact hypersensitivity, where inflamed lymphatic endothelium of the skin promotes the exit of leukocytes from tissue to afferent lymphatic routes through induced expression of the adhesion molecules ICAM-1 and VCAM-1 (Johnson et al. 2006).

The described effect of prolonged cold graft ischemia was further mimicked by intracoronary substitution of mutant VEGF-C if given without any cold ischemia. VEGF-C substitution was also leading to early overexpression of VEGFR-3, adhesion molecules ICAM-1 and VCAM-1 and increased mRNA production of lymphocyte attracting chemokine CXCL-2. These results point out that endogenous VEGF-C is the key protein that induces the early lymphatic activation within hours after the cardiac allograft reperfusion.

These findings also strongly remind on NF-κB mediated induction of lymphangiogenesis in chronic inflammation, which requires the involvement of PROX-1 encoding pathway and needs several days until the stable overexpression of VEGFR-3 (Flister et al. 2010). Differently to chronic inflammation, our data describe an independent phenomenon of rapid VEGF-C dependent activation of lymphatic endothelium without involving PROX-1 upregulation.

In summary, ischemia-reperfusion injury led to rapid activation of cardiac lymphatic endothelium and made it more sensitive to VEGF-C by upregulating its core receptor VEGFR3, and induced the encoding of leucocyte attracting chemokines thus stimulating the initiation of afferent leucocyte traffic. The observed in this experimental setting VEGF-C mediated rapid lymphatic activation seems to repeat the physiological endogenous anti-inflammatory mechanism aimed at limiting edema formation and accumulation of inflammatory cells (Huggenberger et al. 2010). Such a lymphatic activation can be beneficial in acute skin inflammation (Huggenberger et al. 2011) or even inhibit chronic skin inflammation (Huggenberger et al. 2010). Similarly, promotion of lymphangiogenesis is beneficial in the treatment of asthma and other inflammatory airway diseases (Baluk et al. 2005). Thus, it was important to further investigate what is the role of lymphatic endothelial cell activation in the cardiac allograft allogeneic environment.

In conclusion, this is the first study to demonstrate the early acute VEGF-C mediated LEC activation after rat allogeneic heart transplantation.

The schematic overview of the possible VEGF-C pathways, relevant for experimental cardiac transplantation and based on our results, is presented in **Figure 10**.

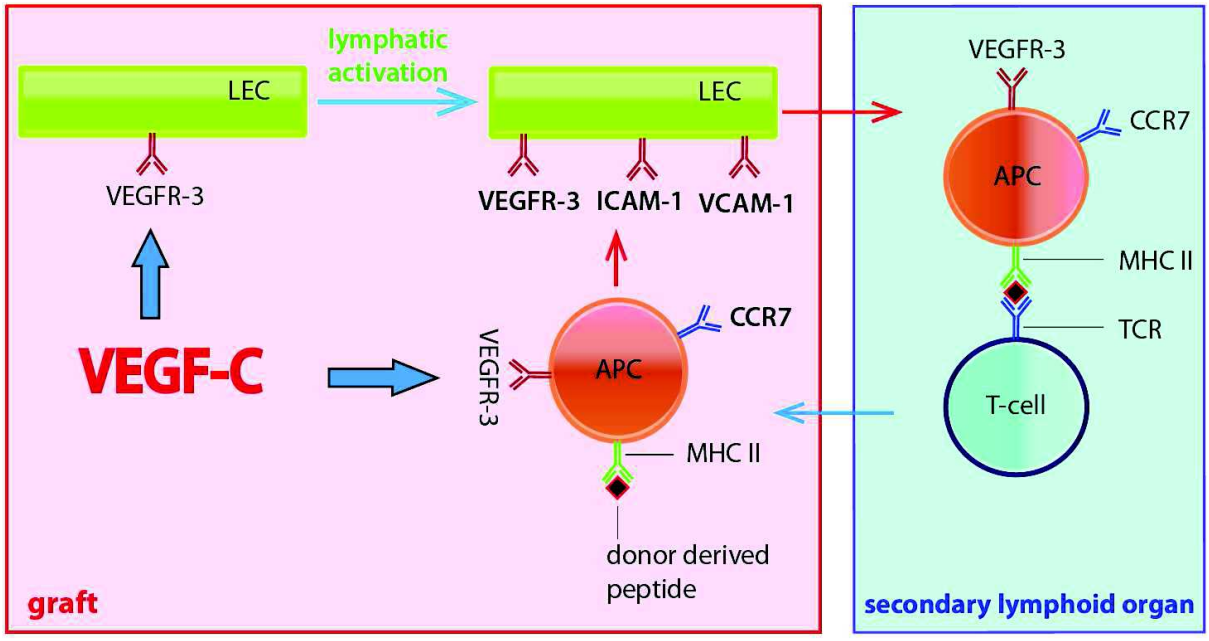

Figure 10. VEGF-C signaling pathways in cardiac transplantation. VEGF-C modifies lymphatic vessel properties by upregulating CCL21 – a chemokine that attracts CCR7+ dendritic cells. VEGF-C also has direct effects on VEGFR-3+ dendritic cells and induces their maturation and unilateral migration through the lymphatic network to secondary lymphoid organs. Thus, VEGF-C plays an important role in the initiation of direct alloimmune recognition through direct effects on lymphatic endothelial cells and antigen-presenting cells. VEGF-C may also have angiogenic effects through VEGFR-2 binding; its role in transplantation remains unclear. BEC, blood endothelial cell; CCL-21, chemokine ligand 21; LEC, lymphatic endothelial cell; CCR-7, C-C chemokine receptor type 7; DC, dendritic cell; MHC II, major histocompatibility complex class II; VEGFR-2, VEGF receptor 2; VEGFR-3, VEGF receptor 3; TCR, T cell receptor. Figure authored by Alexey Dashkevich, copyright by John Wiley and Sons; reprinted

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3 Long-term beneficial effects of VEGF-C inhibition strategies in rat cardiac allografts

Based on the previous data demonstrating the early acute VEGF-C mediated LEC activation, our next goal was to inhibit this endothelial activation and investigate the possible effects in rat cardiac allografts

To prevent the adverse effects of VEGF-C, we treated the donor rat hearts *ex vivo* at the time of organ procurement with intracoronary administration of VEGF-C/D trap. According to our data, VEGF-C inhibition in donor heart prevented the early lymphatic vessel activation as observed previously. Further, the single-time application of the VEGF-C trap prevented the activation of adaptive immune responses and the consequent development of cardiac fibrosis and allograft vasculopathy. These results appear to be of clinical importance, as cardiac allograft vasculopathy and rejection belong to the leading causes of death after heart transplantation today (Stehlik et al. 2012). Especially the long-term beneficial consequences of a single dose of VEGF-C/D trap for cardiac allografts seem to be important.

The mature VEGF-C also binds and activates VEGFR-2, which can be expressed on both: the blood and lymphatic vascular endothelium (Joukov et al. 1996; Makinen et al. 2001). Thus, it was the further goal of the study to dissect whether VEGFR2 might also play a role in the described processes. I showed here that preoperative donor treatment with neutralizing monoclonal VEGFR-3 antibody ameliorated acute rejection response and prolonged cardiac allograft survival. This finding proved that the similar beneficial effects of VEGF-C/D trap are based on specific interaction with VEGFR-3, not involving VEGFR-2 signaling.

Furthermore, conditional gene deletion of *VEGFR3* specifically in lymphatic endothelial cells of heart donors proved that VEGF-C/VEGFR3 pathway in lymphatic endothelium, but not in myelomonocytic cells, is central in the bridging of innate and adaptive immune responses in the cardiac allografts.

Here, the implication of specific conditional gene deletion of *VEGFR3* in the myelomonocytic cell line of cardiac allograft donors would be of specific interest in the future, as specific targeting of VEGFR3 expressing donor antigen presenting cells might represent a separate important therapeutic target.

In summary, our results proved that inhibition of VEGFR3-signaling in heart donor allows preventing lymphatic endothelial cell activation and dendritic cell maturation. It consequently reduced activation of adaptive alloimmune rejection responses and the development of cardiac fibrosis and allograft vasculopathy. Furthermore, single dose donor treatment with a monoclonal VEGFR3 antibody as well as the conditional lymphatic specific VEGFR3 deletion in the donor heart improved graft survival. Importantly, the beneficial treatment did not influence the myocardial damage severity or innate immunity but was exclusively based on the modification of adaptive alloimmune

responses. These results specifically pointed out the pathogenetic relevance of VEGF-C-VEGFR3 signaling axis in the development of adaptive immune responses, thus highlighting a new therapeutic target for prevention of alloimmune responses.

SUMMARY

The lymphatic endothelium is relevant for the pathogenesis of various cardiac and pulmonary diseases. However, the knowledge about the functions and role of lymphatic endothelium in the setting of transplantation is very limited. Therefore it was the main focus of this study.

The study investigated the changes of lymphatic endothelial phenotype in patients with terminal heart failure and during the time course after heart and lung transplantation. These observations of the lymphatic phenotype are the first of their kind and provide the evidence, that acute allograft rejection after heart and lung transplantation in human patients is associated with significant changes in the phenotype of lymphatic endothelium. To show the exact mechanistic role of lymphatic endothelium in acute organ rejection and to clarify the cause-effect relation between allograft rejection and lymphatic endothelium, the experimental studies involving heterotopic heart transplantation in rat and mouse were conducted. The results demonstrated that ischemia-reperfusion injury induced the activation of lymphatic endothelial cells in rat cardiac allografts. The process was mediated by interaction in the VEGF-C-VEGFR-3 axis and had direct consequences for the development of alloimmune responses. Further, specific perioperative single-dose VEGF-C inhibiting strategies demonstrated beneficial effects on lymphatic vessel activation, antigen-presenting cell trafficking and subsequent development of alloimmune responses in rat cardiac allografts. VEGF-C/D trapping in donor heart prevented acute lymphatic vessel activation and led to homing of VEGFR-3+ dendritic cells in cardiac allograft. Intracoronary *ex-vivo* perfusion with VEGF-C/D trap also improved rat cardiac allograft survival and inhibited the development of cardiac fibrosis, allograft vasculopathy and inflammation.

The results of the study, thus, demonstrate the significance of VEGF-C-VEGFR-3 signaling in alloimmunity and suggest VEGF-C/D inhibiting strategies as an alternative clinically feasible immunomodulatory approach targeting lymphatic vessels.

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