CRITICAL ROLE OF ANGIOPOIETIN PATHWAY IN ISCHEMIA-REPERFUSION INJURY IN CARDIAC TRANSPLANTATION

SIMO SYRJÄLÄ

2014
CRITICAL ROLE OF ANGIOPOIETIN PATHWAY IN ISCHEMIA-REPERFUSION INJURY IN CARDIAC TRANSPLANTATION

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Academic dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 3, Meilahti Hospital, Haartmaninkatu 4, on 12th December, at 12 o’clock noon
Helsinki 2014
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ISBN 978-951-51-0390-1

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ORIgINAL PUBLICATIONS

The thesis is based on the following original publications referred to in the text by their Roman numerals:


ABBREVIATIONS

AMR  antibody-mediated rejection
Ang  angiopoietin
AP-1  activation protein 1
APC  antigen-presenting cell
CCL21  chemokine (C-C motif) ligand 21
CCR7  C-C chemokine receptor type 7
CD  cluster of differentiation
CMC  cardiomyocyte
CMV  cytomegalovirus
COMP  cartilage oligomeric matrix protein
COX  cyclooxygenase
CTL  cytotoxic lymphocyte
DA  Dark Agouti rat
DAMP  danger/damage-associated molecular pattern
DNA  deoxyribonucleic acid
DC  dendritic cell
EC  endothelial cell
ECM  extracellular matrix
ELISA  enzyme-linked immunosorbent assay
ET-1  endothelin-1
FITC  fluorescein isothiocyanate
FOXP3  forkhead box p3
HAS  hyaluronic acid synthase
HIF-1  hypoxia-inducible factor-1
HLA  human leukocyte antigen
HMBG1  high-mobility box group 1
ICAM-1  intracellular adhesion molecule-1
IFN-g  interferon gamma
IL  interleukin
IP-10  IFN-g-inducible protein 10
IRI  ischemia-reperfusion injury
i.v.  intravenously
KLF-2  Krüppel-like factor-2
LFA1  leukocyte function antigen 1
MHC  major histocompatibility complex
NF-AT  nuclear factor of activated T cells
NF-κB  nucleic factor kappa B
PAMP  pathogen-associated molecular pattern
PBS     phosphate-buffered saline
PC      pericyte
PMNC    polymorphonuclear cell
Rbc     red blood cell
RhoA    Ras homolog gene family member A
ROR     retinoic acid receptor-related orphan receptor
ROS     reactive oxygen species
RT-PCR  reverse-transcription polymerase chain reaction
s.c.    subcutaneously
SLO     secondary lymphoid organ
SMA     smooth muscle actin
SMC     smooth muscle cell
STAT    signal transducer and activator of transcription
TGF     transforming growth factor
Th      T helper cell
TLR     Toll-like receptor
TNFa    tumor necrosis factor alpha
TnT     troponin T
TOR     target of rapamycin
Treg    regulatory T cell
VCAM-1  vascular endothelial growth factor-1
VE-cadherin  vascular endothelial cadherin
WF      Wistar Furth rat
ABSTRACT

Heart transplant is disconnected from the circulation and preserved in hypothermia before transplantation. Paradoxically, revascularization of the heart transplant results in ischemia-reperfusion injury described as myocardial injury, microvascular dysfunction, and innate and adaptive immune activation. The innate response consists mainly of neutrophils and macrophages and innate lymphoid cells and may lead to sustained adaptive immune response leading to chronic rejection and late graft failure. The heart is especially susceptible for lack of oxygen; therefore, the ischemic time in clinical practice is critical. Prolonged ischemic time – due to long distance between hospitals or technically difficult operation – is an independent risk factor for primary graft dysfunction and chronic rejection.

Angiopoietin-1 and -2 (Ang1 and 2) are vascular growth factors binding to Tie2 receptor with indispensible role in embryonic vascular development, but also in endothelial maintenance in mature vasculature. Vascular supporting cells constantly secrete Ang1, which maintains the endothelium in quiescent state. In contrast, Ang2 is produced and released from the endothelial cells in response to stress stimuli, such as hypoxia and inflammation, destabilizing and activating the endothelium in order to ease inflammatory cell accumulation and transmigration.
This study utilized experimental animal model to describe the effect of cardiac allograft ischemia-reperfusion injury on innate immune activation and adaptive immune responses, such as the development of acute and chronic rejection. This study further investigated the effects of either activating or inhibiting the angiopoietin/Tie2-signaling pathway in this disease process. The results show that prolonged hypothermic preservation enhanced ischemia-reperfusion injury-related innate immune activation and adaptive immune and worsened the prognosis of the cardiac allografts. Analysis of samples from clinical heart transplant recipients revealed increase in peripheral blood Ang2 levels during the first day after the operation. Similar findings were evident in the recipients of rat cardiac allografts.

Ang1 was proven protective when injected into allograft coronaries prior to the preservation: the treatment stabilized the endothelium, reduced myocardial injury and inflammation, and hindered the development of chronic rejection. Donor heart treatment with Ang2-blocking antibody had similar effects on endothelium, but further inhibited the activation of endothelial cells, acute and chronic rejection. Furthermore, recipient treatment with multiple doses of the anti-Ang2 antibody immediately after transplantation significantly prolonged allograft survival and had superior effect when compared to heart donor treatment.
Primary and late graft dysfunction, either due to ischemia-reperfusion injury, or acute and chronic rejection, limit the survival of patients with solid organ transplant. According to this study, targeting Ang/Tie2-signaling prevents early allograft endothelial activation and inflammatory cell accumulation. Of studied treatment protocols, early systemic recipient treatment with anti-Ang2 antibody had the most robust effect in preventing allograft dysfunction. Ang2-targeted antibody treatment would have clinical implications in induction therapy of transplant patients, as the dosage of other immunosuppressive drugs may be lowered and the adverse side effects of these drugs avoided. These results encourage further studies to determine the clinical significance of Ang/Tie2-pathway modification.
INTRODUCTION

Cardiovascular diseases are the leading cause of death in Western countries. After advances in surgical techniques and the introduction of immunosuppressive medication, cardiac transplantation has become plausible treatment for many end-stage heart diseases. The shortage of organ donors limits the availability of heart transplants and presents challenges to donor management.

Acute rejection, primary graft dysfunction, infections, malignancies, and chronic allograft dysfunction limit the survival of cardiac transplant patients. Of these, acute rejection and infections are effectively managed with adjustments in immunosuppressive medication and antibiotics; however, the side effects of immunosuppressive drugs and the development of chronic rejection continue to puzzle the clinicians and scientists.

Angiopoietins are vascular growth factors with indispensable role in embryonic vascular development, but also in the stabilization of mature vasculature. The angiopoietin signaling has been suggested a potential immunomodulatory pathway in regulating microvascular dysfunction and inflammation after cardiac transplantation.

The purpose of this study was to characterize, in experimental rat cardiac transplantation model, the effects of ischemia-reperfusion injury on transplant inflammation and to elaborate the therapeutic potential of angiopoietin-1 supplementation and angiopoietin-2 blocking in this setting.
REVIEW OF THE LITERATURE

1. Clinical heart transplantation

1.1. Background

The first documentations of tissue replacement are based on the work of Gasparo Tagliacozzi – an Italian surgeon performing successful autologous skin transplantations in the 16th century. He also repeatedly failed with allogeneic transplantations, introducing the idea of rejection in his publication *De Curtorum Chirurgia per Instionem* in 1596. Alexis Carrel and Charles Guthrie developed new suturing techniques for transplanting arteries and veins, subsequently enabling vascular anastomosis operations and solid organ transplantation (Carrel and Guthrie 1905). Joseph Murray and J. Hartwell Harrison performed the first technically successful kidney transplantation between identical twins in 1954 (Guild et al. 1955), raising further interest in clinical solid organ transplantation. Eventually, the development of heart-lung machine enabled surgeons to perform open-heart surgery, and Christiaan Barnard to perform the first allogeneic heart transplantation in 1967 (Barnard 1967). The first patient died unfortunately due to pneumonia early after the successful operation. Nevertheless, Barnard inspired others to establish several heart transplantation programs. Acute rejection resulting from tissue type mismatching was fatal to most of the recipients, however, and the hype quickly subsided. The introduction of immunomodulatory drugs – corticosteroids, azathioprine, and most importantly cyclosporine in 1976 (Borel et al.
1976) – enabled long-time survival of recipient and finally made solid organ transplantation a plausible treatment for many end-stage diseases.

1.2. General histology of the heart

Histologically, the heart consists of myocardium, supporting connective tissue, and vascular structures. Vascular structure can be divided into large coronary arteries, veins, arterioles, venules, capillaries, and lymphatic vessels. Lymphatic vessels are responsible for trafficking inflammatory cells from the heart into secondary lymphatic organs. The vasculature feeds the myocardium with oxygen and nutrients, but also enables circulating inflammatory cells to enter the tissue. Mesenchyme derived pericytes (PC), smooth muscle cells (SMC), fibroblasts, and extra-cellular matrix (ECM) form the connective tissue surrounding and supporting the vascular structures and the myocardium. The myocardium is formed by delicately organized and interconnected cardiomyocytes that are responsible for the contractile function of the myocardium.

Endothelial cells (EC) line the inner lumen of the blood vessels, cardiac chambers, and the lymphatics forming a barrier between the circulation and the tissues. EC play important role in inflammation, as inflammatory cells must pass through the endothelium in order to reach target tissue. As a response to inflammation, EC express adhesion molecules on their luminal surface. These adhesion
molecules enable circulating leukocytes to attach to vascular wall, slow down, and eventually transmigrate through the endothelium.

All cells express self-antigens on their cell surface with major histocompatibility complex (MHC) class I receptors. Antigen-presenting cells (APC; macrophages and dendritic cells) also express MHC class II receptors and are able to present foreign peptides – i.e. non-self antigens – to T cells with these receptors. In MHC-mismatched organ transplantation, the donor tissue type differs from the recipient tissue type – the transplant is therefore allogeneic and called an allograft.

1.3. Indications, patient characteristics, and outcome
The International Society of Heart and Lung Transplantation (ISHLT) Registry data show that between 2006 and 2012, 22,318 adult transplantations have been performed around the world with the most common indications being non-ischemic cardiomyopathy (54% of the patients) and coronary artery disease (37%). Other indications were valvular (2.8%), congenital heart disease (2.9%), and retransplantation (2.5%). Over 35% of patients were bridged to the transplantation with mechanical circulatory assist devices. The median age of the heart transplant recipients was 54 years, and the median age of the donors was 35 years. The cause of death for organ donation was head trauma (46%), stroke (24%), or other (30%) (Lund et al. 2013).
The survival of cardiac transplant recipients has changed little during the last 30 years; the median survival of cardiac transplant recipients is 11 years, however, patients surviving the first year after the transplantation have median survival of 14 years (Stehlik et al. 2011). The latest data shows that 1-year survival of all cardiac transplant patients is 81%, and 5-year survival is 69%. Figure 1 demonstrates the median survival of transplant recipients on different decades. The patient mortality is the highest during the first year after the transplantation due to graft failure (36% of deaths), non-CMV infections (12%), acute rejection (4%), or other reasons (46%). After the first year, graft failure, cardiac allograft vasculopathy, and malignancies are the main survival-limiting factors (Lund et al. 2013).

![Figure 1](image_url)

*Figure 1. The median survival of all cardiac transplant patients operated between 1982-2011. Modified from Stehlik et al 2013.*
After the transplantation, the patients usually gain significant improvement of life and can perform normally in daily activities. The Registry data show, however, that only 35% of the recipients are employed 1 year after the transplantation, and 46% at 3 years, possibly due to regional employer-related factors (Lund et al. 2013).

1.4. Risk factors

Immunologic and non-immunologic factors pose risks to allograft and patient survival. The donor-derived non-immunologic factors are the cardiovascular status of the donor, the body-mass index, blood glucose levels and direct donor organ trauma. Immunologic risk factors include donor brain death, tissue-type mismatch between the donor and the recipient, and recipient antibody production. Donor brain death produces systemic cytokine storm making the grafts even more susceptible to IRI and rejection (Takada et al. 1998; Wilhelm et al. 2000).

Due to the acute nature of cardiac diseases leading to transplantation and shortage of organ donors, cardiac transplantations are performed between donors and recipients with mismatching tissue type, or major histocompatibility class (MHC; human leukocyte antigen, HLA, in humans). Furthermore, due to the shortage of organ donors, older and sicker patients are accepted as donors. Episodes of acute rejection are common during the first year after transplantation, as 25% of patients are diagnosed with mild-to-severe acute rejection. However, acute rejection is efficiently
treated with immunosuppressive medication, as acute rejection accounts only 4% of deaths during the first year. The age of the donor has linear correlation with the risk of mortality during the first year after the transplantation. Similarly, prolonged allograft ischemia – especially when exceeding 200 min – is associated with increased early mortality risk. Other recipient-derived risk factors for early mortality are renal dysfunction, and the need for mechanical circulatory support before transplantation. In addition to the 1-year risk factors, risk factors for mortality during the first 5 years after transplantation include episodes of acute rejection, the need for dialysis or treated infection during hospitalization, and the absence of certain immunosuppressive drugs 1 year after the transplantation. Interestingly, donor age and ischemic time continue to affect the survival of the recipients 15 years after transplantation (Stehlik et al. 2012).

Immunosuppression fails, however, to protect the allograft and the recipient from several important risk factors: ischemia-reperfusion injury, chronic rejection, the toxicity of immunosuppressive therapy, and the exposure to infections. Episodes of acute rejection are linked to chronic rejection development, and depending on the severity of rejection, even one acute rejection requiring treatment decreases the overall survival of the patients.

The use of immunosuppressive medication is necessary to prevent allograft rejection, but inhibition of the normal function of T cells
increases the risk of infections. Viral infections pose the greatest risks to solid organ transplant recipients, but also to the allograft itself. Cytomegalovirus (CMV) infection increases the risk of allograft rejection and cardiac allograft vasculopathy. Immunosuppression also increases the risk of malignancies. The incidence of non-skin malignancies increases progressively and accounts for 20% beyond 5 years after transplantation (Stehlik et al. 2012).
2. Ischemia-reperfusion injury

2.1. Ischemia and hypothermia

The procurement of heart transplants requires that the blood flow to the organ be stopped for the transportation and during the surgery. Lack of circulation renders the transplant into oxygen and nutrient deprived ischemic state. Current organ preservation techniques rely on cooling of the allograft (Jacobs et al. 2010). Ischemia results in accumulation of anaerobic metabolites, changes in electrolyte balance, and hypoxic tissue injury (McCord 1985). Ischemic time depends on the distance between the donor hospital and the transplantation center, and with heart transplantation, is approximately 3 hours (Stehlik et al. 2012). The kidney, however, endures ischemia better, and can be transplanted even after 16 hours of ischemia (Southard and Belzer 1995). Transplantation-related ischemia is divided into cold and warm ischemia, of which cold ischemia is regarded as organ protective and warm ischemia organ damaging phase. Physiologically, during hypothermia, the cell metabolism and oxygen consumption are reduced, whereas during warm ischemia, the tissue remains metabolically active but lacks oxygen and nutrients driving itself into anaerobic state. Furthermore, hypothermia protects endothelial cells from apoptosis (Yang et al. 2009). Ischemia, on the other hand, results in mitochondrial damage and release of reactive oxygen species, accumulation of lactate, and downregulation of Krüppel-like factor 2 (KLF2) expression, as endothelial shear stress is diminished (Dekker
et al. 2002). KLF2 is essentially involved in vascular development and sustaining physiological quiescence by negatively regulating vascular inflammation, permeability and angiogenesis (SenBanerjee et al. 2004; Bhattacharya et al. 2005; Dekker et al. 2006; Lin et al. 2006).

Hypoxia-inducible factor-1 (HIF1) is a transcription factor constantly produced in various cell types in response to tissue hypoxia and it is rapidly degraded in normoxia by von Hippel-Lindau protein (Wang and Semenza 1993; Maxwell et al. 1999). HIF1 affects wide range of downstream proteins, most relevant to microvascular dysfunction being vascular endothelial growth factor (VEGF), angiopoietin-1, and angiopoietin-2 (Semenza 2014). VEGF is pro-angiogenic and pro-inflammatory growth factor, partly inducing its effects via increase in endothelial permeability and recruiting smooth muscle cells and endothelial cells to form new vessels (Yancopoulos et al. 2000). VEGF also induces adhesion molecule expression on the luminal surfaces of the EC, luring inflammatory cells to the site of it’s secretion (Kim et al. 2001a). Interestingly, KLF2 plays major role in ischemic allograft as it also regulates HIF1 expression (Kawanami et al. 2009). Figure 2 describes the factors involved and their interplay.

2.2. Reperfusion and re-oxygenation

Revascularization of the transplant is vital for the organ but, paradoxically, results in ischemia-reperfusion injury (IRI). Reperfusion of ischemic tissue with oxygenated blood results in release of lactate, reactive oxygen species (ROS), capillary perfusion and permeability disorder, endothelial activation, and inflammatory
cell influx (Eltzschig and Carmeliet 2011; Lampe and Becker 2011). In allogeneic environment, initial macrophage and neutrophil influx is followed immediately by sustained NK and T cell infiltration (El-Sawy et al. 2004). Therefore, IRI of allogeneic solid organ transplant differs from IRI of other origin, such as revascularization in acute coronary syndrome and in stroke. IRI of transplant is referred as Tx-IRI from now on to emphasize the importance of the difference.

The IRI induces the release of endogenous molecules called danger/damage-associated molecular patterns (DAMP), which are structural proteins normally bound to, or part of the extracellular matrix. These molecules are recognized by the TLR-receptors of the innate immune system cells, which in allogeneic environment may accelerate alloimmune and rejection responses.
Figure 2. The effects of hypoxia and ischemia on microvascular wall in the heart. Ang, angioipoietin; COX, cyclooxygenase; ET-1, endothelin-1; EC, endothelial cell; PC, pericyte; CMC, cardiomyocyte; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; HIF-1, hypoxia-inducible factor-1; Rbc, red blood cell; RhoA, Ras homolog gene family member A; SMA, smooth muscle actin; TLR, Toll-like receptor; TNF-a, tumor necrosis factor alpha; TnT, troponin T, VCAM-1, vascular endothelial growth factor-1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

2.3. Microvascular dysfunction

Hypoxia induces EC instability by formation of cell membrane protrusion and disintegration (Aono et al. 2000). Ischemia and reperfusion activates cytoskeletal modulators of endothelial cells,
but more importantly results in PC constriction reducing microvascular blood flow and tissue perfusion (Yemisci et al. 2009). Rho GTPases Rac1, and RhoA regulate these membrane morphology changes, and PC and SMC constriction. In detail, RhoA and Rac1 have opposite function in hypoxia/reoxygenation, as RhoA regulates endothelial barrier function and stress-fiber formation, whereas Rac1 is required for endothelial recovery. (Wang et al. 2001) Rho-kinase phosphorylates adducin and therefore, phosphorylated adducin may be regarded as a parameter for Rho activity (Fukata et al. 1999).

The perfusion defect worsens allograft function and may inflict fibrosis development. Tx-IRI also induces EC-EC junction disruption and microvascular leakage. Leaky vessels are more susceptible to tissue edema and inflammatory cell influx. IRI affects microvascular endothelium, PC and underlying tissue resulting in microvascular dysfunction, endothelial barrier function disruption and cardiomyocyte damage in cardiac allografts (Tuuminen et al. 2011). The activation of EC during ischemia and Tx-IRI induces expression of endothelial cell adhesion molecules attracting circulating macrophages, neutrophils, NK cells, and T cells. Therefore, microvascular dysfunction results in local inflammation, accumulation of inflammatory cells and innate and adaptive immune activation in cardiac allografts (Carden and Granger 2000; Boros and Bromberg 2006; Dumitrescu et al. 2007).
3. Immunobiology

3.1. Innate immune system

The innate immune system consists of plasma complement system, circulating inflammatory cells, such as neutrophil granulocytes, monocytes, and natural killer (NK) cells, and of circulating and tissue residing macrophages, and dendritic cells (Janeway and Medzhitov 2002). It is congenital first-line defense against invading pathogens but is also responsible for the cleaning and degradation of injured tissue (Xu et al. 2006). The complement system may directly destroy pathogens or help the other inflammatory cells to do so (Müller-Eberhard 1986).

Neutrophils and monocytes/macrophages are phagocytes capable of internalizing and ingesting pathogens and particles. They originate from same common myeloid progenitor cells and subsequently from myeloblasts. Neutrophils are the first-responders to inflammation, and migrate to the site of inflammation within minutes with the help of IL-8- and C5d-mediated chemotaxis. They also need to adhere to the vascular wall and transmigrate through the endothelium by interacting with selectins, integrins, and adhesion molecules – most predominantly P-selectin, LFA-1, ICAM-1, and VCAM-1. Neutrophils have characteristic cytoplasmic granules containing substances, such as myeloperoxidase (MPO), lysozyme, and collagenase that enable them to degrade phagocytized bacteria and obliterate internalized particles (Kolaczkowska and Kubes 2013).
Macrophages participate in host’s first-line defense against invading pathogens, but also take part in scavenging aging cells and debris. Furthermore, macrophages are able to boost adaptive immune response in transplantation by presenting internalized antigens to T cells, but also directly attacking allogeneic T cells (Xu et al. 2006; Liu et al. 2012; Canton et al. 2013).

Toll-like receptors (TLR) of innate immune cells recognize pathogen-associated molecular patterns (PAMP), such as bacterial lipopolysaccharide, lipoproteins, peptidoglycan, and flagellin, viral DNA and RNA (Aderem and Ulevitch 2000). When encountered with appropriate ligand, TLR activates innate immune cells to induce adaptive immune responses. Of 10 identified functional human TLR (13 in mouse and rat), TLR2 and 4 also recognize endogenous structural molecules exposed during tissue injury (Roach et al. 2005; Land 2011). These danger/damage-associated molecular patterns (DAMP) include biglycan, fibrinogen, fibronectin, hyaluronic acid (HA), heat-shock proteins and high-mobility group box 1 (HMGB1) (Smiley et al. 2001; Tsan and Gao 2004; Schaefer et al. 2005; Yu et al. 2006). TLR2 or 4 activation on APC surface results in MyD88-dependent NF-kB and mitogen-activated protein kinase signaling, and innate immune activation (Barton and Medzhitov 2003). Innate immune activation includes DC maturation seen as increased superficial co-stimulatory molecule expression, and release of pro-inflammatory chemokines and cytokines (Figure 3) (Janeway and Medzhitov 2002; Rossi and Young 2005; Kaczorowski et al. 2007).
Figure 3. The activation and maturation of immature dendritic cells upon encountering of danger/damage-associated molecular patterns, and allogeneic, foreign peptides. The DC recognize DAMPs with TLR-receptors and begin expressing proinflammatory cytokines through NF-κB transcription factor activation. CCR7, C-C chemokine receptor type 7; CD, cluster of differentiation; DC, dendritic cell; DAMP, danger/damage-associated molecular patterns; MHC, major histocompatibility complex; NF-κB, nucleic factor kappa B; TLR, Toll-like receptor.

Maturation increases the superficial expression of costimulatory molecules CD80, CD83, CD86, CD40. DC migration to secondary lymphoid organs (SLO), such as lymph nodes and spleen is facilitated by increased expression of CCR7 – a receptor for constantly secreted lymphatic chemokine CCL19 and 21 (Banchereau and Steinman 1998; Förster et al. 2008). Activation of DC is important step in linking innate and alloimmune responses (Figure 4).
**Figure 4.** Cross-linkage of innate and adaptive immune responses during ischemia-reperfusion injury. CCL21, Chemokine (C-C motif) ligand 21; CCR7, C-C chemokine receptor type 7; CD, cluster of differentiation; CMC, cardiomyocyte; DAMP, danger/damage-associated molecular pattern; DC, dendritic cell; EC, endothelial cell; IFN-γ, interferon gamma; IL, interleukin; PMNC, polymorphonuclear cell; Th, T helper cell; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

### 3.2. Alloimmune system

The alloimmune system consist of B and T cells, of which the latter can be further divided into CD4+ T helper cells (Th), and CD8+ cytotoxic lymphocytes (CTL). The CD4+ T cells are further classified into subtypes presented in Table 1. CD4+CD25+FoxP3+ subset of T cells is a crucial cell population for immunological balance and
tolerance. These cells are named regulatory T cells (Treg) for their suppressive properties (Wood et al. 2012; Lazarevic et al. 2013).

In direct allore cognition, the donor-derived passenger DC and macrophages of the allograft travel to SLO and present donor peptides with MHC class I receptors directly to cytotoxic CD8+ T cells, or with MHC class II to naïve CD4+ T-cells. T cells may also recognize foreign peptides directly on allograft endothelial cell MHC class I receptors (Ali et al. 2013).

In indirect allore cognition, once the recipient APC encounter foreign protein, they internalize it, activate and increase their expression of CD80, CD83, CD86, and CCR7 on their surfaces and migrate to SLO to present the internalized foreign material to naïve CD4+ T cells with MHC class II receptors (Janeway and Medzhitov 2002; Rossi and Young 2005; Kaczorowski et al. 2007). The T cells recognize peptides presented in MHC class II receptors with their T cell receptors (TCR), and if accompanied with co-stimulatory signal between CD28 and CD80/CD86, the transcription factor of activated T cells (NF-AT) is activated by calcineurin. This leads to interleukin-2 (IL-2) transcription and, by paracrine signaling through IL-2R, to clonal proliferation of alloreactive T cells (Figure 5) (Lee et al. 1994).

In a relatively lately discovered phenomenon, semi-direct allore cognition, donor-derived peptides are presented unprocessed
to T cells by recipient APC. This constitutes a small minority of allore cognition and probably has little clinical significance.

**Table 1. Different T helper cell subsets.** IL, interleukin; IFN, interferon; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; ROR, retinoic acid receptor-related orphan receptor; FOXP3, forkhead box p3.

<table>
<thead>
<tr>
<th>T cell subtype</th>
<th>Function normally / In transplantation</th>
<th>Transcription factors and hallmark cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Th1</strong></td>
<td>Cellular immune defence; boosts macrophage and CD8+ T cell killing ability / Cell-mediated rejection</td>
<td>T-bet; IL-2, IL-12, IFN-γ, STAT4</td>
</tr>
<tr>
<td><strong>Th2</strong></td>
<td>Humoral immune defence; B cell stimulation / Antibody-mediated rejection</td>
<td>GATA3; IL-4, IL-10, STAT6</td>
</tr>
<tr>
<td><strong>Th3</strong></td>
<td>Mucosal immunity in the gut / unknown</td>
<td>IL-4, IL-10, TGF-β</td>
</tr>
<tr>
<td><strong>Th17</strong></td>
<td>Anti-microbial immunity / Cell-mediated rejection</td>
<td>RORγ, STAT3; IL-6, IL-17, IL-23</td>
</tr>
<tr>
<td><strong>Treg</strong></td>
<td>Immune balance / Tolerance?</td>
<td>FOXP3; IL-10, TGF-b</td>
</tr>
</tbody>
</table>
Figure 5. Antigen presentation and costimulation between dendritic cell (DC) and naïve CD4+ T cell. If accompanied with costimulatory signal, antigen presentation results in clonal expansion of alloreactive T cells. CD, cluster of differentiation; DC, dendritic cell; IL, interleukin; NF-AT, nuclear factor of activated T cells; MHC, major histocompatibility complex.

Alloreactive cytotoxic CD8+ T cells are the prime effector cells responsible for allograft injury and may inflict graft rejection in the absence of CD4+ T cell help. Acute rejection is considered to originate from direct allorecognition and from MHC class-I signaling between CD8+ T cells and allogeneic cells, whereas chronic rejection is indirect allorecognition-mediated (Liu et al. 1993; Rogers and Lechler 2001; Schmauss and Weis 2008).

Th17 T cells produce mainly IL-17A and participate normally in host pathogen defense, but also in multiple autoimmune diseases and chronic inflammation. IL-17A has been linked to neutrophil multiplication and recruitment via granulocyte colony-stimulating
factor and CXC chemokines (Laan et al. 1999; Ley et al. 2006). IL-23 signaling via IL-23R is crucial for Th17 T cells effector function and IL-17 mediated inflammation (Korn et al. 2009). The role of Th17 response in allograft rejection was demonstrated with T-box21-deficient mice, which lack Th1 alloimmune response. The findings of Yuan et al. suggest that the absence of Th1-transcription factor T-box21 (murine analog for Tbet) results in clonal expansion of IL-17 producing T cells and accelerated allograft rejection. (Yuan et al. 2008) Furthermore, in wild-type mice, TLR-signaling promotes IL-6 and IL-17-dependent acute rejection bridging Th17 response and innate immune activation (Chen et al. 2009).

Tregs are a subset of T cells naturally originating from thymus with important role in balancing immune system during everyday life, especially during microbial infections and pregnancy. Disruption in Treg population may result in unwanted conditions such as autoimmune diseases, allergies, and tumor immunity. Furthermore, generation of alloantigen-specific Tregs may induce transplant tolerance by inhibiting costimulatory signals of T cells and thus preventing generation of alloreactive effector T cells. (Sakaguchi 2005; Ochando et al. 2006) Recent findings suggest clinical potential for adoptive transfer of ex vivo-expanded antigen-specific Tregs generated from naïve T cells in prevention of allograft rejection (Takasato et al. 2014).
3.5. Acute rejection

Fully allogeneic transplant is foreign tissue to the recipient and, therefore, is considered a threat. PMC, macrophages, and NK cells are the first allograft-infiltrating inflammatory cells early after the reperfusion. Leukocyte infiltration is physiological response to IRI and occurs both in syn- and allografts, and may inflict early graft injury without antigen processing and allore cognition. In syngrafts, this acute inflammation subsidizes in hours. In allografts, however, the direct and indirect allore cognition produces alloreactive effector T-cells, which invade the allograft. The presence of CD8+ T cells boosts innate immune mediated inflammation and inflicts prolonged neutrophil-mediated response. The CD8+ T-cells are also responsible for direct, MHC class-I-mediated destruction of allogeneic tissue (El-Sawy et al. 2004).

Hyperacute rejection of a solid organ transplant is a rare phenomenon seen in sensitized recipients with donor-specific antibodies, resulting from pre-existing antibodies against incompatible ABO blood group or HLA-antigens. The allograft is destroyed within minutes by thrombosis and devascularization. Hyperacute rejection is the main limitation for xenotransplantation (Williams et al. 1968; Mengel et al. 2012).

Acute cellular rejection results from alloreactive T cell proliferation and infiltration after allore cognition. CD8+ T cells and NK cells attack foreign cells either through foreign peptide encountering with MHC
class I receptor or via “non-self” recognition. CD4+ Th1-type T cells and macrophages are responsible for delayed hypersensitivity and inflammation. CD4+ Th2-type T cells and B cells are responsible for antibody-mediated rejection. (Rogers and Lechler 2001; Lakkis and Lechler 2013) Table 2. describes the 2004 revised grading of acute cellular and antibody-mediated rejection in heart transplants according to ISHLT consensus.

**Table 2. International Society of Heart and Lung Transplantation standardized grading of cardiac biopsy for acute cellular rejection (R) and antibody-mediated rejection (AMR). Modified from Stewart et al. J Heart Lung Transplant, 2005.**

<table>
<thead>
<tr>
<th>Grade 0</th>
<th>No rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1 R, mild</td>
<td>Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage</td>
</tr>
<tr>
<td>Grade 2 R, moderate</td>
<td>Two or more foci of infiltrate with associated myocyte damage</td>
</tr>
<tr>
<td>Grade 3, R severe</td>
<td>Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis</td>
</tr>
</tbody>
</table>

| AMR 0                        | Negative for acute antibody-mediated rejection                               |
|                              | No histologic or immunopathologic features of AMR                            |
| AMR 1                        | Positive for AMR                                                              |
|                              | Histologic features of AMR                                                   |
|                              | Positive immunofluorescence or immunoperoxidase staining for AMR (CD68+, C4d+) |
3.6. Immunosuppression

In order to prevent acute allograft rejection and to prolong allograft survival, the alloimmune response is suppressed with various immunosuppressive drugs (Table 3). Usual clinical protocol with triple-drug maintenance therapy consists of steroids, calcineurin inhibitor cyclosporine A or tacrolimus, and of antimetabolite azathioprine or mycophenolate mofetil, or T cell proliferation inhibitor sirolimus or everolimus. Induction therapy with antithymocyte globulin or with anti-IL2R-antibodies results in profound perioperative immunosuppression. With immunosuppressive medication, acute T-cell-mediated rejection is preventable. The immunosuppressive drugs, however, have undesirable side effects, and require constant monitoring. High level of immunosuppression also increases the risk of opportunistic infections (Lindenfeld et al. 2004a; 2004b; Baran 2013).
**Table 3.** Immunosuppressive drugs, their mechanism of action, and clinical use. AP-1, activation protein-1; IL, interleukin; NF-kB, nucleic factor kappa B; TOR, target of rapamycin.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids</td>
<td>AP-1, NF-kB inhibition</td>
<td>Induction, maintenance, antirejection therapy</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Cell cycle inhibitor of T (and B) cells</td>
<td>Maintenance therapy (to lesser extent)</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Calcineurin (and subsequently IL-2) inhibition</td>
<td>Maintenance therapy</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Calcineurin (and subsequently IL-2) inhibition</td>
<td>Maintenance therapy</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>Inhibitor of T and B cell proliferation</td>
<td>Maintenance therapy</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>TOR-dependent inhibition of lymphocyte proliferation</td>
<td>Maintenance therapy</td>
</tr>
</tbody>
</table>
3.7. Chronic rejection

Chronic rejection in cardiac allografts is described histologically as cardiac allograft vasculopathy (CAV) and clinically as allograft dysfunction resulting probably from prolonged and sustained chronic inflammation driven by ischemic injury and acute rejection, and from subsequent vascular remodeling. The pathogenesis of chronic rejection, however, is poorly understood but prognostic factors include preoperative graft ischemia time, episodes of acute rejection, donor age, and cytomegalovirus infection. In contrast to common coronary artery disease, allograft vasculopathy is histologically described as diffuse luminal occlusion of cardiac arteries and fibrosis development. The incidence of cardiac allograft dysfunction increases over time and limits the survival of transplant patients. Current immunosuppressive treatment fails to prevent the development of vasculopathy and cardiac fibrosis and subsequent dysfunction, and the only effective treatment for the disease is re-transplantation (Tanaka et al. 2005; Stehlik et al. 2012).

The scientific community generally considers indirect allore cognition and sustained Th1- and IFN-g-mediated alloimmune inflammation the driving force behind the development of vasculopathy. Several other factors, however, contribute to vascular inflammation and microvascular dysfunction leading subsequently to graft failure (Figure 6). Therefore, a combination of chronic inflammation and response-to-injury better describes the phenomenon.
Figure 6. The interactions between factors affecting the pathogenesis of cardiac allograft dysfunction and vasculopathy (modified from Schmauss and Weis, Circulation 2008).

Early innate immune response and following alloimmune activation described above are the initiating events in pathological vascular remodeling. Inflammatory cytokines IL-1, IL-6, and TNF-α promote vascular inflammation and SMC proliferation. Microvascular dysfunction after cardiac transplantation results in disruption in endothelial barrier function and microvascular leakage, but also in
no-reflow phenomenon. No-reflow occurs as a response to ischemic insult when the small arterioles and capillaries remain contracted and blood flow to the tissue is impaired, possibly due to contraction of endothelial cells and pericytes. Due to loss of capillaries and myocardial inflammation, the myocardium is gradually replaced with fibrotic tissue, accompanied with impaired contractile function. (Reffelmann et al. 2003; Schmauss and Weis 2008; Tuuminen et al. 2011) Chronic cardiac allograft dysfunction manifests histologically as cardiac fibrosis and allograft vasculopathy, which in turn is characterized clinically by diastolic dysfunction and heart failure (Hollenberg et al. 2001; Haddad et al. 2012).

Pollack et al. (2013) have recently reviewed extensively the diagnostic methods of evaluating CAV. Diagnosis of CAV is somewhat complicated, as the innervation of the heart is interrupted after the transplantation and the recipients do not feel any heart-related chest pain. The progressive intimal stenosis results ultimately in graft failure and, therefore, the clinical symptoms implicating problems appear too late. Current clinical protocols usually embrace routine transvenous biopsies taken regularly after the transplantation. Angiography is the most usual method of evaluating coronary arteries, however, the method is highly susceptible for interpretation error, as the occluded arteries seem to be in better shape than they actually are. Intravenous ultrasound is the most reliable method of evaluating the degree of intimal occlusion. Cardiac magnetic resonance is novel method of
evaluating risk of CAV development and CAV-associated changes, but does not directly detect or determine severity of CAV (Hofmann et al. 2014; Braggion-Santos et al 2014).

The incidence of CAV increases over time. According to the ISHLT Registry data, approximately 10% of patients are diagnosed with CAV one year after the transplantation. At ten years, approximately half of the patients have developed CAV-associated changes. (Stehlik et al. 2012) Therapeutic options in the treatment of CAV remain limited, as coronary dilatation with angioplasty or stenting, and coronary by-pass operations have resulted in high morbidity and are nowadays considered as palliative procedures. (Halle et al. 1995; Bader et al. 2006) Therefore, re-transplantation remains as the only definitive treatment for CAV, however, patients that have undergone re-transplantation have reduced survival after the operation (Srivastava et al. 2000; Johnson et al. 2007).
4. Angiopoietin pathway

4.1. General
The angiopoietins are vascular growth factors consisting of angiopoietin-1, -2, and -4 (Ang1, Ang2, Ang4), of which Ang1 and -2 act through their shared receptor tyrosine kinase Tie2 (Jones et al. 2001). The physiological role of Ang4 has not yet been defined. Tie1 and Tie2 are endothelial cell membrane receptors with high structural homology. Although discovered earlier than Tie2 receptor, little is known about the physiological role of Tie1 receptor. Tie1 seems crucial for endothelial cell survival, but not vasculogenesis (Partanen et al. 1996). Tie1 has been suggested to have blood flow-dependent impact on arteriosclerotic plaque formation, as Tie1 deletion reduces arteriosclerotic lesion formation (Woo et al. 2011). Tie1 and Tie2 both have cytoplasmic tyrosine kinase domains and are composed of two extracellular IgG-like domains, followed by three EGF-like domains, another Ig-like domain, and three fibronectin type III domains (Figure 8).

Angiopoietins regulate physiological microvascular integrity but also pathophysiological dysfunction. Ang/Tie2-signaling is essential for embryonic development, as Tie2-deficiency results in early embryonic lethality (Davis et al. 1996).
Figure 8. The angiopoietin/Tie2 signaling system. Ang1 and 2 both ligate to the same Tie2 receptor tyrosine kinase. Ang2 acts as a context-dependent agonist or antagonist to the receptor. Tie2 phosphorylation results in activation of PI3K/Akt pathway and downregulation of FOXO1 transcription factor, which in turn downregulates Ang2 expression (modified from Augustin et al. Nat Rev Mol Cell Biol 2009).

4.2. Angiopoietin-1

Ang1 has essential role in embryonic vascular development and vessel maturation; Ang1-deficient mice die early during the embryogenesis (Hanahan 1997). Ang1 stabilizes the immature and leaky vessels by promoting interactions between the ECs and perivascular structures (Gamble et al. 2000; Saharinen et al. 2008), and by regulating the endothelial cytoskeleton (Mammoto et al.)
Ang1 is constitutively secreted by endothelial supporting cells, such as pericytes and SMC, but also by fibroblasts, and several types of tumor cells (Davis et al. 1996; Stratmann et al. 1998; Sugimachi et al. 2003).

Tie2 phosphorylation by Ang1 induces signaling cascade that leads to recruitment of p85 subunit of phophoinositide 3-kinase (PI3K), and the activation of transcription factor Akt. Akt signaling promotes survival-associated pathways, and suppresses apoptotic pathways. Interestingly, Akt also inactivates Ang2-targeting forkhead transcription factor FOXO1 resulting in negative feedback loop on Ang2 production. On the other hand, inactivation of PI3K/Akt signaling activates FOXO1 and increases Ang2 production and EC destabilization (Papapetropoulos et al. 2000; Kontos et al. 2002; Daly et al. 2004; DeBusk et al. 2004).

Ang1/Tie2-signaling regulates EC-EC permeability by inhibiting VEGF-dependent adherens junction protein vascular endothelial (VE)-cadherin internalization from junctional complexes (Gavard et al. 2008). In addition, Ang1 and Tie2 form junctional complexes further stabilizing endothelium, but also its binding to extracellular matrix (Saharinen et al. 2008).

Ang1 acts as an anti-inflammatory cytokine as it inhibits leukocyte recruitment by preventing VEGF-induced endothelial adhesion molecule expression and after LPS induced endotoxic shock (Kim et
In addition, Ang1 induces KLF2 expression on endothelial cells counteracting VEGF-mediated VCAM-1 expression and inflammatory cell adhesion (Sako et al. 2009). Furthermore, Ang1/Tie2-signaling inhibits NF-kB-mediated inflammation via A20 binding inhibitor of NF-kB (ABIN) protecting the endothelial cells from inflammation and apoptosis (Hughes et al. 2003; Tadros et al. 2003).

4.3. Angiopoietin-2

Originally discovered 1997 as Ang1 and Tie2 receptor antagonist, the complex mechanism of Ang2 physiology remains somewhat unknown (Maisonpierre et al. 1997; Eklund and Saharinen 2013). Ang2 is not crucial for embryogenesis, as seemingly normal mice are born with Ang2-knockout background. However, the mice lacking Ang2 soon develop severe chylous ascites and die postnatally. Ang2 is normally stored in the Weibel-Palade bodies of EC with endothelin-1 (ET-1) and von Willebrand factor and IL-8, and is exocytosed upon stress, i.e., in ischemia, shear stress, and VEGF. Hypoxia induced RhoA activation and cytoskeletal changes have essential role in Weibel-Palade body exocytosis. (Vischer et al. 2000; Fiedler et al. 2004; Kuo et al. 2008). Recent studies have further shed light on Ang2 secretion by showing that Ang2 is released in exosomes when PI3K and Akt are inhibited, and that the release was regulated by syndecan/syntenin pathway. (Tsigkos et al. 2006; Ju et al. 2014) Ang2 selectively competes with Ang1 in binding to the Tie2 receptor and is considered to have antagonistic function on the
receptor signaling in resting endothelium (Maisonpierre et al. 1997). However, exogenous and endogenous Ang2 has been demonstrated to induce Tie2 phosphorylation under certain conditions (Kim et al. 2000; Daly et al. 2006). Ang2 has, therefore, context dependent function and can enhance neovascularization, promote EC survival, or increase vascular permeability (Oliner et al. 2004; Nag et al. 2005; Gallagher et al. 2007).

Ang2 expression increases in peripheral blood in several types of malignancies. Its role in tumor environment has been suggested to be closely linked to neovascularization, tumor growth and metastasizing. (Etoh et al. 2001; Scholz et al. 2007; Schulz et al. 2011) Furthermore, increased expression of Ang2 has been associated with primary graft failure in lung transplant patients and cardiac allograft vasculopathy development (Diamond et al. 2012; Daly et al. 2013). The inflammatory function of Ang2 partly manifests as sensitization of endothelial cells to TNF-a and by increase of endothelial adhesion molecules (Fiedler et al. 2006).
AIMS OF THE STUDY

The aim of this study was to characterize the effects of hypothermic preservation on ischemia-reperfusion injury and innate and adaptive immune responses in rat cardiac allografts, and to investigate the therapeutic effects of targeting the angiopoietin-1 and -2 signaling with clinically relevant protein and antibody treatment in this animal model.

The specific aims of were:

1. to describe the effects of hypothermic preservation on ischemia-reperfusion injury and innate and adaptive immune responses, and on the development of cardiac fibrosis and allograft vasculopathy;

2. to investigate the effect of donor heart treatment with recombinant and stable variant of native angiopoietin-1 on ischemia-reperfusion injury and subsequent development of innate and adaptive immune responses in cardiac allografts;

3. to investigate the kinetics and mechanisms of angiopoeitin-2 during ischemia-reperfusion injury and adaptive immune responses in rat cardiac allografts.
METHODS

1. Experimental rat cardiac transplantation model

Specific pathogen-free, inbred Dark Agouti (DA, RT1av1) rats weighing 200-250 g were used as cardiac allograft donors. The donor rat heart was perfused with ice-cold heparinized phosphate-buffered saline (PBS), and excised. In the treatment studies, the coronaries were then perfused with 200 µl of anti-Ang2 antibody (MEDI3617; 150 ng/ml, in PBS; MedImmune), or non-specific IgG (150 ng/ml, in PBS; MedImmune), or with recombinant Ang2 (50 µg/ml, in PBS) (Hwang et al. 2005), COMP-Ang1 (10 µg/ml, in PBS) or PBS. The donor heart was preserved in PBS at 4°C (cold ischemia) for 2 to 4 h, depending on the study model (Tuuominen et al. 2011). Warm ischemia was standardized to 1 h. 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, RT1u) recipient rats (Harlan Laboratories, Boxmeer, The Netherlands).

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 chronic rejection groups received cyclosporine A (Novartis, Basel, Switzerland) 2 mg/kg/day s.c. for the first 7 days and 1 mg/kg/day thereafter. For anesthesia and perioperative analgesia, the recipient rats inhaled isoflurane
(Isofluran, Baxter, Deerfield, IL) and received s.c. buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ).

The effect of cold preservation on the integrity of microvascular endothelial cells was analyzed by transmission electron microscopy in the non-transplanted DA donor hearts. After reperfusion, the recipients of cardiac allografts were sacrificed at 30 min to analyze the effect on microvascular perfusion and permeability with *Lycopersicon esculentum* (Tomato) lectin perfusion and modified Miles assay, at 6 h to analyze endothelial activation and inflammatory cell influx with immunohistochemistry, myocardial injury with serum troponin T (TnT) analysis, and innate immune activation with real-time quantitative reverse-transcription PCR, and at 8 weeks to analyze the degree of cardiac fibrosis and allograft vasculopathy with histological stainings.

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).

2. Transmission electron microscopy analysis

The mid-cardiac specimens were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4). The Advanced Microscopy Unit (Haartman Institute, University of Helsinki, Helsinki, Finland) prepared the samples by TEM-routine: samples were post-fixed in
1% osmium tetroxide, then dehydrated in graded ethanol, and embedded in Epoxy resin LX-112. Ultra-thin sections were cut at around 80 nm. Electron staining was performed with uranyl acetate and lead citrate in Leica EM STAIN automatic stainer. The sections were then examined with JEM-1400 TEM (JEOL, Tokyo, Japan) at 80 KV and analyzed the incidence of endothelial cell (EC)-EC junction gaps and EC blebbing with 25000x magnification.

3. Immunohistochemistry and immunofluorescence stainings

Cryostat sections were stained for subsets of inflammatory cells and 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 568 red and Alexa 488 green (Promega, Madison, WI) secondary antibodies.

The following antibodies and dilutions were used: Ang2 (10μg/ml, AF623, R&D Systems); RECA-1 for rat endothelium (50 μg/ml, MCA97, AbD Serotec, Dusseldorf, Germany); FLAG for COMP-Ang1 (10μg/ml, Sigma-Aldrich Co, St. Louis, MO); CD4 for T cells (5 μg/ml, 22021D, BD Pharmingen, San Diego, CA), CD8 for T cells (5 μg/ml, 22071D BD Pharmingen), ED1 for macrophages (5 μg/ml, 22451D, BD Pharmingen); MPO for neutrophils (20 μg/ml, ab9535, Abcam, Cambridge, UK); OX62 for dendritic cells (10 μg/ml, MCA 1029G, Serotec, Oxford, UK); VCAM-1 (10 μg/ml, MMS-141P, Covance, Princeton, NJ); ICAM-1 (10 μg/ml, 1A29, Seikagaku, Tokyo, Japan).
The immunoreactivity was quantified in a blinded manner using 400x magnification.

4. Histological evaluation
Paraformaldehyde-fixed paraffin mid-cardiac cross sections were used for histological stainings. The degree of cardiac fibrosis was determined 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. Cardiac allograft vasculopathy was evaluated 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.

5. Enzyme-linked immunosorbent assay
To quantify Ang1 and Ang2 from human and rat serum samples, ELISA was performed according to the Manufacturer’s instructions with following kits: human Ang1 (DANG10; R&D Systems Inc., Minneapolis, MN); human Ang2 (DANG20; R&D Systems); rat Ang2 (E90009Ra; Uscn Life Science Inc., Wuhan, China).
6. Cell culture assays
Human dermal blood microvascular endothelial cells (BECs) (PromoCell, Heidelberg, Germany) were maintained on fibronectin-coated dishes in Endothelial Cell Basal Medium MV (ECBM, PromoCell) with growth supplements provided by the manufacturer and used in passages 2-6. Hypoxic treatment (1% O₂) was carried out in Invivo₂ 400 hypoxia chamber (Ruskinn Technology, Bridgend, UK). Tie2-GFP retroviral transfected cells were produced as described (Saharinen et al. 2008). Cells were treated with non-specific IgG (2 mg/ml; MedImmune, Gaithesburg, MD) or anti-Ang2 antibodies (MEDI3617; 2 mg/ml; MedImmune) or with COMP-Ang1 (Cho et al. 2004) (50 ng/ml). The cells were fixed, permeabilized, and stained for Ang2, Tie2, or COMP-Ang1.

7. Microvascular leakage and perfusion assay
A modified Miles assay was used to measure extravasation of plasma proteins from the microvasculature into the interstitial space of cardiac allografts: immediately after reperfusion, the recipients were injected i.v. with Evans Blue dye (Sigma-Aldrich; 30 mg/ml diluted in 0.9 % NaCl), and allowed this to circulate for 30 min. To detect perfused vessels 30 min after reperfusion, 50 ml FITC-labeled Lycopersicon esculentum (Tomato) lectin (Vector Laboratories, Burlingame, CA) diluted in 150 ml of 0.9% NaCl was injected to the aortic root a few minutes before removing the allografts. Immediately thereafter, the right auricle was incised and the coronary network was flushed with 5 ml of 1 % PFA in 0.05 M citrate
buffer, pH 3.5. For quantification of extravasated Evans Blue, 100 mg of apical myocardium was incubated into 500 ml of formamide on a shaker at +60 °C for 24 h. The absorbance of dissolved Evans Blue dye was then measured from the formamide by spectrophotometry at 610 nm wavelength. The mean density of FITC positive microvascular vessels was quantified from mid-axial cross sections from 10 random fields of each cardiac cross section by fluorescence microscopy.

8. Gene expression analysis
Total RNA was isolated from tissue samples dissected from the myocardial midpiece of the allografts using RNeasy kit according to the Manufacturer’s instructions (Qiagen, Hilden, Germany) and reverse transcribed by using the High-RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on a RotorGene-6000 (Corbett Research, Doncaster, Australia) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland) and the mRNA quantities of the following factors were measured from each group: innate immune receptors TLR2 and TLR4, their ligands biglycan, HAS1-3, HMGB1, transcription factor NF-kB, dendritic cell (DC) maturation markers CD80, CD86, and CD83, as well as inflammatory cytokines TNF-a, IL-1b, IP-10, TGF-b, IL-6 and IL-10. 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 and therefore all RT-PCR data were normalized against 18SRNA.

9. Heart transplant patients
The data consists of 11 cardiac allograft donors (8 men) and 11 recipients (8 men) operated between 2010 and 2011 in Helsinki University Central Hospital, Helsinki, Finland. Healthy volunteers were used as a control group (n = 24; 9 men). The mean age of the donors was 40.9±14.1 years (range 19-56 years), of the recipients 47.4±11.4 years (range 29-63 years), and of the healthy volunteers 34.9±11.9 years (range 22-61). Blood samples were collected preoperatively from cardiac allograft donors and postoperatively from the recipients 1, 6, and 24 h after transplantation and ELISA was used to measure the serum levels of Ang1 and Ang2. The Ethical Committee of University of Helsinki, Helsinki, Finland approved the use of the patient samples, and each patient gave written informed consent.

10. Statistical analysis
All data are two group comparisons were performed by Mann-Whitney U test; multiple group comparisons were performed by Kruskal-Wallis analysis with Dunn correction, or by repeated measures ANOVA using PASW Statistics 19.0 (SPSS Inc., Chicago, IL). P<0.05 was considered statistically significant.
RESULTS

1. Prolonged ischemic preservation promoted ischemia-reperfusion injury-mediated myocardial injury and inflammation in rat cardiac allografts (I)
Myocardium is highly susceptible to hypoxic injury. Cardiomyocyte injury is therefore inevitable during organ recovery and preservation. Prolonged ischemic time resulted in profound myocardial injury, but also increased the influx of inflammatory cells into the allograft. The acute Tx-IRI phase was characterized by increased expression of innate immune activating endogenous danger molecules and influx of innate immune macrophages and neutrophils alongside with induction of inflammatory cytokines IFN-γ, IL-6, and TNF-α.

2. Prolonged ischemic preservation enhanced chronic rejection (I)
As regards alloimmune activation, allografts transplanted after prolonged ischemic preservation exhibited increased influx of CD4+ and CD8+ T cells, as well as macrophages, 10 days after the transplantation and under subclinical immunosuppression. Interestingly, during Th1-inhibiting CsA immunosuppression, prolonged preoperative ischemic preservation resulted in shifting of the cytokine profile of allograft-infiltrating T cells towards Th17 alloimmune response. This was seen as increased IL-17 and IL-23p19 gene expression. Paradoxically, Treg-associated FoxP3 transcription factor expression was also robustly induced during alloimmune
response. Increased myocardial injury, acute and sustained inflammation reflected as accelerated the development of cardiac fibrosis, loss of functional capillary network, and arterial luminal occlusion.

These findings emphasize the importance of early allograft injury and inflammation in the development of late dysfunction, and suggests early protection of allografts as potential target for treatment with long-lasting effects.

3. Hypoxia induced endothelial cells to release Ang2 (unpublished)
Previous findings indicate that hypoxia regulates Ang2 expression in human umbilical vein endothelial cells (Pichiule et al. 2004). Human blood microvascular endothelial cells were incubated in hypoxic environment for 1, 3, 8, 12, or 24 hours, and the culture medium was analyzed for Ang2 with ELISA. In congruence with previous findings, the data showed 2-fold increase in Ang2 secretion in response to hypoxia during 24-hour incubation, when compared to cells incubated in normoxia (Figure 9).
Figure 9. Ang2 release by human microvascular cells in normoxia or hypoxia.

When Tie2-overexpressing ECs were incubated in hypoxia, the secreted Ang2 localized into EC-EC junctions and associated with Tie2. Application of anti-Ang2 antibody blocked this Ang2/Tie2 co-localization (Figure 9A). Similarly, exogenous Ang1 applied to the culture localized with Tie2 receptor in normoxia, but to lesser extent during hypoxia indicating competitive binding to Tie2 with Ang2 (Figure 10 B-C).
**Figure 10.** Hypoxia induces Ang2 deposition in endothelial cell-cell junctions and inhibits Ang1-Tie2 binding in endothelial cells. **A,** Human dermal blood microvascular cells (BECs) transfected with Tie2-GFP retrovirus were subjected to normoxia or hypoxia (1% O2) for 16 h in the presence of control-IgG or anti-Ang2 antibody (2 μg/ml). Immunofluorescent staining shows the localization of Tie2 (green) and Ang2 (red); DAPI indicates nuclei (blue). **B,** Tie2-GFP retrovirus transfected BECs were cultured in normoxia or hypoxia (1% O2) for 24 h, and stimulated with COMP-Ang1-FLAG (50 ng/ml) for the last 30 min. **C,** fractional area of Tie2/FLAG double-positive pixels from total area quantified from samples incubated in normoxia and hypoxia with or without COMP-Ang1. Immunofluorescent staining shows the localization of Tie2 (green) and COMP-Ang1 (FLAG; red). DAPI indicates nuclei (blue). Scale bars = 20 μm.
4. Cardiac transplantation induces immediate changes in angiogenic growth factor expression in human and in rat (III)

Heart transplant donors and recipients were analyzed for serum levels of Ang1 and Ang2 before organ donation and during the first 24 h after the transplantation. No changes were observed in the serum levels of Ang1 in the donors (Figure 11A). The expression of Ang2 was increased in brain dead heart donors compared to healthy volunteers (Figure 11B). In the recipient patients, heart transplantation induced progressive decrease in Ang1 levels and increase Ang2 expression during the first 24 h (Figure 11C,D). Similarly, in experimental rat cardiac transplantation, allogeneic heart transplantation resulted in serum release of Ang2, but interestingly reduced expression of Ang1 in peripheral blood. Syngeneic transplant recipients, however, showed no changes in serum levels of Ang2.
Figure 11. Peripheral blood analysis for Ang1 and 2 levels in cardiac allograft donors after brain death and in cardiac allograft recipients after transplantation. A and B, Human plasma samples from hemodynamically stable cardiac allograft donors (D; n = 19) before organ procurement and compared to healthy controls (CTRL; n = 24) analyzed by enzyme-linked immunosorbent assay (ELISA). C and D, Human plasma samples from cardiac allograft recipients (n=19) before transplantation (0 h) and 6, and 24 h after transplantation analyzed by ELISA. Data are given as scatter plot where the black bar indicates mean. ***P<0.001 by Repeated ANOVA measurements.

5. Targeting Ang/Tie2 pathway prevented Tie2-dependent endothelial destabilization and microvascular dysfunction induced by ischemic preservation (II-III)

Rat heart procurement and hypothermic preservation resulted in endothelial destabilization, morphological changes in EC, and disruption of EC-EC connections observed with transmission electron microscopy analysis. When the allografts were ex vivo perfused either with recombinant Ang1 or with anti-Ang2 antibody, these changes were absent.
Allograft tissue perfusion was measured from the apex of the heart with laser Doppler velocimetry immediately after reperfusion. *Ex vivo* donor heart treatment either with COMP-Ang1 or with anti-Ang2 antibody resulted in significantly improved myocardial tissue perfusion during the first 10 minutes after reperfusion. This effect was dependent on functional capillaries, as the histologically determined numbers of perfused vessels were increased 30 min after the reperfusion in the treatment groups, whereas the absolute numbers of vessels were indifferent.

Endothelial stability was further assessed by microvascular leakage in modified Miles assay, in which the recipient is perfused with albumin-binding Evans Blue dye, and the extravasation of the dye is assessed 30 min after the reperfusion. Both donor heart-targeted *ex vivo* COMP-Ang1 and anti-Ang2 antibody treatment stabilized the allograft endothelium and preserved functional capillary network, whereas Tx-IRI in control allografts resulted in reduced capillary density and increased Evans Blue extravasation.

*Ex vivo* donor heart treatment with COMP-Ang1 or anti-Ang2 antibody prevented such early inflammatory response by reducing the influx of inflammatory cells as well as downregulating the gene expression of TLR-activating ligands. Importantly, these findings were associated with significantly lower immunoreactivity of endothelial adhesion molecules ICAM-1 and VCAM-1.
6. **Acute rejection can be restrained by endothelial inactivation (III)**

Alloimmune response is imminent after allogeneic solid organ transplantation. Prolonged preoperative ischemia prior to allogeneic cardiac transplantation, however, worsens the acute innate immune response in the absence of immunosuppressive medication. In our experimental model, the fully MHC-mismatched cardiac allografts are rejected in 5 or 6 days, if the recipients are not immunosuppressed. When daily administration of CsA 1mg/kg i.p. was applied, the allograft survival extended to 10-15 days. Recipient treatment with single-dose of anti-Ang2 antibody failed to prolong allograft survival with this background immunosuppression. However, when the administration was continued on days 1, 3, and 5 after the transplantation, the survival was highly significantly prolonged (Figure 12).
Figure 12. The effect of systemic recipient anti-Ang2 antibody treatment on rat cardiac allograft survival. Red dash line represents the survival of allografts transplanted after 4-h hypothermic preservation and without any immunosuppression. Red line represents allograft survival in recipients treated CsA 1 mg/kg s.c. and with non-specific IgG i.p. 4h before the transplantation. Green line represents allograft survival in recipients treated CsA 1 mg/kg s.c. and with anti-Ang2 antibody i.p. 4h before the transplantation. Blue line represents allograft survival in recipients treated CsA 1 mg/kg s.c. and with anti-Ang2 antibody i.p. 4h before, and on days 1, 3, and 5 after the transplantation. Data are presented by Kaplan-Meier survival plot. ***P<0.001 by Log Rank analysis.

7. Cardiac allograft vasculopathy development is correlated with preoperative ischemia time and the number of endothelial cells and pericytes (I-III)
Cardiac allograft vasculopathy was associated with gradual loss of rat endothelial cell antigen-1 (RECA-1) positive capillaries, but also NG2-positive pericytes. Donor heart treatment with COMP-Ang1 inhibited early endothelial activation, but also preserved the numbers of capillaries and the pericytes surrounding them (Figure 13).
Figure 13. The density of RECA-1+ EC (A) and NG2+ pericytes (B) in COMP-Ang1- and PBS-treated allografts at 6 h, 10 d, 56 d. Representative immunofluorescence images from different timepoints (C). Scale bar = 40 µm. Data are given as mean ± SEM. *P<0.05, **P<0.01 by the Mann-Whitney U test.

Ischemic time in clinical cardiac transplantation is an independent risk factor for cardiac allograft vasculopathy development. In a chronic rejection model, rat cardiac allografts were transplanted to recipient rats receiving subclinical immunosuppression to prevent episodes of acute rejection. This treatment protocol enabled chronic rejection associated arterial luminal occlusion and myocardial fibrosis development in 2-month follow-up. Cardiac allograft vasculopathy changes were correlated with allograft preoperative ischemic time (Figure 14).
Figure 14. Cardiac allograft vasculopathy analyzed 56 d after rat allogeneic heart transplantation. A, B, and C, the incidence, occlusion, and representative histological images of arterial luminal occlusion in allografts transplanted after 0, 2, or 4 h hypothermic preservation. D and E, myocardial fibrosis analyzed semi-quantitatively from Masson-Trichrome stainings. Scale bar = 40 μm. Data are given as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 by the Mann-Whitney U test.
Donor heart ex vivo treatment either with recombinant COMP-Ang1 protein, or with anti-Ang2 antibody significantly inhibited the development of aforementioned chronic rejection-associated changes. Systemic recipient treatment with anti-Ang2 antibody had similar effects on allograft vasculopathy development, indicating that early inflammation is crucial.
DISCUSSION

Solid organ transplantation can be regarded as one of the crown achievements of modern surgery. Replacing failing livers, lungs, or hearts with functional grafts from brain dead donors gives second chance to those patients with otherwise virtually no hope for living. The median survival of heart transplant recipients has exceeded a decade many years ago, and it keeps prolonging (Stehlik et al. 2012). Primary graft dysfunction and allograft vasculopathy are important factors limiting short- and long-term survival of cardiac transplant recipients. This study emphasizes the role of hypothermic preservation, and moreover, IRI as an inductor of microvascular dysfunction, and innate and adaptive immune responses leading to primary and late allograft dysfunction after cardiac transplantation. The results presented here shed light on the characteristics behind IRI-induced inflammation and allograft dysfunction, and their impact on the development of cardiac fibrosis and allograft vasculopathy. Tie2 signaling was proven important, as supplementation of recombinant Tie2 agonist COMP-Ang1 or inhibition of Tie2 antagonist Ang2 with specific antibody resulted in significant improvement in microvascular function and allograft survival, as well as inhibition of innate and adaptive immune responses and the development of cardiac fibrosis and allograft vasculopathy.

Donor aspects and preservation

Incredible success of clinical heart transplantation has with increasing numbers of heart transplant recipient candidates resulted
in chronic shortage of donated organs. This shortage has required extending the donor pool to marginal, or extended criteria donors, who are older and sicker than previously accepted. Furthermore, the brain death of organ donor may induce a release of catecholamines, a systemic inflammatory response, microvascular endothelial cell and pericyte dysfunction, and myocardial injury (Venkateswaran et al. 2009). Both longer ischemic time and increased donor age are independent risk factors for 1-year mortality after the transplantation (Lund et al. 2013).

Grafts from donors with extended criteria are especially susceptible to primary graft dysfunction and injury requiring novel treatment strategies to prevent Tx-IRI-induced effects (Iyer et al. 2011; Abecassis et al. 2012). An experimental rat study demonstrated worse cardiac function in grafts from older donor rats during early reperfusion phase compared to grafts from younger donors (Korkmaz et al. 2013). Recipients with grafts from marginal donors have higher risk for primary graft failure and require temporary circulatory support with i.e. extracorporeal membrane oxygenation (Listijono et al. 2011). The use of temporary circulatory support, however, is again a significant risk factor for 1- and 5-year mortality (Lund et al. 2013).

Current clinical organ preservation protocols abrogate Tx-IRI only partially. With current immunosuppressive regimen, the episodes of acute rejection are preventable, but chronic rejection still
significantly limits the survival of cardiac allograft recipients (Lund et al. 2013). According to 2012 ISHLT Registry data, median ischemic time for cardiac transplant is 3.1 hours. At the same time, ischemic time exceeding 200 minutes is an independent risk factor for cardiac allograft vasculopathy development. (Stehlik et al. 2012)

The problems with donors with extended criteria and ischemic time may be manageable with novel preservation techniques, solutions, or treatment options. Ex vivo heart perfusion machines are long studied with little progression regarding the heart (Jacobs et al. 2010). However, Organ Care System for ex vivo perfusion of lung allografts has shown promising potential and is currently further investigated (Warnecke et al. 2012). Novel preservation solutions are investigated in order to preserve grafts longer and in warmer temperatures (Thatte et al. 2009; Lowalekar et al. 2013). Similarly, solution with small interfering RNA against proinflammatory cytokines and complement has proven potential in prolonged allograft preservation (Zheng et al. 2009).

**Reperfusion phase**

Ischemia is not the sole determining factor for myocardial injury in Tx-IRI, as syngeneic rat cardiac transplants with similar preoperative ischemic time exhibit significantly milder cardiomyocyte injury than allogeneic transplants (Keränen et al. 2013). Allorecognition initiates after allogeneic solid organ transplantation regardless of T cell-targeted immunosuppression. The duration of hypothermic
preservation, however, dictates the intensity of innate immune response after reperfusion of the allograft (I).

Tx-IRI is an inevitable factor in solid organ transplantation, but differs greatly from conventional IRI occurring in the treatment of stroke or acute coronary syndrome. Rapid neutrophilic infiltration is resolved in 24 h in syngrafts, whereas infiltrating CD8+ T cells induce sustained neutrophilic influx to allografts (El-Sawy et al. 2004). Furthermore, hypothermic preservation of cardiac allografts during organ recovery and transportation induces ischemic injury to cardiomyocytes, but also inflicts endothelial cell and pericyte detachment from one another and disruption of endothelial barrier (Tuuominen et al. 2011). Longer preservation promotes endothelial and pericyte dysfunction that manifests as myocardial capillary perfusion defect, microvascular leakage, vasoconstriction, and EC activation. Activated EC increase adhesion molecule expression and EC-EC junction disruption allows easier transmigration of inflammatory cells. Together with increased accumulation of inflammatory cells and secreted proinflammatory cytokines, perfusion defect induces significantly worse cardiomyocyte injury after prolonged preservation. (II-III)

**Immunological issues**

Foreign tissue transplanted to allogeneic recipient is, without immunosuppression, rapidly rejected by innate immune cells such and NK cells and adaptive immune response involving antigen-
presenting cells and the generation of allograft-specific IFN-γ producing Th1-type T cells (Lehmann et al. 1997). Endothelial cells are extremely important in the course of immunological recognition, inflammatory cell recruitment, and myocardial perfusion. EC express both MHC class I and class II molecules on their cytoplasmic surfaces, and therefore, can communicate with both CD4+ and CD8+ T cells and induce IL-2 production. EC have, however, been shown to stimulate only memory, but not naïve CD8+ T cells to become cytolytic T cells. (Hancock et al. 1982) Activated EC may drive sustained inflammation behind allograft rejection by inducing local IFN-γ secretion by T cells (Salomon et al. 1991; Tellides and Pober 2007). Type I activation of EC is gene transcription-independent and results from histamine or thrombin, and manifests as nitric oxide or prostaglandin secretion, or Weibel Palade body exocytosis, resulting in vasodilatation, increased perfusion, and permeability disorder. Type II activation is described as transcription-dependent adhesion molecule upregulation induced by cytokines such as TNF-α, and IL-1 (Pober and Sessa 2007). Activated EC promote leukocyte activation and transmigration by secretin cytokines and chemokines, i.e. PECAM-1, IL-8, IP-10, MCP-1, and RANTES (Al-Lamki et al. 2008).

Endothelium is the physiological first line barrier and defence between circulating inflammatory cells and the allograft. Therefore, injury to EC greatly contributes to cardiac allograft vasculopathy development. In allogeneic environment, macrophages, cytotoxic CD8+ T cells and natural killer cells induce EC apoptosis by non-self
recognition, Bax-induction, death receptor-signaling, or by TNF-related apoptosis-inducing ligand (Zheng et al. 2000; Hsieh et al. 2002; Bleackley 2005; Wyburn et al. 2005). EC are, however, resistant to injury and apoptosis by upregulating anti-apoptotic zinc finger protein A20, heme oxygenase-1, Bcl-2, or the PI3k/Akt pathway. (Soares et al. 1999; Madge and Pober 2000)

Our findings suggest that early EC activation may be a key mediator of chronic inflammation, and that preventing this activation results as significant improvement in allograft function and survival. (II-III)

Cardiac allograft vasculopathy and late graft dysfunction
The early innate immune response following solid organ transplantation is described by infiltration of inflammatory cells consisting mainly of macrophages, neutrophils and NK cells depending on cellular adhesion molecules (El-Sawy et al. 2004; 2005). Dendritic cells and macrophages rapidly recognize foreign, allogeneic antigens, but also allograft-derived TLR-activating danger molecules result in innate and alloimmune activation (Goldstein 2004). Immunosuppressive drugs mainly target IL-2-mediated T cell activation and proliferation; corticosteroids also inhibit but fail to completely prevent innate immune activation. Of inflammatory cytokines, IL-6 is extremely important in early CD4+ T cell mediated inflammation and allograft rejection. In allogeneic murine cardiac transplant model, recipient anti-IL-6 antibody treatment results in prolonged survival (Lei et al. 2010). In a similar experimental setting,
but with recipient mice depleted of CD8+, IL-6 inhibition results in virtually non-existent rejection, even when administered 6 d after transplantation (Booth et al. 2011).

The answer to the pathophysiology of cardiac allograft vasculopathy remains yet to be fully elucidated. On the cellular level, T cell driven alloimmune response may be considered the main driving force behind allograft vasculopathy development. Accelerated Th17 alloimmune response results in allograft rejection in Th1-deficient mice (Yuan et al. 2008), and also seemed to promote chronic rejection development in our experimental model (I). Different T cell depletion strategies are used in clinical practice mainly as an induction therapy in kidney transplantation, but to lesser extent in other organs. Rapid release of cytokines, opportunistic infections, lymphoproliferative diseases, and T cell repopulation are most common problems, and despite of effectively promoting immunosuppression early after transplantation, T cell depletion fails to induce tolerance (Page et al. 2013).

As described above, EC have high potential in inducing or regulating inflammatory responses after allogeneic solid organ transplantation. Based on results presented in papers II and III, endothelial stabilization and inactivation are crucial for allograft survival and evasion from rejection. With inactivated endothelium, the need for T cell depletion-mediated induction therapy may reduce.
**Angiopoietins in solid organ transplantation**

Angiogenic growth factors are intensively studied in the field of cancer science, as angiogenesis is crucial for tumor growth and metastasis (Welti et al. 2013). Interestingly, the same factors that promote tumor growth elicit vascular instability in solid organ transplantation.

Endothelial detachment is essential for neovascularization and VEGF is a potent angiogenic growth factor inducing rapid and prominent vascular leakage (Nagy et al. 2012). VEGF activates EC in transplantation setting, and neutralization of VEGF has anti-inflammatory effect in cardiac allografts by inhibiting the expression of endothelial cell adhesion molecules and the production of several chemokines (Lemström et al. 2002; Reinders et al. 2003). These VEGF mediated pro-inflammatory effects are possibly downstream of Ang2 signaling, and preventable by Ang2 inhibition (III). Another study shows that neutralization of Ang2 reduces endothelial adhesion molecule expression in murine ischemic limb model (Tressel et al. 2008). Rat cardiac allograft treatment *ex vivo* with anti-Ang2 antibody reduces VEGF and TGF-b mRNA expression after the transplantation and subsequently inhibits vascular permeability and perfusion disorder, and fibrosis development (III).

Ang1 elicits vascular stability and homeostasis during normal physiology. Ang1 signaling through Tie2 receptor promotes anti-apoptotic signals particularly by activating PI3k/Akt pathway and
ABIN (Papapetropoulos et al. 2000; Tadros et al. 2003). Furthermore, type I endothelial activation may be avoided by Ang1, which prevents VEGF-induced plasma leakage and enhances EC-EC connections at cell junctions. Ang1 also reinforces blood vessel coating by pericytes and surrounding extracellular matrix. (Thurston et al. 2000; Saharinen et al. 2008) Donor heart-targeted Ang1 treatment stabilized microvascular endothelium of the allografts and inhibited innate and alloimmune responses (II). Maintaining EC viability and quiescence is therefore of great importance for allograft survival.

**Future prospects**

**A. Donor perspective**

Currently, transplant units suffer from chronic shortage of donated organs. Recognizing potential organ donors is a key factor in increasing the numbers of solid organ transplants. In addition, due to the shortage of organ donors, older and sicker patients are accepted as organ donors. This makes donor management important issue. Brain death of heart transplant donors causes systemic cytokine storm. Circulating inflammatory cytokines may prime the heart for subsequent inflammation and injury. Current clinical protocols include donor treatment with systemic steroids to prevent the adverse effects of this cytokine storm, but more targeted treatment may be advantageous. Donor preoperative simvastatin treatment in experimental rat cardiac transplantation model protects the allograft from post-operative problems, such as
Tx-IRI and chronic rejection development (Tuuminen et al. 2011). Our studies have pointed Ang2 to be increased in the donor peripheral blood before organ procurement, and therefore a potential target for donor treatment.

B. Allograft (preservation) perspective

The allograft is subjected to hypothermia and ischemia during the preservation. Organ preservation solutions are designed to protect the allograft from adverse effects of hypoxia, acidosis, low glucose levels, and edema (Jacobs et al. 2010). Antioxidants are included also in some. Potential treatment targets are the oxygen metabolism producing reactive oxygen species during ischemic preservation, endothelial protection and inactivation, or prevention of allorecognition. Solution with small interfering RNA against TNF-α, C3, and Fas has shown promising potential in prolonged allograft preservation (Zheng et al. 2009). Also, novel preservation solutions are designed to enable the allografts to be preserved in milder hypothermia, or even normothermia (Thatte et al. 2009; Lowalekar et al. 2013). Restoration of circulation with perfusion system after allograft recovery has been studied for decades, however, the costs and perfusion system management issues have resulted this technique to be concluded as inferior to static hypothermic preservation of the grafts (Cobert et al. 2008; Jacobs et al. 2010).
C. Recipient perspective

Current immunosuppressive protocols mostly target IL-2-mediated adaptive immunity. Novel immunomodulatory strategies that target anything else may reduce the need for classical immunosuppressants. Recipient treatment with statins has long been standard treatment for their anti-inflammatory properties (Kobashigawa et al. 1995; Shimizu et al. 2003). Angiopoietins play crucial role in maintaining EC quiescence. The endothelium acts as the definitive barrier between the transplant and the effector cells of alloimmune system, and therefore it may be hypothesized that the alloimmune response may be absent if the endothelium is preserved intact and non-adhesive to inflammatory cells. Our results show that Ang1 substitution or Ang2 neutralization inhibit early influx of inflammatory cells, and also the early alloimmune response, as the gene expression of hallmark cytokines IL-2, IL-6, IL-12 were downregulated. Ang1 has therefore potential in allograft protection during the preservation. Ang2 competes with Ang1 in binding to the Tie2 receptor, promotes inflammation and endothelial activation, and acts as a chemokine to inflammatory cells. Neutralization of Ang2 may therefore be beneficial during the preservation, but also during the acute phase of inflammation after the reperfusion.
Limitations
The experimental rat cardiac allograft model used in this study is a non-functional and heterotopic model. Therefore, the model represents clinical cardiac transplantation mainly immunologically. The model excludes donor brain death, which produces systemic cytokine storm and induces early innate immune response in clinical patients. The treatment agents used in this study are not in clinical use, but are currently in clinical trials.

Conclusions
This study emphasizes the importance of ischemia and reperfusion on endothelial integrity in cardiac allografts. With functional and intact endothelium, the key mediators of inflammation and alloimmune response are unable to reach the allograft. This results in delayed allorecognition and subsequently reduced development of cardiac allograft vasculopathy. Angiopoietin signaling was proven important in endothelial stabilization, either by supplementation of Ang1 or inhibition of Ang2. Further studies are warranted to investigate the clinical potential of Ang1 and inhibition of Ang2 in human solid organ transplant recipients. In human patients, recombinant Ang1 may be added to cardioplegia solutions and anti-Ang2 antibody may be beneficial in induction therapy of the allograft recipient.
YHTEENVETO


Angiopoietiini-1 ja -2 (Ang1 ja 2) ovat Tie2-reseptoriin sitoutuvia verisuonikasvutekijöitä, joilla on tärkeä merkitys verenkiertoelimistön sikiöaikaisessa kehityksessä. Verisuonien tukisolut erittää jatkuvasti Ang1:tä, joka ylläpitää verisuonen seinämän tasapainoa kehittyneessä verisuonistossa. Ang2 puolestaan vapautuu verisuonen seinämän endoteelisoluista hapenpuutteen tai tulehduksen vaikutuksesta ja houkuttelee paikalle tulehdussoluja sekä muuttaa verisuonen seinämän läpäiseväämmäksi.

Tutkimuksessa rotan sydänsiirtomallissa siirteen sepelvaltimoihin irrotuksen yhteydessä ruiskutettu Ang1-proteiini stabiloi siirteen verisuoniston seinämän ja Ang2-vasta-aine esti iskemia-reperfuusiovaurioon liittyvän tulehduksen ja sydänlihasvaurion, sekä hiussuoniston läpäisevyys- ja perfuusiohäiriön. Tämä alku vaiheen suojavaikutus hillitsi myös siirteiden kroonisen hyljinnän muutoksien kehittymistä. Sydänsiirteen vastaanottajalle systeemisesti annettu vasta-aine ei vaikuttanut verisuoniston läpäisevyteen, mutta esti siirteen tulehduksen, akuutin hyljintäreaktion ja kroonisen hyljintäreaktion kehittymisen.

Elinäintopotilaan ennustetta rajoittavat merkittävästi siirteen varhaisvaiheen pettäminen ja kroonisen hyljintäreaktion aiheuttama

Tässä tutkimuksessa osoitettiin koe-eläinmallissa, että estämällä angiopoietiini-2 (Ang2) vaikutus vasta-aineella paikallisesti sydänsiirteessa tai siirteen vastaanottajassa vähennetään siirteen varhaisvaiheen sydänlihasvauriota ja tulehdusvastetta. Varhaisvaiheen hoidolla oli myös pitkäaikainen hyöty, sillä hoidetut siirteet selvisivät pidempään ilman akuuttia ja kroonista hyljintäreaktiota.
ACKNOWLEDGMENTS

This study was carried out in the Transplantation Laboratory, Haartman Institute, University of Helsinki, and Helsinki University Central Hospital. This work was supported by grants from the Helsinki University Central Hospital Research Funds, the University of Helsinki, the Sigrid Juselius Foundation, the Academy of Finland, the Finnish Foundation for Cardiovascular Research, the Päivikki and Sakari Sohlberg Foundation, the Paulo Foundation, the Aarne Koskelo Foundation, the Ida Montin Foundation, and the Emil Aaltonen Foundation.

I wish to express my sincere gratitude to my Supervisor Professor Karl Lemström for his enthusiastic guidance and passion for science. You have allowed me to quite freely fulfill myself scientifically, yet you have kept my feet on the ground and persistently demanded top performance and hard work. I feel privileged to have been able to work for and with you.

I also wish to return special thanks to:

The Reviewers of this work appointed by the Faculty of Medicine, Professor Timo Paavonen and Professor Hannu Sariola for your advice and insight over the topic.

Professor and Dean of the Faculty of Medicine Risto Renkonen, the Head of the Transplantation Laboratory, for introducing me to the academic world and to my Supervisor, and for constant encouragement and support.
Professor Emeritus Pekka Häyry for establishing our laboratory, and for inspiring stories of science, traveling, and the dawn of transplantation medicine.

My co-authors Kari Alitalo for sharing invaluable ideas and connections around the world; Gou Young Koh for providing recombinant Ang1 and 2; Ching Ching Leow from MedImmune for providing anti-Ang2 antibody; Pipsa Saharinen for sharing your expertise of angiopoietin signaling; Markku Tammi for your help with hyaluronic acid; Antti Nykänen for your help, friendship, and excellent discussions; Raimo Tuuminen, Alireza Raissadati, Alexey Dashkevich for your help and friendship throughout the years; Ralica Arnaudova for your help in lab work; Rainer Krebs for being the wizard you are; Mikko Keränen for introducing me to the everyday life of the Transplantation Laboratory, for your help, friendship, and shared experiences around the world.

The other members of our research group: Eeva Rouvinen for teaching me laboratory practice and for your extremely invaluable technical assistance; Jussi Tikkanen, Jussi Ropponen, and Janne Jokinen for your friendship, support, and for our shared journeys over the years.

Leena Saraste for your humor, assistance with everyday problems, and for reviewing our manuscripts, letters, and presentations.

The staff of Transplantation Laboratory and H3 Animal Unit for your kind help and support. The heart and lung transplant coordinators
Marja-Liisa Hellstedt and Catharina Yesil from Helsinki University Central Hospital for obtaining the clinical samples.

All my friends for support, encouragement, and for the extracurricular activities: Joonas, Kalle, Sandor, and Tomi; all the friends in the medical school, especially Tuomas J., Jussi, Juhani, Ora, Antti, and Lauri, and also Ilkka, Tuomas K., Juhana, Timo, Kapo, Vera, Julia, and the late Petri for sharing your time during (scientific!) lunch, tennis, work, skiing, or whatever reason; all the friends at the Blood Service, especially Aleksi, Heidi, John, Tea K., and Tea S.

My family: Satu and Martti, Ossi and Rina, Mikko and Anna for your love, support, and encouragement throughout my life; and all my other relatives for your support. Tintti and Hanski, Hessu and Anna, Harri and Hannis for your support and good times spent together.

My deepest gratitude I owe to the most important person in my life, Heli, thank you for your support, love, and patience during this project.

In Helsinki, October 2014
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