Therapeutic strategies targeting vascular endothelial growth factors and early kidney allograft injury in the prevention of chronic rejection in the rat

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ABSTRACT

Chronic rejection is an irreversible fibrotic process that is driven by an alloimmune response and which leads to progressive graft dysfunction and eventually to the graft loss. In addition to the alloimmune response many nonimmunological factors also contribute to the development of chronic allograft injury and dysfunction. Although modern immunosuppressive therapy efficiently prevents acute rejection it has a limited effect on the development of chronic allograft injury. Many side effects also complicate the long-term use of current immunosuppressive regimens. Thus more targeted therapies are needed.

Vascular endothelial growth factors (VEGFs) VEGF-A and VEGF-C are essential to the angiogenesis and lymphangiogenesis during development. VEGFs also have direct pro-inflammatory functions and VEGF-A is involved in the development of atherosclerosis. In renal transplants VEGF-A expression has been linked to chronic rejection. Lymphatic vessel proliferation also occurs in kidney allografts, but its significance is unclear.

Ischemia-reperfusion injury and the subsequent inflammatory microenvironment predispose the graft to later fibrosis. Thus treatments that limit ischemia-reperfusion injury may also inhibit the development of chronic allograft injury. Cholesterol-lowering agents statins, have been shown to protect from renal ischemia-reperfusion injury in multiple rodent studies. Activins are cytokines that belong to the transforming growth factor -β superfamily. They regulate the onset of acute and chronic inflammatory responses in addition to renal fibrosis.

The first aim of this research was to investigate the role of VEGF-A and VEGF-C in the development of chronic renal allograft injury in the rat and to evaluate their potential as possible targets for intervention. The second aim was to examine treatment strategies that could inhibit IRI and acute inflammation in renal allografts and thus limit the development of chronic allograft injury. The therapeutic potential of perioperative simvastatin treatment and activin inhibition was investigated.

According to our results monocyte/macrophages express VEGF-A and its receptor VEGFR-1 during acute and chronic rejection in renal allografts. Inhibition of VEGF-A signaling by the tyrosine kinase inhibitor PTK787 limited fibrosis of the graft. Macrophages along with CD4+ T cells also produced lymphangiogenic factor VEGF-C during chronic rejection. Extensive lymphatic vessel proliferation occurred in cyclosporine treated, but not in sirolimus treated allografts. Newly formed lymphatic vessels were associated with nodular inflammatory cell infiltrates and the development of chronic allograft injury.

Perioperative simvastatin treatment given to the transplant recipient and to the donor limited renal allograft inflammation, improved graft function and limited the development
of chronic allograft injury. Paradoxically simvastatin treatment impaired graft function and increased proteinuria, when only given to the transplant donor. Activin A expression was found in the glomeruli and the interstitial cells of the allografts during acute rejection. Activin inhibition by a soluble activin receptor limited the innate immune response and the induction of early fibrotic signaling in kidney allografts.

In conclusion, this study provides experimental evidence that VEGF and activin inhibitors in addition to perioperative simvastatin treatment may have therapeutic potential in the prevention of chronic kidney allograft injury. Thus these molecules are interesting targets for further investigation. Our data also suggest that immunosuppressive regimen sirolimus can be used to inhibit lymphatic vessel proliferation in renal transplants.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


The publications are hereafter referred to in the text by their roman numerals.
ABBREVIATIONS

ABMR  antibody-mediated rejection
ActRII  type 2 activin receptor
AEC  3-amino-9-ethylcarbazole
AKI  acute kidney injury
ALK  activin receptor-like kinase
APC  antigen presenting cell
ATN  acute tubular necrosis
CADI  chronic allograft damage index
CAI  chronic allograft injury
CAN  chronic allograft nephropathy
CCL  chemokine ligand
CCR  chemokine receptor
CNI  calcineurin inhibitor
CsA  cyclosporine A
C4d  complement factor 4d
DA  Dark Agouti (rat)
DAMP  damage associated molecular pattern
DGF  delayed graft function
DSA  donor specific antibody
GFR  glomerular filtration rate
EGF  epidermal growth factor
ELISA  enzyme-linked immunosorbent assay
FSH  follicle-stimulating hormone
HIF-1  hypoxia inducible factor -1
HLA  human leukocyte antigen
HMGB1  high-mobility group box 1
HMGC0A  3-hydroxy-3-methylglutaryl coenzyme A
IF/TA  interstitial fibrosis and tubular atrophy
IL  interleukin
IL-2R  interleukin-2 receptor
IFN-γ  interferon-γ
i.p.  intraperitoneally
IRI  ischemia-reperfusion injury
KIM-1  kidney injury molecule -1
LYVE-1  lymphatic vessel endothelial hyaluronan receptor -1
MHC  major histocompatibility complex
mTOR  mammalian target of rapamycin
MyD88  myeloid differentiation primary response gene 88
NF-κB  nuclear factor kappa B
NGAL  neutrophil gelatinase -associated lipocalin
NRP  neuropilin
PDGF  platelet-derived growth factor
PDGFR  platelet-derived growth factor receptor
PEG  polyethylene glycol
PIGF placental growth factor
ROS reactive oxygen species
sActRIIB-Fc soluble type 2 activin receptor
s.c. subcutaneously
SRL sirolimus
TCMR T cell mediated rejection
TCR T cell receptor
TGF-β transforming growth factor beta
Th cell  T helper cells
TLR toll-like receptor
TLT tertiary lymphoid tissue
TNF-α tumor necrosis factor –α
Treg T regulatory cell
VEGF vascular endothelial growth factor
VEGFR vascular endothelial growth factor receptor
WF  Wistar Furth (rat)
INTRODUCTION

Kidney transplantation is the treatment of choice for patients with terminal uremia in terms of the patient’s outcome and cost of treatment. The short-term results of kidney transplantation have significantly improved in the past decades due to effective immunosuppression and infection prophylaxis. Despite the advances in preventing short-term graft loss, there has been only slight improvement in the long-term graft survival (Matas et al. 2013).

Chronic rejection is a major reason for late allograft loss. It is an irreversible fibrotic process that is characterized by transplant glomerulopathy, vasculopathy, tubular atrophy and interstitial fibrosis. Chronic rejection results in progressive graft dysfunction and eventually in the loss of the graft itself. Chronic rejection essentially is the chronic allograft injury (CAI) caused by alloimmune response. However, the development of chronic allograft injury and dysfunction is often a multifactorial process including both immunological and nonimmunological factors.

Currently used immunosuppressive drugs have been primarily designed to target T- and B- cell mediated adaptive immune responses. Although modern immunosuppressive therapy is effective in preventing acute rejection it has a limited effect on the development of chronic allograft injury. Other major problems associated with the long-term use of immunosuppressive drugs are their many metabolic, cardiovascular and infectious side effects. Immunosuppression also accelerates malignancy rates in renal transplant patients. Thus new more targeted therapies against chronic rejection are needed.

Graft macrophage and monocyte infiltration early after transplantation and during acute rejection is a risk factor for the later graft fibrosis and graft loss (Croker et al. 1996, Pilmore et al. 2000).

Thus monocytes/macrophages may have an important role in the pathogenesis of chronic allograft injury. Amongst other functions, they are a major source of growth factors. Vascular endothelial growth factors (VEGFs) are a family of growth factors that regulate angiogenesis and lymphangiogenesis during development and also under pathological conditions. VEGFs also have direct pro-inflammatory functions and VEGF-A is involved in the development of atherosclerosis. The increased expression of VEGF-A in renal allografts has been linked to chronic allograft injury (Pilmore et al. 1999). The formation of new lymphatic vessels also occurs in renal allografts. New lymphatic vessels may be involved in the organization of chronic inflammation and maintain local immunoresponse in renal allografts but its significance is still unclear (Kerjaschki et al. 2004).

Macrophages and other cells of the innate immune system are first activated as a result of tissue injury during ischemia and reperfusion of the kidney transplant. Ischemia-reperfusion injury and the subsequent inflammatory microenvironment predispose the
graft to later fibrosis (Nankivell et al. 2004). Cholesterol-lowering drugs, statins, known for their pleiotropic effects have been shown to protect from renal ischemia-reperfusion injury in multiple experimental studies (Joyce et al. 2001, Gueler et al. 2002). Activins are members of the transforming growth factor -β (TGF-β) superfamily of cytokines. They regulate the onset of acute and chronic inflammatory responses such as septicemia and asthma (Hedger and de Kretser 2013). Experimental studies also suggest that activins are involved in the development of renal fibrosis (Yamashita et al. 2004, Maeshima et al. 2014).

The first aim of this study was to investigate the role of VEGF-A and VEGF-C in the development of chronic renal allograft injury in the rat and to evaluate their potential as possible targets for intervention. The second aim was to evaluate the potential treatment strategies that could inhibit ischemia-reperfusion injury and early inflammation in preventing the development of chronic allograft injury. The therapeutic potentials of perioperative simvastatin treatment and activin inhibition were examined.
REVIEW OF THE LITERATURE

1. Clinical kidney transplantation

A kidney transplant, often much anticipated, transforms the life of a patient with end-stage renal disease. Without a kidney transplant the patient is restricted to heavy kidney replacement therapy. Kidney transplantation is the treatment of choice for end-stage renal disease patients. It replaces all renal functions and offers superior outcomes in terms of mortality when compared to dialysis (ERA-EDTA Registry Annual Report 2012). Primary indications for kidney transplantation are diabetes, hypertension, glomerulonephritis, and polycystic kidney disease (Matas et al. 2013). A total of 2573 adult patients in Finland had a functioning kidney transplant at year 2013 (Finnish Registry for Kidney Diseases Report 2013).

The first successful kidney transplantation was performed between identical twins by Joseph Murray in Boston at Peter Bent Brigham Hospital in 1954 (Guild et al. 1955). Transplantation of tissues from a donor that genetically differs from the graft recipient, however, is more complicated since it induces an alloimmune response in the recipient. The alloimmune response leads to acute rejection, which if not controlled will destroy the graft. In the early 1960s corticosteroids and azathioprine were used as immunomodulatory therapy, but acute rejection with graft tenderness and fever still remained common. The introduction of a powerful immunosuppressive regimen, cyclosporine (CsA) (Borel et al. 1976), finally made solid organ transplantation a plausible treatment for end stage renal disease in the 1980s.

1.1 Outcomes

The short-term results of kidney transplantation have significantly improved during the past decades due to advances in immunosuppression and infection prophylaxis. At first year post-kidney-transplant over 96% of transplant recipients are alive with a functioning kidney transplant (Matas et al. 2013). Primary graft dysfunction and acute rejection are the major reasons for the graft loss during the first year after the transplant (El-Zoghby et al. 2009). With modern immunosuppression the risk of acute rejection within one year after transplantation is less than 15%, and it is often treatable. Despite the advances in preventing short-term graft loss, there has been only marginal improvement in 10-year graft survival (Matas et al. 2013).

After one year the most important reason for the loss of graft is death with a functioning graft, which accounts for 47% of transplant losses. Major reasons for death are cardiovascular diseases, malignancies and infections (El-Zoghby et al. 2009, Pilmore et al. 2010, Matas et al. 2013). The death rate for these conditions is accelerated in
transplantation patients. For example, cardiovascular mortality in transplantation patients is 20-fold that of the general population (Pilmore et al. 2010). Current immunosuppressive medication is problematic for these diseases. Immunosuppressive medication increases blood pressure, causes dyslipidemia and increases the risk of diabetes (Svensson et al. 2012). It also reduces the ability of the immune system to eradicate pathogens, which makes the patient more vulnerable to infections. Immunosuppression also accelerates malignancy rates especially in cancer types associated with oncogenic viruses (Grulich et al. 2007). A more targeted therapy approach against rejection is needed to limit these adverse effects.

The kidney graft itself can be lost in both immune and non-immune mechanisms. Some transplants are lost due to one obvious reason whereas other grafts will accumulate damage by several mechanisms each of which contributes to functional impairment (Nankivell and Kuypers 2011). One of the leading causes for late allograft loss is ongoing alloimmune response induced injury, chronic rejection. Other major reasons for chronic allograft dysfunction include recurrent and de novo glomerular diseases and infections of the graft (El-Zoghby et al. 2009). Paradoxically, immunosuppressive regimens, calcineurin inhibitors (CNI) CsA and tacrolimus themselves are also nephrotoxic and are one reason for late allograft loss (Naesens et al. 2009). The renal transplants from living donors have superior outcomes when compared to transplants from deceased donors (Matas et al. 2013). This difference reflects the significance of early graft injury to the transplant survival in long-term.

Regardless of the insult, scarring of the graft is the final consequence of progressive injury. Typical histopathological findings in chronic allograft injury are obstructing vascular and glomerular changes in addition to interstitial fibrosis and tubular atrophy (IF/TA). Both alloimmune independent factors and alloimmune related factors create a risk for allograft loss. See table 1 for a list of risk factors and reasons for allograft loss.
Table 1.

Risk factors and causes of renal transplant failure

**Alloimmune independent**

- Deceased donor, non-heart beating donor
- Donor age, female donor sex, donor cardiovascular diseases
- Ischemia-reperfusion injury (extended ischemia time)
- Delayed graft function
- Ascending urinary tract infection and graft pyelonephritis
- Transplant ureteric obstruction
- Cytomegalovirus infection
- Polyoma virus nephropathy
- Calcineurin inhibitor nephrotoxicity
- Recurrent renal disease or de novo glomerulonephritis
- Recipient hypertension, hyperlipidemia, smoking, diabetes

**Alloimmune related**

- Recipient’s age
- Histoincompatibility (HLA mismatches)
- Donor specific antibodies or recipient presensitization
- Acute rejection, especially severe, steroid-resistant, vascular, antibody-mediated or late occurring
- Subclinical rejection
- Chronic active rejection (T cell mediated or antibody mediated)
- Non-compliance

Human leukocyte antigen (HLA). The table is modified from Nankivell and Kuypers 2011.
2. Ischemia-reperfusion injury

2.1 Ischemia

The donor kidney has to remain viable without blood flow during the organ retrieval, preservation and transplantation procedure. Although tolerance to hypoxia varies amongst cell types, the extended periods of imbalance between oxygen supply and metabolic demand inevitably leads to cellular death. Prolonged cold ischemia in kidney transplantation is known to increase the incidence of delayed graft function (DGF) and acute rejection and to reduce graft survival (Ojo et al. 1997, Salahudeen et al. 2004).

The lack of circulation leads the kidney to oxygen and nutrient deprived ischemic state. The hypoxia causes an accumulation of reactive oxygen species (ROS) and anaerobic metabolites and changes the electrolyte balance that lead to hypoxic cell injury. Hypoxia also activates transcription factor nuclear factor kappa B (NF-κB) and hypoxia inducible factor -1 (HIF-1) signaling (Koong et al. 1994, Baan et al. 2003). The lack of blood flow also entails the loss of vascular endothelial shear stress, which is an important signal sustaining endothelial quiescence during physiological conditions (Dekker et al. 2006). Thus, ischemia itself damages the graft and also produces a proinflammatory state that increases tissue vulnerability to further injury upon reperfusion. The prime protective technique against ischemic damage is to cool the organ during the preservation. Hypothermia reduces cell metabolism, oxygen consumption and the rate of apoptosis, which thus limits the injury (Lampe and Becker 2011, Yang et al. 2009).

2.2 Reperfusion

When the organ is implanted and the blood flow is subsequently restored, this paradoxically amplifies the ischemic damage and results in ischemia-reperfusion injury (IRI). The supply of oxygen to ischemic tissue releases an excessive burst of ROSs, which damage cells and eventually leads to cell death (McCord 1985). IRI also induces microvascular dysfunction, which is characterized by increased vascular permeability, perfusion disturbances, thrombosis and vasoconstriction (Ogawa et al. 1990, Conger et al. 1991).

IRI triggers both the innate and the adaptive immune responses. The activation of endothelial cells during ischemia and reperfusion induces adhesion molecule expression, which attracts circulating inflammatory cells to the graft. The increased vascular permeability further facilitates the leukocyte extravasation (Ogawa et al. 1990). In addition, the tissue injury itself directly activates the innate immune response through pattern recognition. The resulting inflammatory response further amplifies the tissue damage.
2.3 Delayed graft function

Prolonged cold ischemia is associated with a higher incidence of DGF (Ojo et al. 1997). DGF is an acute kidney injury (AKI) after kidney transplantation histologically characterized by acute tubular necrosis (ATN). Matas and colleagues analyzed data of the United network of organ sharing database and reported that DGF occurred in 21.3% of all US patients that received a kidney transplant in 2008 (Matas et al. 2013). The incidence of DGF in Finland is almost 40% (Hollmen et al. 2011). DGF causes need for dialysis, lengthens hospitalization and increases the cost of treatment. ATN is thought to be reversible, but delayed graft function may predispose the graft to increased risk for acute rejection and reduced graft function and affect the long-term graft survival (Ojo et al. 1997).

3. Innate immune response

Innate immunity is the first-line defense against invading pathogens. It is also involved in the cleaning, degradation and repair of injured tissue and activates adaptive immune response through antigen presentation.

Complement activation enhances leukocyte extravasation, facilitates phagocytic activity and causes direct cell injury during innate immune response (Cravedi and Heeger 2014). Natural killer cells destroy cells by releasing cytolytic molecules (Trapani et al. 2000). Neutrophils, monocytes and macrophages destroy pathogens and particles by phagocytosis. Neutrophils have specific cytoplasmic granules containing myeloperoxidase, lysozyme and collagenase that enable them to efficiently degrade phagocytosed particles (Kolaczkowska and Kubes 2013). In addition to their phagocytic activity, macrophages are able to produce a large number of pro-inflammatory cytokines including interleukin (IL)-1β and tumor necrosis factor -α (TNF-α) (Nau et al. 2002, Sica and Mantovani 2012). All these functions are important in host defense, but may also induce tissue damage in a renal transplant.

Both dendritic cells and macrophages function as professional antigen presenting cells (APCs) and are capable of internalizing, processing and presenting antigens to T cells. Mature dendritic cells express high levels of co-stimulatory molecules on their cell surface and thus can effectively present antigens to both naïve and primed T cells (Banchereau and Steinman 1998). Thus, dendritic cell activation is an important link between innate and alloimmune responses.
3.1 Toll-like receptors

In 1989 Janeway suggested that APCs are normally quiescent and are activated during infection via pattern-recognition receptors in response to pathogen-associated molecular patterns (Janeway 1989). The role of innate immunity in transplantation began to resolve after Polly Matzinger suggested in 1994 that not only pathogens, but also tissue injury is sensed through pattern recognition receptors. During tissue injury pattern-recognition receptors sense the presence of damage associated molecular patterns (DAMPs), which are endogenous structural molecules that are released and exposed during tissue injury (Matzinger 1994). Thus, the innate immune system could also be activated directly by injured organ transplant. DAMPs released and produced during renal ischemia reperfusion include high-mobility group box 1 (HMGB1), hyaluronan, and biglycan (Wu et al. 2007).

Toll-like receptors (TLR) are widely expressed pattern recognition receptors. They are classically expressed in dendritic cells and macrophages, but several other cell types including podocytes and renal epithelial and tubular cells also express TLRs (Leventhal and Schroppel 2012). TLR2 and TLR4 have been suggested to have an important role in the initiation of the inflammatory response during renal IRI in mice (Leemans et al. 2005, Wu et al. 2007). TLR2 and TLR4 activation in antigen presenting cells lead to myeloid differentiation primary response gene 88 (MyD88) -dependent NF-κB signaling, which has various pro-inflammatory downstream effects including: increased cytokine production and generation of reactive oxygen species, upregulation of co-stimulatory molecules and enhanced antigen presentation (Barton and Medzhitov 2003). A deficiency of MyD88 increases renal allograft survival in mice (Wu et al. 2012, Lerret et al. 2015), which suggests that innate immune system is essential to the development of alloimmune response.
Brain death and ischemia-reperfusion injury damage the allograft and lead to the production of reactive oxygen species (ROS). Stressed allograft cells actively secrete DAMPs to signal danger. DAMPs are also passively released from injured cells and generated from damaged extracellular matrix. DAMPs such as high-mobility group box -1 (HMGB1), biglycan and hyaluronan are recognized by the toll-like receptors (TLR) -2 and -4 on the surface of immature dendritic cells (DC). After recognition, TLR-mediated signals convert immature DCs into potent antigen-presenting cells (APC) that are capable of stimulating the alloantigen-specific naive T-cells. Alloantigens presented to T-cells are released from injured allograft cells and endocytosed by antigen-presenting cells (indirect antigen presentation). (Modified from Land 2012)
4. **Alloimmune response**

Sir Peter Medawar in his seminal study in 1944 was the first to describe alloimmune response. He discovered that skin transplants grafted to British Second World War pilots did not survive, since they were recognized as foreign (Medawar 1944). The graft is recognized at the immunological level as being foreign during the encounter of activated APCs and the alloreactive lymphocytes (allorecognition). Allorecognition initiates the alloimmune response, which, without proper immunosuppression, destroys the graft through cell-mediated and humoral mechanisms.

4.1 **Antigen presentation**

After transplantation both the donor’s passenger APCs that were in the graft at the time of organ retrieval and the recipient’s APCs recruited to the transplant internalize antigens and travel to secondary lymphoid organs, namely the spleen and lymph nodes, to present them to T cells.

The antigen is presented to the T cell receptor (TCR) on the T cell surface in the groove of major histocompatibility complex (MHC) on the APC surface. The first step in T cell activation is the antigen-MHC complex interaction with the TCR complex. In addition a co-stimulatory signal, B7 (CD80 or CD86) on APC binding to CD28 on T cell surface, is needed (Turka et al. 1992). Without sufficient co-stimulation T cells become unresponsive to further stimulation, a state called anergy (Schwartz 1990).

MHC-I molecules are expressed in almost all nucleated cells and they present proteins from inside the cell to CD8+ cytotoxic T cells (Daar et al. 1984a, Norment et al. 1988). MHC-II molecules mainly expressed by professional APC present antigens that have been endocytosed, processed and then brought to the cell surface with MHC-II molecule to the CD4+ T helper (Th) cells (Daar et al. 1984b, Doyle and Strominger 1987).

4.2 **Allorecognition**

For an antigen to trigger an alloimmune response, it has to be sensed as nonself. An allogeneic nonself is any antigen expressed by donor but not by recipient tissues that is responsible for graft rejection. The MHC-molecules are themselves highly polymorphic and thus can differ greatly between individuals. In addition they are ubiquitously expressed. A mismatch between MHC-molecules elicit an unusually intense rejection and MHC molecules are thus called major antigens (Snell 1948). Other polymorphic molecules are called minor antigens. One example of a minor antigen are Y chromosome encoded proteins in male to female transplantation (Zelenika et al. 1998).
Three ways of allorecognition has been described to date. First, the direct allorecognition, the TCR recognize donor passenger APC MHC-peptide complexes through cross-reaction (Yin and Mariuzza 2009). The TCR that would normally bind self-MHC+nonself-peptide complex, in turn, binds the nonself-MHC+peptide complex. Cytotoxic T cells can also recognize endothelial cell MHCI molecules in the graft through cross-reaction (Chalasani et al. 2002). The direct antigen presentation elicits a vigorous alloimmune response early after transplantation, which then dwindles gradually as the passenger dendritic cells are lost (Liu et al. 1993, Garrod et al. 2007, Lechler and Batchelor 1982). Second, the indirect allorecognition, the recipient’s APCs present donor specific antigens in their own MHC-molecules to the T cells (Auchincloss et al. 1993). The indirect allorecognition is according to current knowledge responsible for later rejection episodes and chronic T cell mediated rejection (Hornick et al. 2000). Third, the semidirect allorecognition, the donor’s APC transfers the whole MHC-complex through exocytosis or cell-to-cell contact to the recipient’s APC. (Herrera et al. 2004).

4.3 T cell activation and clonal proliferation

Antigen presentation with sufficient co-stimulation leads to the activation of the calcium-calciineurin pathway (Borel et al. 1976). This initiates the transcription of interleukin-2 (IL-2), which regulates T cells in an autocrine and paracrine fashion (Kirkman et al. 1985). Interleukin-2 receptor (IL-2R) and subsequent cytosolic receptor mammalian target of rapamycin (mTOR) signaling initiates the cell cycle progression and the clonal proliferation of alloreactive T cells (Dumont et al. 1990). The cytokine milieu defines the subtype direction in which the Th cells specialize. The Th cell subtypes include Th1, Th2, Th17 and T regulatory cells (Treg).

4.4 T cell subtypes

Th1 cells are the classical proinflammatory T cells, that are central to delayed-type hypersensitivity responses (Safinia et al. 2010). The signature cytokine produced by Th1 cells is interferon-γ (IFN-γ), which activates proinflammatory functions in macrophages (Dalton et al. 1993). In addition Th1 cells boost killing ability of cytotoxic T cells. Alloreactive cytotoxic T cells recognize MHCI molecules on target cells and then release cytotoxic granules that containing perforins, granzymes and Fas ligand (Trapani et al. 2000). The Th17 cells produce highly proinflammatory cytokine IL-17, which is linked to neutrophil recruitment (Laan et al. 1999).

Th2 cells are part of a humoral immune response. The hallmark cytokines produced by Th2 cells are IL-4 and IL-10. Th2 cells activate B cells to produce antibodies. (Boom et al. 1988, Zheng and Flavell 1997) The process is complex and usually takes place in the germinal centers of the secondary lymphoid organs. The transplant recipient may also
have pre-existing antibodies against the transplant due to exposure to donor antigens during pregnancy, blood transfusion, or previous transplantation.

The Tregs modulate the immune system and maintain tolerance to self-antigens (Bennett et al. 2001, Wildin et al. 2001). Tregs have induced both CD8⁺ and CD4⁺ T cell hyporesponsiveness to donor target cells in mice. Thus, Tregs may limit the alloimmune response and induce tolerance against organ transplant (Graca et al. 2002).

**Figure 2** Antigen presentation and activation of T cells. The antigen-presenting cell (APC) travels to the secondary lymph node to present the antigen to the CD4⁺ T cell. The antigen is presented in the groove of the major histocompatibility complex (MHC) to the T cell receptor (TCR). A co-stimulatory signal, B7 binding to CD28, is also needed. T cell recognizes alloantigen as a foreign (indirect antigen presentation). If the APC is a donor passenger, the T cell can also recognize the non-self-MHC+antigen complex as foreign (direct antigen presentation). After allore cognition interleukin-2 (IL-2) signaling initiates the cell-cycle progression and clonal proliferation of the T cell. Cytokine milieu defines the direction the T helper (Th) cell differentiates. Th cells include Th1, Th17, Th2, and T regulatory (Treg) cells. The signature cytokines produced by these Th cell subtypes are presented in the figure. (Modified from Nankivell and Alexander 2010).
4.5 Acute rejection of renal allograft

Acute rejection is characterized by an abrupt rise in serum creatinine and typically occurs during the first year after transplantation (Matas et al. 2015). Banff classification is used to diagnose acute rejection in kidney transplant biopsies. Acute rejection is divided to acute T cell mediated (TCMR) and antibody-mediated rejection (ABMR) depending on the histopathological finding. The diagnosis of acute ABMR requires serological evidence for donor specific antibodies to be present. Acute TCMR and ABMR may occur simultaneously (Haas et al. 2014). Subclinical rejection can be identified in the graft biopsy, but does not cause clinical symptoms. Hyperacute rejection, caused by preformed donor-specific antibodies (DSA), destroys the graft within minutes or hours after transplantation (Patel and Terasaki 1969). Improvements in cross-matching techniques that can better detect DSA before transplantation have made hyperacute rejection cases extremely rare.

Pathologically acute TCMR is manifested by the accumulation of CD4+ and CD8+ T cells and macrophages in the graft interstitium, which is accompanied by an inflammation of the tubules and sometimes of the arteries as well (Sis et al. 2010). Circulating antibodies during ABMR bind to graft endothelium, which subsequently leads to activation of the complement and coagulation system, recruitment and stimulation of leukocytes and endothelial cell necrosis (Colvin 2007). Typical pathological finding of acute ABMR is accumulation of neutrophils and monocytes in the peritubular and glomerular capillaries (Tinckam et al. 2005, Fahim et al. 2007, Haas et al. 2014). Complement activation can often be detected by complement factor 4d (C4d) staining in the peritubular capillaries (Feucht et al. 1993, Collins et al. 1999). C4d is an inactive fragment of the classic complement pathway. In the more severe cases of acute ABMR microthrombi, hemorrhage, necrosis of the arterial walls, and infarction may occur (Colvin 2007).

4.6 Immunosuppression

To prevent acute rejection, the alloimmune response is suppressed by immunosuppressive regimens. The immunosuppressive medication mainly target different steps of T cell activation. The maintenance therapy usually consists of corticosteroid, CNI (tacrolimus or CsA) and anti-metabolite (mycophenolate mofetil or azathioprine) or mTOR inhibitor (sirolimus or everolimus). Other possible treatments include co-stimulation blockade with belatacept and induction therapy with anti-ILR2 and anti- thymocyte globulin antibodies (Masson et al. 2014, Hardinger et al. 2013). Treatment options against humoral rejection are limited. Plasmapheresis, immunoadsorption, intravenous immunoglobulin and anti-CD20 antibody rituximab can be used as desensitization therapy to recipients with high levels of donor specific antibodies and to ABO-incompatible patients (Macklin et al. 2014, Clatworthy 2011).
Figure 3  The molecular targets of immunosuppressive maintenance therapy. Current medication mainly inhibits different steps of T cell activation. Cyclosporine inhibits the calcineurin pathway. Calcineurin inhibition limits the translocation of transcription factor nuclear factor of activated T cells (NF-AT) to the nucleus thus preventing the gene transcription of interleukin-2 (IL-2). Sirolimus inhibits mammalian target of rapamycin (mTOR). mTOR inhibition causes inactivation of p70S6 kinase thus limiting the production of ribosomal components necessary for protein synthesis and IL-2 driven lymphocyte division. Antigen presenting cell (APC), glucocorticoid receptor (GR), interleukin -2 receptor (ILR2) major histocompatibility complex (MHC), mycophenolate mofetil (MMF), T cell receptor (TCR). (Modified from Lindenfeld et al. 2004)
5. Chronic rejection

Chronic rejection of renal transplant particularly means the fibroproliferative response to ongoing alloimmune injury. Histologically chronic rejection manifests as chronic inflammation, transplant glomerulopathy, peritubular capillaropathy, arteriopathy and IF/TA. In general the pathogenesis of chronic rejection is thought to involve (i) tissue injury and activation, (ii) recruitment of inflammatory cells, (iii) release of fibrogenic cytokines and growth factors, (iv) activation, proliferation and/or recruitment of fibroblasts, myofibroblasts, mesangial cells and smooth muscle cells, and (v) production of excess extracellular matrix (Torres et al. 2014). Clinically chronic rejection results in progressive deterioration of the graft function and eventually loss of the graft itself. Hypertension and proteinuria may also be present.

Alloantigen-dependent factors such as acute rejection episodes, the degree of MHC mismatch and DSA are known risk factors for chronic rejection. Multiple alloimmune independent factors such as IRI and infections damage the graft and not only predispose the graft to alloimmune response but also directly contribute to the development chronic injury and dysfunction. Thus, the development of chronic allograft dysfunction is often multifactorial (table 1) (Nankivell and Kuypers 2011). Earlier a term chronic allograft nephropathy (CAN) was used to describe chronic allograft injury regardless of the cause. Diagnostic guidelines have, however, recommended avoiding the term CAN already for a decade and instead aim at specific diagnosis and optimal treatment for every condition (Solez et al. 2007).

Chronic rejection is diagnosed histologically in graft biopsies. An allograft biopsy is recommended for all patients with declining kidney function of unclear cause (Heemann et al. 2011). Regularly updated Banff classification is used in the clinical evaluation of graft histopathology (table 2) (Sis et al. 2010, Haas et al. 2014). Another grading system, chronic allograft damage index (CADI), was developed to show the intensity of chronic changes in the transplant as a single numerical figure (Isoniemi et al. 1994). CADI is useful for scientific purposes because of its numerical format.

5.1 Treatment of chronic rejection

Although there is no specific treatment for chronic rejection, there are several strategies to improve long-term graft survival. Both immunologic and nonimmunologic risk factors can be treated in terms to prevent the development of chronic allograft injury.

Organ cold storage and optimized storage solutions are used to reduce IRI. Since MHC-mismatches increase the risk of chronic allograft injury, it would be logical to find a perfectly matching donor for each recipient in order to reduce the alloimmune response. However, both ischemia time and organ shortage have to be taken into account for organ allocation. The efficient treatment of hypertension in the general population, prevents the
progression of chronic kidney disease. There is little reason to believe that this would not apply to chronic kidney allograft injury and thus effective treatment of hypertension is recommended (Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group 2009).

The optimal use of current immunosuppressive regimens in the prevention of chronic allograft injury is still undetermined. Earlier CNI nephrotoxicity was considered to be one of the main causes of chronic renal allograft injury (Nankivell et al. 2003). This assumption has resulted in the development of a variety of CNI sparing treatment strategies with a possible risk of increased acute rejection rate. Recent studies suggest that some of the adverse outcomes that have been attributed to the use of CNIs, may actually be explained by other causes of chronic injury (El-Zoghby et al. 2009, Rush 2013). Thus the optimal use of CNIs in renal transplant patients remains to be determined.

One of the most intensive areas of research to reduce CNI exposure has been the use of mTOR inhibitors. Conversion of CNI to mTOR inhibitor at three to six months after transplantation appears to have renoprotective effects in the long-term (Lebranchu et al. 2009, Budde et al. 2015, Witzke et al. 2016). Earlier switch to mTOR inhibitor exposes the graft to acute rejection (Mjornstedt et al. 2012, Chadban et al. 2014) whereas later conversion does not usually improve graft function unless the baseline graft function is good (Schena et al. 2009, Holdaas et al. 2011). Multiple adverse effects limit the clinical use of mTOR inhibitors. The mechanism behind their graft protecting effect, however, remains of interest for further drug development.

5.2 Pathogenesis and histological features of chronic rejection

The typical histological changes of chronic allograft injury were described during the early days of clinical kidney transplantation (Hume et al. 1955). There was a general understanding in the past that long-term graft destruction is an alloimmune-mediated process that slowly destroys the graft. The multifactorial pathogenesis of chronic allograft injury was established in the 1990s (Hayry et al. 1993, Halloran et al. 1999). The major contribution of alloantibodies in the pathogenesis of chronic allograft dysfunction has only recently been acknowledged (El-Zoghby et al. 2009, Sellares et al. 2012). Chronic rejection as defined by the Banff classification is divided into T cell mediated and antibody mediated chronic active rejection (Sis et al. 2010, Haas et al. 2014).

Chronic allograft arteriopathy is the characteristic feature of chronic TCMR, but arterial fibrosis can also be present in chronic ABMR. The physiological intima is the innermost layer of the arterial wall and consists of a monolayer of endothelial cells that is supported by a thin layer of elastic tissue. In transplant arteriopathy, infiltration and proliferation of the vascular smooth muscle cells and accompanying T cells and
macrophages within the intima and the production of extracellular matrix leads to diffuse intimal thickening and finally vascular occlusion (Sis et al. 2010, Haas et al. 2014).

Specific histological features of chronic active ABMR include transplant glomerulopathy and peritubular capillary multilayering (Haas et al. 2014). In chronic ABMR pre-existing or de-novo DSA, particularly against MHCII antigens, damage and activate the endothelium in the glomeruli and peritubular capillaries (Gloor et al. 2007, Wavamunno et al. 2007, Hidalgo et al. 2009). The activation of the endothelium is followed by glomerulitis and capillaritis (Wavamunno et al. 2007). Eventually the chronic active ABMR leads to progressive endothelial basement membrane duplication and glomerular mesangial matrix expansion, which occlude these vascular structures (Roufosse et al. 2012). The prognosis for transplant glomerulopathy is poor. Within 5 years of diagnosis, the death-censored graft survival rate is only 20% (John et al. 2010).

Both TCMR and ABMR cause interstitial fibrosis and tubular atrophy of the graft. The ongoing inflammatory response is an important driving force for fibrosis. The combination of inflammation and IF/TA reduces graft survival, but IF/TA alone, is a stable condition (Park et al. 2010). Interstitial fibrosis replaces the functional parenchyma, which leads to gradual loss of graft function.

5.3 Molecular mechanisms in renal fibrosis

Macrophages and myofibroblasts have a central role in the development of renal fibrosis. Myofibroblasts are persistently activated fibroblasts, which can be derived from resident fibroblasts, from circulating progenitors, or by epithelial-to-mesenchymal transition (Djamali and Samaniego 2009). Myofibroblasts produce extracellular matrix. Macrophages are a rich source of growth factors that become involved in fibrosis. The macrophages themselves are divided into proinflammatory M1 macrophages and M2 macrophages that are involved in fibrotic processes (Kwan et al. 2014). This M1/M2 classification is, however, an oversimplification and macrophages most plausibly form a continuum of different phenotypes (Murray et al. 2014). Early intragraft myofibroblast and macrophage expression is linked to poor renal graft outcome (Croker et al. 1996, Pilmore et al. 2000).

TGF-β is a key molecule in renal fibrosis. TGF-β together with other profibrotic cytokines and downstream growth factors induce a full repertoire of fibrotic responses. It directly enhances fibroblast proliferation and transdifferentiation into myofibroblasts and increases extracellular matrix production (Djamali and Samaniego 2009). Both experimental and clinical evidence supports the role of TGF-β in the pathogenesis of chronic kidney allograft injury (Shihab et al. 1995, Shihab et al. 1996).
Other growth factors especially platelet-derived growth factor (PDGF) have also been linked to kidney transplant rejection (Fellstrom et al. 1989, Savikko et al. 2003). Amongst other things, PDGF stimulates the chemotaxis and proliferation of fibroblasts and smooth muscle cells. PDGF also induces the production of several extracellular matrix proteins. (Ostendorf et al. 2014) Inhibition of growth factor signaling by tyrosine kinase inhibitors imatinib (Savikko et al. 2003), sunitinib (Rintala et al. 2015) and erlotinib (Rintala et al. 2014) has been shown to limit chronic renal allograft rejection in a rat model. Imatinib inhibits PDGF signaling (Buchdunger et al. 2000). Sunitinib inhibits PDGF, and also VEGF signaling at similar concentrations (Mendel et al. 2003). Erlotinib is a specific inhibitor of epidermal growth factor (EGF) receptor (Pollack et al. 1999).
Table 2. Banff diagnostic categories for chronic allograft rejection, IF/TA and other causes (According to Banff ’97 criteria and taking into account the Banff ’09 and Banff ‘13 updates)

<table>
<thead>
<tr>
<th>Category</th>
<th>Criteria</th>
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<tr>
<td><strong>Chronic, active antibody mediated rejection</strong></td>
<td>All three features must be present for diagnosis:</td>
</tr>
<tr>
<td></td>
<td>1. Morphologic evidence of chronic tissue injury: transplant glomerulopathy (if no evidence of chronic thrombotic microangiopathy) and/or severe peritubular capillary basement membrane multilayering and/or arterial intimal fibrosis of new onset, excluding other causes.</td>
</tr>
<tr>
<td></td>
<td>2. Evidence of current/recent antibody interaction with vascular endothelium: linear C4d staining in peritubular capillaries and/or at least moderate microvascular inflammation and/or increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury.</td>
</tr>
<tr>
<td></td>
<td>3. Serologic evidence of DSAs (HLA or other antigens)</td>
</tr>
<tr>
<td><strong>Chronic, active T-cell mediated rejection</strong></td>
<td>‘Chronic allograft arteriopathy’: arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of the neo-intima</td>
</tr>
<tr>
<td><strong>Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology (IF/TA)</strong></td>
<td>Grade I. Mild interstitial fibrosis and tubular atrophy (&lt;25% of cortical area).</td>
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<tr>
<td></td>
<td>Grade II. Moderate interstitial fibrosis and tubular atrophy (26-50% of cortical area)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Changes not considered to be due to acute or chronic rejection</td>
</tr>
<tr>
<td></td>
<td>All the categories may coincide</td>
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<td>DSA (donor-specific antibody)</td>
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</table>
6. The VEGF family

The VEGF family belongs to the platelet-derived growth factor/vascular endothelial growth factor superfamily (Keck et al. 1989). The members of VEGF family are the most important regulators of vasculogenesis, angiogenesis and lymphangiogenesis. VEGF (VEGF-A) was first discovered in 1979 due to its ability to induce vascular permeability in tumor cell supernatants (Dvorak et al. 1979). Hence, it was first named as vascular permeability factor. The VEGF family currently consists of five known mammalian ligand members: VEGF (VEGF-A), VEGF-B (Olofsson et al. 1996), VEGF-C (Joukov et al. 1996, Lee et al. 1996), VEGF-D (Achen et al. 1998) and placenta growth factor (PIGF) (Maglione et al. 1991). VEGF ligands bind to three tyrosine kinase receptors: VEGFR-1/flt-1 (de Vries et al. 1992), VEGFR-2/flk-1 (Terman et al. 1992) and VEGFR-3/flt-4 (Pajusola et al. 1992, Joukov et al. 1996). The VEGF family members also bind to neuropilins NRP-1 and NRP-2, which are considered to function as co-receptors for the VEGF receptors (Soker et al. 1998). This study concentrates on the investigation of VEGF-A and VEGF-C due to their central roles in angiogenesis and lymphangiogenesis. The key functions of all mammalian family members are listed in table 3.

6.1 VEGF-A

VEGF-A binds to VEGFR-1, VEGFR-2, NRP-1 and NRP-2 and is a central molecule for the regulation of physiological and pathological angiogenesis. VEGF-A stimulates endothelial cell proliferation, migration and survival through VEGFR-2, in addition to its ability to increase vascular permeability (Shibuya and Claesson-Welsh 2006). Mice that have only one deficient VEGF allele die in utero due to deficiencies in early vasculature, which demonstrates the crucial role of VEGF in vascular development (Carmeliet et al. 1996). VEGF-A also has many proinflammatory properties. VEGF enhances the expression of chemokines and adhesion molecules, and is a chemoattractant for monocytes and T cells (Clauss et al. 1990, Barleon et al. 1996, Kim et al. 2001, Reinders et al. 2003).

VEGF-A is expressed in several isoforms that are generated by alternative exon splicing of the VEGF gene. The four main human isoforms include VEGF$_{121}$, VEGF$_{165}$, VEGF$_{189}$ and VEGF$_{206}$, of which VEGF$_{165}$ is the predominant form (Tischer et al. 1991, Houck et al. 1992). The isoforms differ in their heparin binding affinities, which affects their bioavailability.

Many cell types including endothelial cells, smooth muscle cells, T cells, and macrophages produce VEGF-A (Ferrara et al. 1991, Berse et al. 1992, Freeman et al. 1995, Melter et al. 2000). The production of VEGF-A is upregulated by the HIF-1
transcription factor system during hypoxia (Shweiki et al. 1992, Liu et al. 1995). In addition, various proinflammatory cytokines and growth factors including IL-1, IL-6, TNF-α, IFN-γ, platelet-derived growth factor (PDGF) and TGF-β have been shown to increase VEGF production \textit{in vitro} (Ben-Av et al. 1995, Pertovaara et al. 1994, Ryuto et al. 1996, Cohen et al. 1996, Li et al. 1995, Finkenzeller et al. 1997).

VEGF has been linked to atherosclerotic plaque progression and instability (Celletti et al. 2001a, Celletti et al. 2001b). VEGF increases macrophage migration to the atherosclerotic plaque and plaque endothelial cell content. Moreover, VEGF induces smooth muscle cell migration directly (Grosskreutz et al. 1999) and indirectly by inducing matrix metalloproteinase production (Wang and Keiser 1998). VEGF also mobilizes and recruits vascular progenitor cells that may subsequently transdifferentiate into smooth muscle cells (Hattori et al. 2001, Simper et al. 2002). Thus, VEGF could also attract smooth muscle cells to the atherosclerotic plaque.

Inhibition of VEGF signaling has reduced acute rejection (Reinders et al. 2003) and the development of cardiac allograft arteriosclerosis in animal models of heart transplantation (Lemstrom et al. 2002). Accordingly overexpression of VEGF within cardiac allografts accelerates the development of allograft vasculopathy (Lemstrom et al. 2002). Increased macrophage VEGF expression in kidney transplants has been described in biopsies with chronic rejection (Pilmore et al. 1999).

6.2 VEGF-C

VEGF-C binds to VEGFR-2, VEGFR-3, NRP1 and NRP2. VEGF-C is the major regulator of lymphatic vessel development (Joukov et al. 1996, Karpanen et al. 2006) and VEGFR-3 is the key receptor that mediates this effect (Jeltsch et al. 1997, Karkkainen et al. 2004). Proteolytic processing regulates the biological activity and receptor specificity of VEGF-C (Joukov et al. 1997). The loss of one VEGF-C allele causes an impairment in lymphatic growth, whereas homozygous VEGF-C knockout mice die \textit{in utero} due to tissue edema that is caused by the resulting lack of lymphatic vessels (Karkkainen et al. 2004). VEGF-C also has effects on the vascular endothelium and on angiogenesis, although at higher concentrations than VEGF-A (Joukov et al. 1997, Pepper et al. 1998).

In addition to embryogenesis, formation of new lymphatic vessels occurs during inflammatory situations (Baluk et al. 2005). At sites of inflammation, lymphangiogenesis is induced by macrophages and dendritic cells, which produce VEGF-C (Cursiefen et al. 2004, Baluk et al. 2005). Proinflammatory cytokines including TNF-α, IL-1α and IL-1β increase VEGF-C production (Ristimaki et al. 1998). VEGF-C also promotes macrophage and dendritic cell migration and can thus recruit them at the inflammatory sites (Skobe et al. 2001, Chen et al. 2004).
VEGF-C/VEGFR-3 pathway also induces chemokine ligand (CCL) 21 signaling (Issa et al. 2009, Nykanen et al. 2010). CCL21 is a chemokine that is produced by stromal cells in the secondary lymphoid organs and also in the lymphatic endothelial cells (Luther et al. 2000, Kriehuber et al. 2001). It facilitates the entry of the chemokine receptor (CCR) 7+ dendritic cells and T cells into the secondary lymphoid organs (Gunn et al. 1998, Saeki et al. 1999, Gunn et al. 1999). VEGFR-3 inhibition suppresses dendritic cell traffic into the secondary lymphoid organs and thus limits allograft rejection in experimental transplantation models (Chen et al. 2004, Nykanen et al. 2010). This limiting action may be explained by consequent inhibition of the lymphatic endothelial cell CCL21 production (Nykanen et al. 2010).

6.3 Lymphangiogenesis in kidney transplantation

Newly formed lymphatic vessels are seen in 61–74% of renal protocol biopsies taken from kidney transplantation patients (Stuht et al. 2007). Similar to that which occurs in other inflammatory situations, VEGF-C expressing macrophages in kidney transplant biopsies have been detected in association with newly formed lymphatics (Kerjaschki et al. 2004). A retrospective study of graft biopsies taken for graft dysfunction reported that almost all graft losses were concentrated in patients with a high lymphatic vessel density (Kerjaschki et al. 2004). However a one-year follow-up study of normally functioning kidney transplants found that high lymphatic vessel density was actually associated with better graft outcome (Stuht et al. 2007). Thus the significance of lymphangiogenesis in kidney transplants is still unclear.

New lymphatic vessels in renal transplants are associated with tertiary lymphoid tissue (TLT) formation (Kerjaschki et al. 2004). TLT is organized lymphatic tissue that forms at the site of chronic inflammation (Prineas 1979). TLT is present in 10% of kidney allografts with dysfunction (Kerjaschki et al. 2004). TLTs have the same microarchitecture as germinal centers including high endothelial venules, lymphatic vessels, B and T cell compartments and follicular dendritic cells (Ruddle 2014). TLT may promote the development of chronic rejection by maintaining the local immune response and alloantibody production (Thaunat et al. 2005).
Table 3.  
The key functions of mammalian VEGF family ligands

<table>
<thead>
<tr>
<th>VEGF ligand</th>
<th>Binds to</th>
<th>Biological function</th>
<th>Knockout phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>VEGFR-1</td>
<td>-Vasculogenesis</td>
<td>- Homozygous: die in utero, impaired blood island formation and angiogenesis</td>
</tr>
<tr>
<td></td>
<td>VEGFR-2</td>
<td>-Angiogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP1</td>
<td>-Vascular permeability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP2</td>
<td>-Inflammation</td>
<td>-Heterozygous: die in utero, deficient early vasculature (Carmeliet et al. 1996)</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>VEGFR-1</td>
<td>-Normal heart function</td>
<td>-Homozygous: reduced size of heart, impaired lipid uptake (Bellomo et al. 2000, Hagberg et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>NRP1</td>
<td>-Blood vessel survival</td>
<td></td>
</tr>
<tr>
<td>VEGF-C</td>
<td>VEGFR-3</td>
<td>-Lymphangiogenesis</td>
<td>-Homozygous: die in utero, edema due to lack of lymphatic vessels</td>
</tr>
<tr>
<td></td>
<td>VEGFR-2</td>
<td>-Angiogenesis</td>
<td>-Heterozygous: not lethal, impaired development of lymphatics (Karkkainen et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>NRP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-D</td>
<td>VEGFR-3</td>
<td>-Lymphangiogenic</td>
<td>-Homozygous: healthy, functional lymphatic system (Baldwin et al. 2005)</td>
</tr>
<tr>
<td>(VEGFR-2)</td>
<td></td>
<td>-Angiogenic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIGF</td>
<td>VEGFR-1</td>
<td>-Pathological angiogenesis</td>
<td>-Homozygous: normal embryogenesis, subtle vascular defects (Carmeliet et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>NRP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRP2</td>
<td></td>
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</table>

(Modified from Krebs 2014)
6.4 Medications that interfere VEGF family signaling

Angiogenesis is essential for tumor progression, and the process is highly dependent on VEGF-A signaling (Ferrara et al. 2003). Consequently, numerous drugs that target VEGF pathway have been developed, of which the monoclonal antibody, bevacizumab, was the first to become clinically available. Monoclonal antibodies and tyrosine kinase inhibitors are now used in clinical practice as VEGF inhibitors to treat cancer patients (Hurwitz et al. 2004, Escudier et al. 2007). Anti-VEGF-A therapy is also an effective treatment for wet-related macular degeneration (Rosenfeld et al. 2006).

The mTOR inhibitors interfere with VEGF family signaling (Guba et al. 2002). Both sirolimus (SRL) and everolimus form a complex with the FK binding protein complex -12. The complex efficiently binds to mTOR. This interaction causes an inactivation of p70S6 kinase, which when activated stimulates the production of ribosomal components that are necessary for protein synthesis and cell cycle progression. Thus mTOR inhibitors effectively block IL-2 driven lymphocyte division, which is the mechanism for their immunosuppressive effect (Wiederrecht et al. 1995) (Figure 3).

The mTOR inhibitors also inhibit spontaneous and growth factor induced proliferation of many other cell types including endothelial cells, fibroblasts and smooth muscle cells (Akselband et al. 1991, Cao et al. 1995). Similarly the PI3K-p70S6 kinase pathway is required for VEGF-A and VEGF-C signaling in vascular and lymphatic endothelial cells (Vinals et al. 1999, Yu and Sato 1999). The mTOR inhibitors also reduce VEGF production (Guba et al. 2002). Consequently, mTOR inhibitors have been shown to inhibit both angiogenesis and lymphangiogenesis (Guba et al. 2002, Huber et al. 2007). The possible graft protecting effect of mTOR inhibitors, when compared to CNI, has often been attributed to the elimination of CNI toxicity. However, it may also be linked to direct actions of mTOR inhibitors including the inhibition of VEGF pathways.
7. The activin family

The activins are members of the TGF-β superfamily of cytokines. The activins were initially named after their ability to activate the release of follicle-stimulating hormone (FSH) from the anterior pituitary. Activins are dimers of inhibin β chains, whereas the inhibins that inhibit the release of FSH are heterodimers of α and β chains (Ling et al. 1986, Vale et al. 1986). The literature has best described activin A and B. Activin A is a homodimer of βA chains and activin B of βB chains, but βE and βE chains also exist (Ling et al. 1986, Vale et al. 1986, Htten et al. 1995, Fang et al. 1996). Activins bind to one of two type 2 activin receptors (ActRIIA and ActRIIB) on the cell surface, which dimerise with a type 1 activin receptor (activin receptor-like kinase, ALK) (Attisano et al. 1996, Willis et al. 1996) to activate SMAD 2/3 (Shimizu et al. 1998) and mitogen-activated protein kinase signaling pathways (Zhang et al. 2005a, Huang et al. 2006). Activin A signaling is mediated through ALK4, but activin B can also use ALK7 as well as ALK4 (Tsuchida et al. 2004, Bernard et al. 2006).

Follistatin and inhibins are endogenous regulators of activin activity (Nakamura et al. 1990, Vale et al. 1986). Follistatin is a powerful activin binding protein. After follistatin has bound to activin, the complex is attached to the cell surface and removed by a lysosomal degradation pathway (Hashimoto et al. 1997). Inhibins are mainly produced by gonads and circulate as hormones (Bilezikjian et al. 1993). The inhibin α chains efficiently compete with the β chains to reduce the formation of activin homodimers (Pangas and Woodruff 2002). Inhibins are also able to bind to the type 2 activin receptor through a co-receptor transforming growth factor beta receptor 3 and prevent the dimerization of the type 2 and type 1 receptor that is necessary to initiate intracellular signaling of activins (Lewis et al. 2000).

7.1 Activins in inflammation

Activins are important regulators of inflammation (Hedger and de Kretser 2013, Sideras et al. 2013). Increased activin A production has been described in many acute and chronic inflammatory diseases (Hedger and de Kretser 2013). Treatment with follistatin has been shown to reduce inflammation in experimental models of septicemia, allergic asthma and colitis: a result which indicates proinflammatory role for activins (Dohi et al. 2005, Hardy et al. 2006, Jones et al. 2007). Furthermore the overexpression of activin A in the murine lung has been shown to cause lung damage that simulates acute respiratory distress syndrome (Apostolou et al. 2012). Various inflammatory cells including monocytes, macrophages, dendritic cells and neutrophils are able to produce activin A (Eramaa et al. 1992, Robson et al. 2008, Chen et al. 2011). Activin A production is stimulated by inflammatory mediators lipopolysaccharide, TNF-α and IL-1β (Shao et al. 1992, Eramaa et al. 1992).
7.2 Activins and macrophages


7.3 Activins in experimental kidney disease


Results from an in vitro experiment in renal fibroblasts (Yamashita et al. 2004) and an in vivo study in rat unilateral urethral obstruction model (Maeshima et al. 2014) suggest that activin A produced by renal fibroblasts regulates renal fibrosis. Activin A enhances renal fibroblast transdifferentiation into myofibroblast and the formation of extracellular matrix (Yamashita et al. 2004, Maeshima et al. 2014). Activin expression is induced by TGF-β in renal fibroblasts and blockade of activin action with follistatin reduces TGF-β induced type I collagen expression. This may indicate that activin A, in its own part, mediates the fibrogenic effects of TGF-β (Yamashita et al. 2004). Similar pro-fibrotic role in glomerular pathology has been suggested for activin A. Glomerular activin A expression is markedly increased in rats with anti-Thy1 glomerulonephritis. Incubation of mesangial cells with activin A stimulates TGF-β1, plasminogen activator inhibitor -1 and connective tissue growth factor expression and increases the production of extracellular matrix proteins. (Gaedeke et al. 2005)

In experimental kidney transplantation high activin levels have been shown to be associated with profibrotic (M2) macrophage phenotype independent of IL-4 and IL-13.
This finding suggests that activins may regulate the M2 response in kidney allografts (Famulski et al. 2008, Famulski et al. 2010).

**Figure 4**  
The regulation of activin A during tissue injury, inflammation and fibrosis. During infection and tissue injury pathogen- and damage-associated molecular patterns such as lipopolysaccharide (LPS) and high-mobility group box 1 (HMGB1) stimulate toll-like receptor (TLR) signaling. TLR signaling and proinflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) increase activin A production. Transforming growth factor-β (TGF-β) is known to be a key regulator of fibrosis and it also stimulates activin A production. Follistatin is an endogenous inhibitor of activin activity. (Modified from de Kretser et al. 2012).
8. HMG-CoA reductase inhibitors

Statins inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and they are used in the primary and secondary prevention of cardiovascular diseases. The group contains several molecule, which include atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin. Inhibition of HMG-CoA results in a reduced synthesis of cholesterol and isoprenoids. Isoprenoids modulate the protein prenylation. Important prenylated proteins affected by statins include Rho GTPases that modulate cell cytoskeleton and Ras proteins that are involved in intracellular signaling (Greenwood et al. 2006). Statins also have an HMG-CoA independent effect through lymphocyte function-associated antigen-1 inhibition (Weitz-Schmidt et al. 2001). Statins, thus have numerous pleiotropic effects in addition to their ability to lower blood cholesterol in patients with hyperlipidemia. Amongst other things, statins regulate nitric oxide synthesis (Pahan et al. 1997), cell proliferation and apoptosis (Weiss et al. 1999), Nf-κB signaling (Guijarro et al. 1996), production of free oxygen radicals (Bokoch and Prossnitz 1992), blood coagulation, platelet aggregation (Mayer et al. 1992) and tissue remodeling (Essig et al. 1998).

8.1 Statins in acute kidney injury

Several investigators have studied the protective mechanism of preoperative statin treatment against renal IRI in rat and mouse experimental models and obtained promising results (Table 4). Lately, the clinical application of statin use in the prevention of AKI has gained a lot of attention. Statin pretreatment protects the kidney from contrast-induced nephropathy in patients undergoing percutaneous coronary intervention, which may indicate a desirable new role for statins use in the future (Patti et al. 2011, Quintavalle et al. 2012, Leoncini et al. 2014). However, this protective effect may not apply to AKI of other causes, since it might be specifically related to inhibited reabsorption of contrast media from the urinary space. Some recent clinical trials even suggest that daily use of high statin doses may induce AKI per se. For example, a retrospective study found that high daily statin doses caused increased hospitalization for AKI in patients who had no previous history of chronic kidney disease (Dormuth et al. 2013). A randomized clinical trial compared rosuvastatin and atorvastatin in patients with diabetes and progressive renal disease. The authors found that the risk of AKI associated particularly with high doses of rosuvastatin. (de Zeeuw et al. 2015)

8.2 Statins in chronic renal disease

The role of statins in the prevention of renal fibrosis has also been studied in experimental rodent models. Statins inhibit fibrosis in a model of unilateral ureteral obstruction in rat and mouse (Moriyama et al. 2001, Vieira et al. 2005). Statins also reduce glomerular
mesangial cell proliferation and matrix expansion in anti-thymocyte antibody induced experimental glomerulonephritis in the rat (Yoshimura et al. 1998). A current large meta-analysis on the use of statins under a clinical setting in chronic kidney disease patients shows that long-term use of statins does not impact on the decline of renal function, but may reduce proteinuria (Palmer et al. 2014).

8.3 Statins in kidney transplantation

Donor statin pretreatment in kidney transplantation models in the rat have shown controversial results. Donor atorvastatin pretreatment (50 mg/kg) in the isogeneic kidney transplantation model reduced tubular apoptosis, monocyte infiltration and improved graft function five days after transplantation (Gottmann et al. 2007). The protective effect was linked to the inhibition of aldose reductase activity. The same study also found that treatment of the donor with atorvastatin before allogeneic transplantation inhibited early inflammation, but it did not improve graft function or affect long-term graft survival (Gottmann et al. 2007). Atorvastatin (50 mg/kg) pretreatment did not inhibit inflammation or improve renal allograft histology in a brain dead donor model analyzed at 10 days after transplantation (Hoeger et al. 2012). However, simvastatin (5mg/kg) donor pretreatment reduced microvascular permeability, tubular cell apoptosis, activation of the innate and adaptive immune responses and improved graft function during a 5-day follow-up after allogeneic transplantation (Tuuminen et al. 2013).

Although statins showed anti-inflammatory, vasculoprotective, anti-fibrotic and anti-apoptotic effects in experimental studies, the graft protecting effects in clinical kidney transplantation are still inconclusive. Fluvastatin (40mg) treatment for 120 days after renal transplantation does not inhibit acute rejection episodes (Holdaas et al. 2001). Statin treatment in a large clinical trial “ALERT”, in which fluvastatin (80mg) was initiated six months after transplantation or later, inhibited cardiovascular deaths but did not affect chronic allograft injury (Fellstrom et al. 2004). However in the ALERT study the statin treatment may have been started too late to protect from developing alloimmune response and fibrosis. A smaller trial in which fluvastatin (80mg) was administered for six months following transplantation, found that fluvastatin reduced graft vasculopathy as defined by Banff-criteria although it did not affect arterial intimal volume fraction, which was the primary efficacy variable (Seron et al. 2008)
<table>
<thead>
<tr>
<th>Statin pretreatment studies in rodent renal ischemia-reperfusion models</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Statin</strong></td>
</tr>
<tr>
<td>Atorva</td>
</tr>
<tr>
<td>Prava</td>
</tr>
<tr>
<td>Simva</td>
</tr>
<tr>
<td>Ceriva</td>
</tr>
<tr>
<td>Atorva</td>
</tr>
<tr>
<td>Prava</td>
</tr>
<tr>
<td>Sabbatini et al. 2004</td>
</tr>
<tr>
<td>Yokofo et al. 2003</td>
</tr>
<tr>
<td>Gueler et al. 2002</td>
</tr>
<tr>
<td>Joyce et al. 2003</td>
</tr>
</tbody>
</table>

Note: ATN = acute tubular necrosis, GFR = glomerular filtration rate, NOS = nitric oxide synthase, IL = interleukin, HO-1 = heme oxygenase-1, NF-κB = nuclear factor-kappa B, ERK = extracellular signal-regulated kinase, AP-1 = activator protein-1.
Abbreviations: activator protein-1 (AP-1), acute tubular necrosis (ATN), day (d), endothelial nitric oxide synthase (eNOS), extracellular-signal-regulated kinase (ERK), glomerular filtration rate (GFR), heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), nitric oxide (NO), nuclear factor kappa B (NF-kB), preoperatively (preop.). (Modified from Tuuminen 2014).
AIMS OF THE STUDY

The aim of this study was to investigate the role of VEGF A and C in an experimental model of chronic allograft injury in the rat. Treatment strategies that could inhibit ischemia-reperfusion injury and early inflammation in terms to prevent the development of chronic allograft injury were also investigated.

The specific aims of this study were:

1. To describe the intragraft expression, localization and kinetics of VEGF-A, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3 and lymphatic vessels in renal allografts during the development of chronic allograft injury;

2. To investigate the effect of VEGFR-1/VEGFR-2 inhibition on chronic renal allograft injury;

3. To investigate the effect of sirolimus on VEGF family, lymphangiogenesis and chronic allograft injury in renal transplants;

4. To investigate the effect of perioperative simvastatin treatment on chronic allograft injury;

5. To examine the effect of activin inhibition on acute kidney injury, inflammation and activation of fibrinogenesis in renal allografts.
METHODS

1. Experimental rat models

Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. All animals received care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy (ISBN, 0-309-15400-6, revised 2011), in addition to being in full compliance with other specific national laws relevant to animal experimentation.

1.1 Kidney transplantation model (I-IV)

Inbred fully MHC-mismatched Dark Agouti (DA) (AG-B4, RT1a) male rats and Wistar Furth (WF) (AG-B2, RT1b) male rats (Harlan, Horst, Netherlands) weighing 250–350 g were used. Allogeneic transplantations were performed between DA and WF rats and syngeneic control transplantations between DA rats.

Transplantations were performed using a modified microsurgical technique described by Fisher and Lee (Fisher and Lee 1965). The donor right kidney was perfused with 5 ml cold PBS containing 50 IU/ml heparin and removed with a segment of the aorta and the vena cava. The transplant was stored in the same heparin solution at +4°C until used for transplantation. The kidney was transplanted heterotopically to the recipient’s abdominal aorta and the inferior vena cava with end-to-side aortic and vena caval anastomosis. The ureter was anastomosed end-to-end. The right native kidney was removed at the time of transplantation. Total ischemia time was standardized between 30 to 50 min. The left native kidney of the recipient was removed seven days after transplantation to allow the transplant's function to recover. Thereafter, the recipient was dependent on the transplanted kidney. Operations were performed under anesthesia, intraperitoneal chloral hydrate (240 mg/kg) (I) or inhaled isoflurane (Isoflurane Baxter, Deerfield, III) (II-IV). Buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ) was used for postoperative analgesia (I-IV).

Transplants were recovered at 1, 3, 5, 7 and 14 days after transplantation to study acute rejection and also at 30, 60 and 90 days after transplantation to study chronic rejection. CsA was used as the immunosuppressive agent in allogeneic transplantation. Rats with syngeneic transplant were treated with CsA (I) or did not receive any immunosuppression (II, III).
1.2 Renal ischemia-reperfusion model (IV)

Renal ischemia was induced in the WF rats. The rats were anesthetized by isoflurane and subsequently a midline abdominal incision was performed. Both renal pedicles were clamped for 45 minutes. After clamp removal the kidneys were inspected for a recovery of the blood flow. Buprenorphine was used for postoperative analgesia. The right kidney was removed three days after the procedure for further analysis.

2. Drug regimens

2.1 Cyclosporine A (I-IV)

CsA (Novartis, Basel, Switzerland) was diluted in intralipid (Fresenius Kabi, Uppsala, Sweden) to final concentrations of 1 mg/ml, 1.5 mg/ml, and 2 mg/ml. CsA was administered subcutaneously (s.c.). Two different CsA treatment strategies were used: (i) CsA dosing 1.5 mg/kg once a day. In this model moderate to strong histopathological changes of chronic rejection develop within 90 days (I-IV). (ii) CsA was administered at 2 mg/kg/day for one week, 1 mg/kg/day for two weeks, and subsequently discontinued to allow maximal histological changes of chronic rejection to develop as early as 60 days after transplantation (I). CsA level in the whole blood were measured using radioimmunoassay (Sandimmun-Kit; Novartis).

2.2 PTK787 (I)

PTK 787 (kindly provided by Novartis) is a protein tyrosine kinase inhibitor, which blocks the signaling of both VEGFR-1 and VEGFR-2. High concentrations of PTK787 can also inhibit other class III kinases, such as platelet-derived growth factor receptor (PDGFR) - beta tyrosine kinase, c-Kit, and c-Fms (Wood et al. 2000). PTK 787 was diluted in polyethylene glycol (PEG, molecular weight 300; Sigma-Aldrich, St. Louis, MO) to a final concentration of 50 mg/ml and was given 100 mg/kg once a day orally using an orogastric tube. PEG alone was used as the vehicle control. All transplant recipients received also CsA as described above.

2.3 Sirolimus (II)

Sirolimus (kindly provided by Wyeth, Madison, NJ, USA) was diluted in PEG and given 2 mg/kg once a day orally using an orogastric tube. All transplant recipients also received CsA 1.5 mg/kg/day for the first seven days after transplantation to overcome acute
rejection. Sirolimus treatment was compared to CsA treatment 1.5mg/kg/day. Whole blood concentrations of sirolimus were determined using an immunochemiluminometric assay.

2.4 Simvastatin (III)

Simvastatin (Merck Research Laboratories, Whitehouse Station, NJ) was diluted in PEG to final concentrations of 5 mg/ml for donor treatment and 2 mg/ml for recipient treatment and was given orally through an orogastric tube. Simvastatin was given as a pretreatment to the transplant donor and/or to the recipient two hours before transplantation and/or as daily recipient treatment starting from the first postoperative day. The study groups are presented in more detail in table 5.

Table 5. Simvastatin treatment in the study groups (III).

<table>
<thead>
<tr>
<th></th>
<th>Donor pretreatment</th>
<th>Recipient pretreatment</th>
<th>Treatment 2mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/ kg</td>
<td>2 mg/kg</td>
<td>days 1-90 after</td>
</tr>
<tr>
<td>1. No-statin</td>
<td></td>
<td></td>
<td>transplantation</td>
</tr>
<tr>
<td>2. Dpre</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Rpost</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>4. Dpre+Rpost</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>5. Dpre+Rpre+Rpost</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Note: The donor and recipient simvastatin pretreatment was administered 2 hours before transplantation. All transplant recipients received cyclosporine 1.5 mg/kg/day s.c. All the above groups were compared to each other in the statistical analysis.

2.5 sActRIIB-Fc (IV)

A soluble activin receptor sActRIIB-Fc was used to inhibit activin A and B signaling in experimental models of transplantation and ischemia-reperfusion injury. The recombinant fusion protein that contains the ectodomain of human ActRIIB fused to the Fc domain of human IgG1 (sActRIIB-Fc) was produced in-house (Apostolou et al. 2012, Myllarniemi et al. 2014). sActRIIB-Fc was administered intraperitoneally (i.p.). Control human IgG1-Fc fragment (BioXcell, West Lebanon, NH) was used as specificity control for sActRIIB-Fc activity. The study groups are shown in table 6.
Table 6. The study groups investigating the effect of sActRIIB-Fc (IV)

<table>
<thead>
<tr>
<th>Transplantation model</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CsA 1.5 mg/kg/day s.c.</td>
<td>sActRIIB-Fc 2 mg/kg i.p. for transplant recipient two hours before transplantation + CsA 1.5 mg/kg/day s.c.</td>
<td>IgG1-Fc 2 mg/kg i.p. for transplant recipient two hours before transplantation + CsA 1.5 mg/kg/day s.c.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IRI model</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td>sActRIIB-Fc 2 mg/kg i.p. two hours before the procedure</td>
</tr>
</tbody>
</table>

3. Immunohistochemistry and immunofluorescence (I-IV)

Paraffin-embedded and frozen specimens were cut into four µm thick sections. Epitopes were retrieved by heating the paraffin sections in a microwave oven for 20 min in a sodium citrate buffer (pH 6.0) and then they were allowed to cool at room temperature for 20 min.

Immunohistochemical stainings were accomplished using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) with 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). First, the sections were incubated with an appropriate nonimmune serum. Then the sections were incubated with a primary antibody. Incubation time varied from 30 min at room temperature to overnight at +4°C depending on the antibody. After primary antibody incubation ended, endogenous peroxidase activity was blocked by incubating the sections in a 1% hydrogen peroxidase phosphate-buffered saline solution for 20 min. Next, the samples were incubated with biotinylated secondary antibody for 30 min, followed by avidin-biotinylated horseradish complex for 30 min. Finally, the reaction was revealed by staining with AEC containing 0.1% hydrogen peroxidase. The sections were counterstained with hematoxylin and coverslips were mounted with aquamount (BDH Ltd., Poole, UK). When staining lymphatic vessel hyaluronan receptor-1 (LYVE-1) tyramide signal amplification system was used to enhance the signal (TSA indirect; PerkinElmer, Waltham, MA, USA) (II).

EnVision™ G|2 System/AP kit, Rabbit/Mouse (Permanent Red; DAKO, Glostrup, Denmark) was used in selected immunohistochemical stainings. The system is biotin-free, which reduces the non-specific tubular staining that results from endogenous avidin-biotin activity. The Envision system was particularly useful in optimizing growth factor staining.
specificity. Levamisole (DAKO) was used to reduce endogenous alkaline phosphatase activity. (II)

Immunofluorescence stainings were performed on frozen sections using a sequential approach with Alexa Fluor 488 (green) and Alexa Fluor 568 (red) fluorescent secondary antibodies (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Slides were covered with a mounting medium including DNA stain 4′,6-diamidino-2-phenylindole (Vectashield HardSet Mounting Medium with DAPI; Vector Laboratories). (I, II)

4. **Histological evaluation**

The paraffin-embedded specimens were cut into 2-μm thin sections and stained with Mayer's hematoxylin-eosin, Masson's trichrome, diastase-periodic acid-schiff, methenamine silver PAS, and Unna-Pappenheim stains.

Tubular necrosis, dilatation, flattening and casts were all graded on a semiquantitative scale from 0 to 3 as follows: grade 0 = no damage, grade 1 = mild damage, grade 2 = moderate damage, grade 3 = severe damage. (IV)

Chronic changes were scored according to the Chronic Allograft Damage Index (CADI). The CADI value is the sum of interstitial inflammation and fibrosis, tubular atrophy, glomerular mesangial matrix increase, glomerular sclerosis, and arterial intimal proliferation, scored from 0 to 3 and leading to CADI values that ranged from 0 to 18 (Isoniemi et al. 1994). The CADI grading of fibrosis and tubular atrophy from 1 to 3 are analogous with the BANFF-score (Banff 2010, category 5 grades I, II, and III) (Sis et al. 2010). However, the CADI score is numerical unlike the BANFF-score, which makes it possible to monitor statistical differences between study-groups both in individual histological parameters and also in total rejection-score and for this reason it is used here. (I, II, III)

5. **Kidney function (II, III)**

The serum creatinine of the transplantation model was measured once a week until the rats were sacrificed. Serum creatinine was measured 24 and 72 hours after the procedure in the IRI model. The blood was collected from tail vein and the serum was subsequently frozen at -20°C until further analysis (II-IV). 24-hour urine protein output was measured at 60 and at 90 days after transplantation. Urine collection was done in metabolic cages (III).
6. **Enzyme-linked immunosorbent assay (IV)**

Enzyme-linked immunosorbent assays (ELISAs) were used to measure serum concentrations of AKI markers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) and key proinflammatory cytokines IL-1β, IL-6, and TNF-α. The analyses were carried out according to the manufacturers instructions. The following kits were used: rat NGAL (KIT046, BioPorto Diagnostics, Gentofte, Denmark), rat TIM-1/KIM-1/HAVCR (RKM100, R&D systems, Minneapolis, MN), rat TNF-α (RTA00, R&D systems), rat IL-6 (R6000B, R&D systems), rat IL-1 beta/IL-1F2 (RLB00, R&D systems).

7. **Statistics (I-IV)**

The results are expressed as mean ± standard error of the mean and the probabilities of <0.05 were accepted as statistically significant. The following statistical tests were used: (i) Student’s t-test for two group comparisons of mean parametric results, (ii) analysis of variance (ANOVA) with least significant difference for multiple group comparisons of quantitative results, (iii) Mann-Whitney U-test for non-parametric comparisons of distributions of two groups, and (iv) Kruskal-Wallis with Dunn’s test for non-parametric comparisons of the distributions of more than two groups. In addition ANOVA for repeated measures was used to analyze results that were obtained from multiple time points. Linear regression analysis was applied to evaluate a possible relation of VEGF, VEGFR-1 and LYVE-1+ lymphatic vessel expressions to the CADI score (I, II).
RESULTS

1. **Syngeneic transplantation has little effect on VEGF ligand and receptor expression and it does not induce lymphatic vessel proliferation (I and II)**

The VEGF ligand and receptor protein expression patterns in normal kidneys and syngrafts are summarized in the table 7. Syngenic transplantation induced a mild inflammatory cell VEGF-C expression measured at 30 and 60 days after transplantation. Syngenic transplantation did not affect the expression of other studied VEGF ligands or their receptors. Normal kidneys and syngrafts LYVE-1+ lymphatic vessels were detected only around large vascular structures.

Table 7. *VEGF ligand and receptor expression in normal kidneys and syngrafts (I, II).*

<table>
<thead>
<tr>
<th>VEGF/VEGFR</th>
<th>Glomeruli</th>
<th>Vascular wall</th>
<th>Tubular cells</th>
<th>Inflammatory cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)*</td>
<td>II</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>++</td>
<td></td>
<td>++</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>II</td>
</tr>
</tbody>
</table>

Vascular VEGF-A expressing cells were further identified as vascular endothelial cells and VEGFR-1 expressing cells were identified as smooth muscle cells (I). *VEGF-C expression was detected in a few inflammatory cells in syngenic transplants 30 and 60 days after transplantation.

2. **Monocyte-macrophages express VEGF-A and VEGFR-1 during acute and chronic rejection in renal allografts (I)**

VEGF-A and VEGFR-1 expression in CsA-treated allografts was significantly induced during acute rejection when compared to their expression from syngrafts. The expression levels also remained high during the development of chronic allograft injury. The major source of increased VEGF-A and VEGFR-1 expression were the graft-infiltrating monocyte-macrophages. VEGF-A and VEGFR-1 expression in other renal structures remained at the same levels as those obtained from the syngrafts (Table 7). VEGF-A and VEGFR-1 monocyte-macrophage expression correlated with the degree of chronic allograft injury (CADI score) in linear regression analysis. Allogeneic transplantation did
not modulate VEGFR-2 expression, which was constant in glomeruli and vascular structures (Table 7).

3. **VEGF-C and VEGFR-3 expression is induced during the development of chronic renal allograft injury (II)**

VEGF-C and VEGFR-3 expression levels during acute rejection remained low in CsA-treated allografts. However, their expression was strongly induced during the development of chronic allograft injury measured at 30 and 60 days after transplantation. VEGF-C was produced by monocyte-macrophages and also by CD4+ T cells. Induced VEGFR-3 expression was detected in both vascular and lymphatic vessel endothelial cells.

4. **Allogeneic transplantation induces significant lymphatic vessel proliferation (II)**

Strong lymphatic vessel proliferation in CsA-treated allografts was detected at 60 and 90 days after transplantation, but not earlier. Newly formed lymphatic vessels expressed inflammatory cell chemokine CCL21. Most of the lymphatic vessels were located close to nodular inflammatory cell infiltrates.

5. **VEGFR tyrosine kinase inhibitor limits chronic renal allograft injury (I)**

VEGFR-1 and VEGFR-2 inhibition by the tyrosine kinase inhibitor PTK787 efficiently reduced the development of chronic renal allograft injury in CsA-treated allografts when compared to the vehicle reference. PTK787 treatment reduced the total CADI score and profoundly inhibited the development of the interstitial fibrosis.

6. **Sirolimus reduces VEGF-A and VEGF-C expression and lymphatic vessel proliferation in renal allografts (II and unpublished results)**

SRL efficiently reduced both total intragraft and also inflammatory cell VEGF-A expression in the CsA-treated allografts at 7 and 90 days after transplantation (Table 8, unpublished results). The effect of SRL on VEGF-C expression was only modest. SRL at three days after transplantation reduced the total intragraft VEGF-C expression. The VEGF-C expression for SRL-treated allografts at three days was below the expression
level seen in normal kidneys. Later SRL did not affect VEGF-C expression. SRL had no apparent effect on VEGFR-3 expression.

SRL efficiently reduced the development of new lymphatic vessels in the renal allografts. The lymphatic vessel density of the SRL-treated allografts was only one-third of the density obtained for the CsA-treated allografts. SRL also reduced the amount of nodular inflammatory infiltrates, which in CsA-treated allografts co-localized with the lymphatic vessels. Linear regression analysis revealed a significant correlation between the CADI score and lymphatic vessel density.

Table 8.  

<table>
<thead>
<tr>
<th>Inflammatory cells</th>
<th>Total intragraft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CsA</td>
</tr>
<tr>
<td>3 days</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>7 days</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>90 days</td>
<td>2.1±0.1</td>
</tr>
</tbody>
</table>

The allograft VEGF-A expression was detected by immunohistochemistry. Inflammatory cell VEGF-A expression was scored from 0 to 3. Total intragraft score is the sum of the glomerular, tubular, vascular and interstitial VEGF-A expression that are all scored from 0 to 3. The results are expressed as mean ± standard error of the mean.
7. Sirolimus improves graft function and inhibits chronic allograft injury in renal allografts (II)

Treatment with SRL did not reduce acute inflammation when compared to allografts that received only CsA. However, SRL significantly attenuated the development of chronic renal allograft injury when compared to CsA-treated allografts. The serum creatinine values of SRL-treated allografts were significantly lower when compared to those of the CsA-treated allograft. According to the CADI score SRL-treated allografts had significantly less inflammation, fibrosis and arterial intimal proliferation than the CsA-treated allografts.

8. Perioperative simvastatin treatment limits graft inflammation and reduces chronic kidney allograft injury (III)

Donor and recipient simvastatin pretreatment combined with daily recipient simvastatin treatment improved graft function and decreased the CADI score in CsA-treated allografts. It also reduced the number of graft-infiltrating CD4+ and CD8+ T cells, dendritic cells and macrophages at 90 days. Daily recipient statin treatment alone, started after transplantation, reduced the number of CD4+ T cells and dendritic cells in the CsA-treated allografts at 90 days, but it did not affect graft function or the CADI score.

9. Donor simvastatin treatment alone paradoxically induces acute kidney injury (III)

Donor simvastatin pretreatment alone accelerated the rate of primary graft dysfunction in CsA-treated allografts. It also increased serum creatinine. The serum creatinine had already increased by the beginning of the experiment and stayed at elevated levels until the end of the study at 90 days. In addition, donor simvastatin treatment enhanced proteinuria 60 days after transplantation. Donor simvastatin treatment did not affect proteinuria or graft histology in the end of the experiment at 90 days. If donor simvastatin treatment was combined with recipient treatment started after transplantation, it did not have an apparent effect on any of the measured parameters.
Table 9. The effect of different simvastatin treatment strategies on CsA-treated allografts (III)

<table>
<thead>
<tr>
<th></th>
<th>Serum creatinine*</th>
<th>proteinuria</th>
<th>CADI</th>
<th>Inflammatory cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dpre</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dpre+Rpost</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rpost</td>
<td></td>
<td>↓</td>
<td></td>
<td>CD4⁺</td>
</tr>
<tr>
<td>Dpre+Rpre+Rpost</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td>ED3⁺</td>
</tr>
</tbody>
</table>

Dpre = Donor pretreatment 5mg/kg two hours before transplantation.
Rpre = Recipient pretreatment 2 mg/kg two hours before transplantation.
Rpost = Recipient treatment 2 mg/kg/d days 1-90 after transplantation.
* The serum creatinine levels in all allograft groups treated with simvastatin were elevated from the syngraft level.

10. Activin A expression is induced in the glomeruli and the interstitium during acute rejection (IV)

Only very mild activin A protein expression was detected in the glomeruli and tubuli of normal kidneys. Activin expression was induced in both glomerular and interstitial cells three days after transplantation in CsA-treated allografts. Treatment with a soluble activin receptor sActRIIB-Fc limited glomerular activin A expression in CsA-treated allografts. Bilateral renal IRI did not affect the expression of activin A three days after the procedure. Activin B expression was non-existent in all studied kidneys and allografts.

11. Activin inhibition does not affect acute kidney injury after bilateral renal ischemia-reperfusion injury (IV)

The sActRIIB-Fc treatment did not affect the serum concentrations of the kidney injury markers NGAL or KIM-1 after bilateral renal ischemia-reperfusion injury. Activin inhibition did not affect serum creatinine nor limit acute tubular necrosis in histological analysis. The inflammation found in the IRI model was mild for both control and sActRIIB-Fc treated kidneys three-day timepoint after the procedure.
12. Activin inhibition reduces the innate immune response and limits fibrotic processes in renal allografts (IV)

Activin inhibition with sActRIIB-Fc reduced the number of infiltrating dendritic cells, macrophages and neutrophils found in the CsA-treated allografts three days after transplantation, but it did not affect CD4+ or CD8+ T cell infiltration. Activin inhibition also reduced serum IL-1β and induced serum IL-6 concentrations.

Activin inhibition did not affect acute parenchymal cell injury after transplantation as measured by serum NGAL and KIM-1 levels and histopathological analysis of the grafts. However, it reduced the number interstitial fibroblasts in the graft three days after transplantation.
DISCUSSION

1. VEGF-A produced by macrophages is involved in the development of renal allograft fibrosis (I)

According to our results graft-infiltrating monocyte-macrophages expressed both VEGF-A and VEGFR-1 during acute rejection and the development of chronic allograft injury in CsA-treated allografts. Allogeneic transplantation did not modulate VEGFR-2 expression. The expression pattern we found suggests that VEGF-A functions as an autocrine/paracrine regulator of monocyte-macrophages through VEGFR-1 in renal transplants. VEGF-A is known to stimulate monocyte migration and activation through VEGFR-1. Clinical renal transplant biopsies have shown that macrophages express VEGF-A (Pilmore et al. 1999) and increased VEGF-A expression positively correlates with the macrophage infiltration (Ozdemir et al. 2005). VEGFR-1 inhibition by neutralizing antibody has accordingly been shown to reduce macrophage infiltration into the graft in experimental heart transplantation in the rat (Raisky et al. 2007).

Our results show that VEGF-A and VEGFR-1 inflammatory cell expression correlated with the degree of chronic allograft injury. Moreover VEGFR-1/VEGFR-2 inhibition with the tyrosine kinase inhibitor PTK787 reduced chronic allograft injury and particularly the development of the interstitial fibrosis. Thus our results indicate that increased VEGF-A produced by macrophages enhances renal allograft fibrosis. Increased allograft VEGF-A expression has been linked to increased interstitial fibrosis and accelerated graft loss also in renal transplant biopsies (Ozdemir et al. 2005). Macrophages are important players in the development of fibrosis and thus the profibrotic effect might be related to the VEGF-A/VEGFR-1 signaling in macrophages. VEGF-A may also promote renal fibrosis by inducing the production of other growth factors. VEGF-A has been shown to enhance PDGF and epidermal growth factor (EGF) expression in endothelial cells and smooth muscle cells (Arkonac et al. 1998, Lemstrom et al. 2002). Tyrosine kinase inhibitors that limit PDGF (imatinib), PDGF and VEGF (sunitinib), and EGF (erlotinib) signaling are effective in reducing chronic allograft injury in the rat (Savikko et al. 2003, Rintala et al. 2014, Rintala et al. 2015). Imatinib has been shown to completely abrogate the accelerated obliterative bronchiolitis that is caused by transgenic overexpression of VEGF-A in mouse tracheal allografts. The result strongly suggests that VEGF-A drives fibroproliferative responses by increasing the expression of PDGF (Krebs et al. 2005).
2. Chronic inflammation drives lymphatic vessel proliferation into renal allografts (II)

Our finding that lymphatic vessel proliferation does not occur in syngrafts indicates that the disconnection of lymphatic vasculature during transplantation procedure does not induce lymphatic vessel proliferation. Lymphatic vessel proliferation on CsA-treated allografts did not occur during acute rejection, but extensive lymphatic vessel proliferation was detected during the development of chronic allograft injury. Our results indicate that chronic inflammation drives lymphatic vessel proliferation in renal allografts.

Our study showed that new lymphatic vessels expressed lymphocyte chemokine CCL21 and co-localized with the nodular inflammatory cell infiltrates. CCL21 expressing lymphatic vessels surrounded by nodular inflammatory cell infiltrates have also been described in human biopsy samples obtained from chronically rejecting kidney allografts. Furthermore, cells in these nodular infiltrates have been shown to express CCR7, receptor to CCL21, suggesting that lymphatic vessels have an active role in the inflammatory cell traffic in renal transplants (Kerjaschki et al. 2004). Earlier studies have further identified these nodular infiltrates as tertiary lymphoid organs, which may be involved in the maintenance of local alloimmune response (Kerjaschki et al. 2004, Thaunat et al. 2005). However the studies that investigated the influence of new lymphatics on chronic allograft injury have shown contradictory results (Kerjaschki et al. 2004, Stuht et al. 2007). In our study lymphatic vessel density positively correlated with the degree of chronic allograft injury supporting the knowledge that lymphatic vessel proliferation is associated with poor graft outcome.

We found that macrophages and CD4\(^+\) T cells produced VEGF-C in the CsA-treated allografts during chronic inflammation. The VEGF-C receptor VEGFR-3 was expressed in the newly formed lymphatic vessels. Our result suggests that VEGF-C/VEGFR-3 signaling is involved in the regulation of lymphatic vasculature of renal allografts. Earlier studies in rodent models of tracheal and cardiac allogeneic transplantation suggest that VEGF-C/VEGFR-3 signaling could either drive lymphangiogenesis or regulate the activity of newly formed lymphatic vessels. The overexpression of VEGF-C in tracheal allografts enhanced and VEGF-C inhibition prevented lymphatic vessel proliferation (Krebs et al. 2012). Inhibition of VEGF-C signaling in cardiac transplantation, however, did not reduce lymphatic vessel proliferation. Instead, it inhibited the ability of new lymphatics to transport dendritic cells to the secondary lymphoid organs (Nykanen et al. 2010). Thus, other factors may also contribute to the development of lymphatic vessels in solid organ transplantation. Although VEGF-A does not regulate lymphangiogenesis directly, it drives lymphatic vessel proliferation by attracting macrophages to the site of inflammation (Cursiefen et al. 2004). Macrophages are essential for the development of lymphatic vessels during inflammation. Macrophage depletion inhibits the proliferation of lymphatic vessels (Cursiefen et al. 2004). Macrophages produce VEGF-C, but may also regulate lymphangiogenesis in other ways. It has even been suggested that macrophages
are the source of lymphatic endothelial cells during inflammation (Maruyama et al. 2005).

3. **Sirolimus inhibits VEGF-family signaling, lymphangiogenesis and chronic renal allograft injury (II, unpublished results)**

SRL-treatment in our study resulted in a powerful inhibition of lymphatic vessel proliferation in renal allografts when compared to CsA. This supports the earlier studies in a mouse skin-flap model and in tumour metastasis, which demonstrated the inhibitory effect of SRL on lymphatic vessel growth (Huber et al. 2007, Kobayashi et al. 2007). Our results suggest that SRL could also be used as an anti-lymphangiogenic therapy in renal transplants. The study with mouse skin-flap model demonstrated how the anti-lymphangiogenic effect of SRL is mediated by the inhibition of p70S6 kinase phosphorylation downstream of VEGF-C (Huber et al. 2007). Treatment with SRL in our study had only mild a inhibitory effect on VEGF-C expression, which suggests that inhibition of VEGF-C downstream signaling also played an important role in our study. In addition, sirolimus has a well documented inhibitory effect on VEGF-A expression and its downstream signaling (Guba et al. 2002). Our results also showed that SRL efficiently inhibited VEGF-A expression. Thus SRL might have inhibited lymphangiogenesis by limiting VEGF-A signaling.

The present study also found that SRL-treated allografts had significantly better graft function than CsA-treated allografts. Clinical study data shows that the change from CNI to mTOR treatment improves graft function in the long-term, when the swich is made either three to six months after transplantation or even later, if the baseline graft function is good (Lebranchu et al. 2009, Schena et al. 2009, Holdaas et al. 2011, Budde et al. 2015, Witzke et al. 2016). Our results show that SRL also improves graft histology, which suggests that improved graft function is not a hemodynamic effect but is instead associated with the inhibition of chronic allograft injury.

A very early (<2 months) conversion of CNI to mTOR inhibitor exposes the graft to the risk of acute rejection and should not be undertaken (Mjornstedt et al. 2012, Chadban et al. 2014). Accordingly, SRL treatment alone was not sufficient to overcome acute rejection in our study and therefore SRL-treated allografts were also treated with CsA for the first seven days after transplantation. Even then the early inflammation was similar for the SRL-treated allografts and those allografts that received only CsA. However, our results also show that sirolimus reduces the amount of inflammatory infiltrates associated with lymphatic vessels during the chronic allograft injury. Sirolimus is known to inhibit the signaling of VEGF-A and other growth factors, which may explain the inhibition of chronic inflammation (Akselband et al. 1991, Cao et al. 1995). Our results support the
contention that it is also possible that sirolimus limits chronic inflammation by inhibiting lymphatic vessel proliferation.

4. Perioperative statin treatment protects against chronic renal allograft injury (III)

The impact of early statin use on chronic allograft injury has been studied in one clinical trial. In that study fluvastatin 80mg was used for six months after transplantation. Fluvastatin treatment did not reduce the primary efficacy variable, which was the arterial intimal volume fraction, but limited graft vasculopathy according to the Banff-criteria (Seron et al. 2008). Our results show that when statin treatment for recipients was commenced immediately after transplantation it had a subtle inhibitory effect on chronic inflammation in CsA-treated renal allografts, but it did not affect chronic allograft injury. Thus, our results support the understanding that early statin use after transplantation alone does not limit chronic allograft injury despite its limited anti-inflammatory effects. However, experimental studies in rodents suggest that statin pretreatment limits AKI and inflammation after renal ischemia-reperfusion injury. Accordingly, the anti-inflammatory effect of simvastatin in our study was amplified, when the statin treatment was commenced prior to transplantation as both donor and recipient pretreatments. It efficiently limited the number of macrophages, T cells, and dendritic cells in the graft 90 days after transplantation. The intensive perioperative statin treatment also improved graft function and limited the histopathological changes of chronic allograft injury.

The serum creatinine levels in all allograft groups treated with simvastatin were clearly elevated from the syngraft level and this effect was especially prominent during the first weeks after transplantation. This suggests that despite the perioperative simvastatin treatment having anti-inflammatory effects, this alone did not prevent acute rejection from occurring. The intensive perioperative simvastatin treatment, however, limited the histopathological changes of chronic rejection. Graft macrophage infiltration early after transplantation and during acute rejection is a risk factor for the later graft fibrosis and predicts graft loss (Croker et al. 1996, Pilmore et al. 2000). Thus the inhibition of chronic rejection may be linked to the inhibitory effect the intensive perioperative statin treatment had on macrophage infiltration.

Paradoxically donor statin pretreatment alone increased serum creatinine values already early after transplantation and induced proteinuria at 60 days. This was a surprising finding since earlier experimental studies had suggested that donor statin pretreatment has beneficial or neutral effects on acute inflammation and injury after transplantation. However, there is increasing clinical evidence that high statin doses, similar to the one used in our study to treat transplant donors, may actually induce AKI. A retrospective study suggested that high dose statin (≥10 mg rosuvastatin, ≥20 mg atorvastatin, and ≥40 mg simvastatin) causes increased hospitalization for AKI during the first 120 days of treatment in patients without a history of chronic kidney disease
A randomized trial, PLANET I, compared renal effects of atorvastatin and rosuvastatin on diabetic patients with progressive kidney disease. The results indicate that AKI and doubling of serum creatinine are more common in high dose rosuvastatin users (40mg) than with low dose rosuvastatin users (10mg) or in patients using atorvastatin (80mg) (de Zeeuw et al. 2015). The nature of the renal injury, however, was not established. The link between our study and the clinical evidence is only speculative, since in our study the donors received only a single high simvastatin dose. Nevertheless, caution should be exercised with donor pretreatment especially with high doses until the mechanism between statins and AKI has been clarified more extensively.

5. Activins regulate innate immune response in renal allografts (IV)

Our results showed that activin A, but not activin B, production is induced in CsA-treated renal allografts. A soluble type 2 activin receptor (sActRIIB-Fc), that inhibits both activin A and B, reduced neutrophil, dendritic cell, and macrophage infiltration into the graft. Activin inhibition with sActRIIB-Fc also modulated IL-1β and IL-6 serum concentrations after transplantation. This may reflect the effect of activin inhibition on macrophages, since macrophages are a rich source of cytokines and activins are known to regulate macrophage cytokine production (Hedger and de Kretser 2013, Kwan et al. 2014). The inhibition of innate immune cell infiltration into the graft and altered cytokine production suggest that activin A regulates the innate immune response in renal allografts. Earlier studies especially in experimental lung injury models also indicate that activin A regulates the innate immune response. Overexpression of activin A in mouse airways stimulates marked pulmonary macrophage and neutrophil infiltration (Apostolou et al. 2012). Accordingly follistatin, an endogenous inhibitor of activin activity, efficiently inhibits neutrophil and macrophage infiltration into the airways of mice during experimental cystic fibrosis (Hardy et al. 2015). In vitro studies also suggest that activin A is able to enhance monocyte, dendritic cell and macrophage migration (Petraglia et al. 1991, Salogni et al. 2009, Ogawa et al. 2000). Moreover, activins have been shown to induce HMGB-1 production, which is a central DAMP that is involved in initiating the TLR signaling and innate immune response after renal IRI (Wu et al. 2007, Apostolou et al. 2012).

Even though activin inhibition reduced the innate immune response, it did not limit T cell infiltration into the graft. Furthermore it did not inhibit AKI although earlier studies suggest that activin inhibition could directly protect the kidney from hypoxic tubular cell injury (Maeshima et al. 2001, Maeshima et al. 2002). Earlier experimental studies in mice with deficient TLR signaling suggest that reduced innate immune response has limited effects on acute rejection or on AKI. Although TLR-2 and TLR-4 deficiency protects the kidney from ischemia-reperfusion injury, the effect is primarily mediated by impaired TLR signaling in the parenchymal cells that results in reduced tubular cell apoptosis (Leemans et al. 2005, Wu et al. 2007). Moreover impaired TLR signaling in
renal transplant recipients does not prevent acute rejection (Lerret et al. 2015). Cytotoxic memory CD8+ T cells are able to induce vigorous allograft injury early after transplantation before T cell priming (Chalasani et al. 2002, El-Sawy et al. 2004). Thus, earlier knowledge and also our results suggest that T cells and direct hypoxic cell injury are able to produce acute renal allograft injury although the innate immune response is reduced. However, impaired TLR signaling in mice has been shown to promote the development of graft tolerance later on and to limit chronic inflammation. In addition it has been shown to reduce renal transplant fibrosis and most importantly to improve graft survival (Wang et al. 2010, Lerret et al. 2015).

Activins may also be involved in renal transplant fibrosis. Activin inhibition in our study not only limited the macrophage infiltration into the graft, but also reduced the number of interstitial fibroblasts. Activin inhibition with follistatin has also been shown to reduce fibrosis also in the unilateral urethral obstruction model in the rat (Maeshima et al. 2014). In vitro activin A stimulates fibroproliferative responses in both fibroblasts and glomerular mesangial cells (Yamashita et al. 2004, Gaedeke et al. 2005). TGF-β1 stimulates activin A production in renal fibroblasts and activin inhibition reduces the fibrogenic effects of TGF-β1, which suggests that activins are essential intermediates in TGF-β1 signaling (Yamashita et al. 2004, Wada et al. 2004). High activin transcript levels have also been shown to be associated with a profibrotic M2 macrophage phenotype in mouse renal allografts (Famulski et al. 2008, Famulski et al. 2010). Thus, activins may function in multiple levels to promote renal fibrosis. Activin inhibition could provide a novel pathway to prevent chronic allograft injury.
The results obtained from this series of studies increase the knowledge about the regulation and function of macrophages during the development of chronic renal allograft injury. The acute inflammatory response that occurs after transplantation results in the production of activin A. Activin A attracts yet more macrophages, neutrophils and dendritic cells to the renal allograft. Activin A may also be involved in the regulation of macrophage cytokine production and promote the activation of fibroblasts (IV). Macrophages also produce VEGF-A during acute and chronic rejection, which may attract more monocyte/macrophages into the graft through VEGFR-1. VEGF-A itself is involved in signaling, which leads to graft fibrosis (I). Finally, the macrophages along with CD4+ T cells produce VEGF-C and drive lymphangiogenesis during the development of chronic renal allograft injury. Lymphatic vessels may be involved in the maintenance of nodular inflammatory infiltrates by producing the lymphocyte chemokine CCL21 (II).

These results also indicate the potential for possible therapeutic regimens to prevent macrophage recruitment and function and most importantly chronic allograft injury. Activin inhibition reduces early innate immune cell recruitment into the graft and limits the induction of fibroproliferative responses (IV). PTK787, the tyrosine kinase inhibitor, limits graft fibrosis by inhibiting VEGF-A signaling (I). Both PTK787 and sActRIIB-Fc are experimental molecules as yet, though VEGF-A inhibitors are already in wide clinical use for other indications and activin inhibitors are currently in phase II development.

The results also show that the graft protecting effects of sirolimus might be linked to the inhibition of VEGF-family signaling, lymphangiogenesis and the formation of nodular inflammatory infiltrates (II). This raises the possibility of using sirolimus as anti-lymphangiogenic therapy when needed.

Perioperative donor and recipient simvastatin pretreatment combined with daily recipient simvastatin treatment improved graft function and limited chronic allograft injury. These results indicate that perioperative recipient simvastatin treatment has anti-inflammatory effects that may protect the graft in the long-term (III). A clinical application of our findings could be the early restart for the ongoing statin treatment after transplantation surgery to maximize the anti-inflammatory effect.

In conclusion, this study deepens the understanding about the molecular mechanisms involved in the rejection process of kidney transplants and highlights the potential for new candidate drugs for further development to prevent chronic allograft injury in clinical kidney transplantation.


Verisuonikasvutekijät VEGF-A ja –C ovat keskeisiä verisuonien ja imusuonien kasvun säätelijöitä sikiöaikana. VEGF -perheen kasvutekijöillä on myös suoria tulehdusta edistäviä vaikutuksia ja lisäksi VEGF-A osallistuu ateroskleroosin syntyyn. Munuaissiirteissä VEGF-A:n lisäöntynyt tuotanto on liitetty krooniseen hyljintään. Munuaissiirteisiin syntyy myös uusia imusuonia, mutta tämän ilmiön merkitys on vielä epäselvä.


Tämän tutkimuksen tavoitteena oli selventää VEGF-A:n ja –C:n roolia kroonisen hyljinnän syntymisessä munuaissiirteissä. Tutkimme, voisiko näihin verisuonikasvutekijöihin vaikuttavilla lääkemolekyyleillä vähentää kroonista hyljintää. Toisena tavoitteena oli etsiä hoitomuotoja, jotka suojavat siirrettä iskemia-reperfuusiovauriota ja selvittää estävätko ne samalla myös kroonista hyljintää. Tässä osassa väitöskirjaa tutkittiin liukoisen aktiivinreseptorin ja simvastatiinin vaikutusta siirteeseen.


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"Niina Palin

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