Determinants of acute and chronic renal allograft injury
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Chapter 1

General introduction and outline of the thesis

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1. The kidney, renal replacement therapy and renal transplantation

The kidneys' main function is the maintenance of the milieu intérieur. During each heartbeat, ± 20% of the cardiac output passes through the highly vascularized kidneys where the blood encounters the nephrons, its basic structural units. On average, an individual carries around 900,000 to 1,000,000 nephrons per kidney, although large variations among subgroups exist. It is believed that after 36 weeks of gestation, no new nephrons are being formed, which suggests that the biggest variation in nephron number is determined during development (Bertram et al., 2011). Figure 1 is a schematic visualization of the anatomic structure of the kidney with its functional unit the nephron.

The most proximal part of the nephron is the glomerulus, a capillary tuft with an incoming afferent arteriole and an outgoing efferent arteriole, both responsible for

![Figure 1 | Renal anatomy and functional unit: the nephron](image)

The figure depicts one of the functional units of the kidney called the nephron. The proximal tubular pole with the Bowman's capsule and the glomerular tuft is visualized in detail. The figure was adapted from Kurts et al. (Kurts et al., 2013).
the glomerular filtration rate (i.e. vasoconstriction of the afferent arteriole reduces whereas vasoconstriction of the efferent arteriole increases glomerular filtration). The glomerular filter is composed of an inner endothelial layer, a glomerular basement membrane and an outer epithelial cell layer (podocytes). Fenestration of the endothelium, the physico-electrical properties of the glomerular basement membrane and the podocyte's foot processes are all important in the barrier function to large intravascular proteins and cells (e.g. erythrocytes and leukocytes). In the centers of glomeruli, one can find myo-epithelial cells called mesangium, which are important in providing support to the capillaries and via constriction and relaxation, they are involved in glomerular filtration as well. Glomerular filtration is a highly dynamic process that allows the kidneys to adapt to external stimuli in order to maintain homeostasis (Peti-Peterdi et al., 2015). After filtration, the pre-urine enters the tubular pole of the Bowman’s capsule where proximal convoluted tubules start. Proximal tubular epithelial cells are the most metabolically active cells of the nephron, which makes them susceptible to ischemic damage. The proximal tubules are responsible for bulk resorption of electrolytes, proteins and water in order to reabsorb a large part of the ± 180 liters of blood that is filtered daily. The pre-urine passes through the loop of Henle into the medulla and back to the level of the vascular pole of the glomerulus where it forms the macula densa (MD). In the loop of Henle, various electrolyte and water channels (aquaporins) create a concentration gradient in the renal medulla via countercurrent exchange (Lassiter, 1975; Mount, 2014). Contrary to the proximal tubular epithelial cells in the corticomedullary zone, the renal epithelial cells deep in the medulla are very resistant to local hypoxia, change in osmolar states and constant uremia (Neuhofer and Beck, 2005). The MD is the primary site of tubuloglomerular feedback via the renin-angiotensin-aldosterone system (Damkjær et al., 2013; Friis et al., 2013; Schnermann and Briggs, 2008; Sipos et al., 2010), but possibly also beyond the MD, the distal and connecting tubules bear a feedback mechanism to the local vasculature (Komlosi et al., 2009; Wang et al., 2010). The distal tubules and collecting ducts are responsible for fine-tuning of urinary concentration via the mineralocorticoid hormone aldosterone, the neurohypophysial hormone vasopressin (antidiuresis) and the cardiac and brain natriuretic peptides (natriuresis) (Bie et al., 2004; Staruschenko, 2012; Zois et al., 2014). Finally, the ureter brings the processed urine to the bladder where it is stored before voiding (Lewis, 2000). Besides water and electrolyte homeostasis,
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the kidney is important in the acid-base balance, excretion of water-soluble toxins, blood pressure regulation (via water and electrolyte homeostasis and the renin-angiotensin-aldosterone), calcium-phosphate homeostasis and erythropoiesis via the production of erythropoietin (Lassiter, 1975). Lastly, the kidney is an important player in innate immunity by providing a first barrier to urinary microbes (Kurts et al., 2013; Leemans et al., 2014).

Both intrinsic renal disease and systemic diseases with involvement of the kidneys can lead to end-stage renal disease (ESRD). In order to maintain the abovementioned functions, there are two methods for renal replacement therapy: dialysis (peritoneal and hemodialysis) and renal transplantation. The latter method, the scope of this thesis, has a more favorable outcome with regards to morbidity and mortality for the majority of patients with ESRD (Tonelli et al., 2011; Wolfe et al., 1999). During kidney transplantation, a donor kidney is attached to the recipient’s vasculature and urinary tract and strict immunosuppression subsequently prevents the donor kidney from being rejected (Halloran, 2007). There are two types of donors: donor kidneys harvested from living donors (either related or unrelated to the recipient) and kidneys from deceased donors (either after brain death or circulatory arrest). Kidneys from deceased donors carry an increased risk for early and late graft dysfunction due to a prolonged cold ischemia time: a longer cold ischemia time is a major determinant of delayed graft function (van der Vliet et al.), which in turn increases the risk of both T cell-mediated and antibody-mediated rejection (Wu et al., 2015).

2. Post-transplant renal injuries relevant to this thesis

The current thesis comprises studies that have investigated transplant renal diseases from the early post-transplant period, during which the effects of the transplantation procedure inherently lead to ischemia-reperfusion injury (IRI), particularly but not exclusively in kidneys acquired from deceased donors with prolonged ischemia times. During this early post-transplant period, effects of titrating immunosuppressive medication in patients with a partly recovering renal function can vary between under-immunesuppression with a risk of rejection on the one hand to over-immunesuppression with a risk of nephrotoxicity and infections on the other hand. It is believed that calcineurin inhibitors (cyclosporine A and tacrolimus),
both potent immunosuppressants via inhibition of interleukin-2 production, can lead to graft dysfunction via (reversible) vasoconstriction of the pre-glomerular arterioles resulting in a decline in glomerular filtration as well as direct toxic effects on tubular epithelial cells. Besides drug toxicity, over-immunesuppression renders patients more susceptible to viral infections of which BK polyomavirus infection (BKPyV) and to a lesser extent cytomegalovirus (CMV) directly affect parenchymal cells of the grafted kidney. These post-transplant pathologies can all lead to a final common pathway of sclerosing nephropathy with loss of renal function and finally the need for retransplantation or return to dialysis. As an example, Figure 2 depicts the incidence probability distribution trajectories of the histological diagnoses at the University of Alberta in Edmonton, Canada. The figure provides the reader with an idea when the aforementioned disease entities could be encountered after transplantation, although center-to-center differences occur.

**Figure 2 | Distribution trajectories of post-transplant disease entities**
The prevalence and incidence of renal allograft disease entities differ depending on the post-transplant time period. Here, the distribution trajectories are plotted from data of biopsies for cause at the University of Alberta, Edmonton, Canada. Figure adapted from Sellarés et al. (Sellarés et al., 2012)
2.1. Ischemia-reperfusion injury and delayed graft function
In the majority of kidney transplantations, there is immediate urination after vascular anastomosis. However, in a part of the transplantations, renal function remains inadequate as a consequence of acute kidney injury and in these patients, graft renal function needs to be supported by dialysis (referred to as delayed graft function; DGF). DGF can be caused by various pathologies, but ischemia-reperfusion injury from a prolonged cold ischemia time, especially in the context of deceased donation, plays a major contribution to the pathogenesis. Ischemic injury primarily develops in the cells that have the highest metabolic activity, i.e. proximal tubular epithelial cells of the corticomedullary area. Longer cold ischemia times will also affect tubular epithelial cells that are less susceptible to ischemia and one can observe that during reperfusion, the tubular necrosis migrates to more cortical nephrons and eventually also the medulla. In the reperfusion phase, there is a stereotypical innate inflammatory response that is thought to cause collateral damage of the still vital parenchyma followed by phagocytosis of the debris, regeneration of the architecture, restoration of renal function and finally resolution of the inflammatory response. A detailed review on ischemia-reperfusion injury and delayed graft function is provided by Siedlecki et al. (Siedlecki et al., 2011) and Perico et al. (Perico et al., 2004).

2.2. Rejection
The transplanted kidney is under constant pressure from its environment in an interplay between alloimmunity, immunosuppression and infections (Halloran, 2007). The alloimmune response is defined as the immune response that is specifically directed against donor epitopes that are not shared with the recipient (and are therefore not recognized as “self”), although an autoantibody response directed against “self” epitopes that contribute to antibody-mediated rejection has been reported (Porcheray et al., 2010). The early transplant period is of high importance for priming the alloimmune response that needs to be inhibited by potent immunesuppressive regimens in order to maintain graft function. Dendritic cells are the primary sentinel immune cells that encounter alloantigens within the graft, but also macrophages and B cells are able to act as antigen-presenting cells (APCs) in this case (Kurts et al., 2013; Zhuang and Lakkis, 2015).
Ischemia-reperfusion injury, as outlined in the previous section, leads to necrosis of various donor renal cells, which leads to the release of a wide palette of donor-specific proteins together with cytoplasmic, mitochondrial and nuclear danger-associated molecular proteins (DAMPs) (Leemans et al., 2014; Siedlecki et al., 2011). APCs of donor or recipient origin process donor antigens, leading to direct and indirect alloantigen recognition, respectively (Ali et al., 2013). Concomitantly, various pattern recognition receptors (PRRs) on APCs can recognize DAMPs during reperfusion resulting in APC activation and migration to lymph nodes. Presentation of donor antigen by HLA class I and II on APCs to the T cell receptor (TCR) on respectively CD8+ and CD4+ naive T cells in conjunction with specific co-stimulation will lead to the generation of CD8+ cytotoxic T cells and CD4+ T-helper cells (T_h1, T_h2, T_h17 and FOXP3+ T cells) (Ali et al., 2013). CD8+ cytotoxic T cells are the main cell types responsible for T cell-mediated rejection (TCMR), whereas T_h1-cells are important for the germinal center reaction and the formation of donor-specific antibodies (DSAs) that drives antibody-mediated rejection (ABMR). Histologically, TCMR is characterized by lymphocytotoxic tubulointerstitial inflammation and variably glomerulitis and intima arteritis (Einecke et al., 2006; Halloran, 2010; Mannon et al., 2010; Mengel et al., 2009; Racusen et al., 1999). ABMR is principally a mixed inflammatory response (neutrophils, macrophages, natural killer cells, lymphocytes) directed to the allograft’s vasculature and therefore characterized by glomerulitis, peritubular capillaritis and intima arteritis. In the latest Banff classifications, ABMR is defined as a trias of donor-specific antibodies that are able to bind endothelial cells, endothelial activation (e.g. complement activation) and vascular damage (e.g. inflammation or thrombotic microangiopathy) (Amore, 2015; Haas et al., 2014; Mengel et al., 2012; Stites et al., 2015; Westall et al., 2015). Although visualization of complement activation by C4d staining is the current gold-standard in the diagnosis of ABMR, evidence is accumulating that the majority of cases of ABMR are in fact complement-independent and different classifications are being developed to more accurately diagnose C4d-negative ABMR (Einecke et al., 2009; Haas et al., 2014; Sis and Halloran, 2010).

### 2.3. BK polyomavirus-associated nephropathy

Over the past decades, immunosuppressive regimens have become more and more effective at reducing the incidence of rejection. However, the aspecific nature
of the current medicaments results in failure of physiological immunosurveillance that is necessary to combat microbial infections. BK polyomavirus-associated nephropathy (BKPyVAN) is an opportunistic infection of the grafted kidney that is characterized by tubulointerstitial inflammation. BKPyV resides in the urothelium of immunocompetent hosts as an asymptomatic infection, but in the immunosuppressed graft, BKPyV can migrate via the medulla into the cortex of the kidney to cause acute allograft injury (van Aalderen et al., 2012; Shah, 1996). The current gold-standard for the diagnosis of BKPyVAN requires a renal allograft biopsy in order to visualize intra-epithelial viral replication (Ahuja et al., 2001; Hirsch et al., 2005; Masutani et al., 2012). The differential diagnosis between TCMR and BKPyVAN can be difficult due to similarity in histological appearance and therefore novel approaches that include intragraft and serum/urinary biomarkers are emerging (Hirsch et al., 2013; Latif et al., 2007; Li et al., 2013; Mannon et al., 2005). Contrary to the treatment of TCMR, BKPyVAN is currently treated by weaning off immunosuppression in order to re-establish the host-pathogen immune response (Hirsch et al., 2013).

2.4. Calcineurin-inhibitor toxicity

The serine/threonine phosphatase calcineurin is crucial for the early lymphoproliferative response in cytotoxic T cells via activation of interleukin-2 and co-stimulatory receptors like CD40L (Baksh and Burakoff, 2000). Autocrine interaction of interleukin-2 with the interleukin-2 receptor provides lymphocytes with a pro-survival signal that is necessary for a full-blown cytotoxic response. The calcineurin inhibitors, therefore, are potent immunosuppressive medicaments that prevent the development of (especially T cell-mediated) rejection (Baksh and Burakoff, 2000; Halloran, 2007, 2010). Unfortunately, both the calcineurin inhibitor cyclosporine A (via binding to cyclophilin) and tacrolimus (via binding to FK506 binding protein-12) are toxic to native (Gonwa et al., 2001) and transplanted kidneys (Halloran, 2007; Rowshani et al., 2006). It is thought that calcineurin inhibitors are toxic to the kidney via vasoconstriction of the afferent glomerular arteriole leading to a reduced glomerular filtration, tissue hypoperfusion and ischemia (Liptak and Ivanyi, 2006; Zhang and Victor, 2000) and via direct effects on tubular epithelial cells, leading to stress of the endoplasmic reticulum, epithelial dedifferentiation and fibrosis (Galichon et al., 2011; Pallet et al., 2008). Therefore, randomized trials aiming at minimization or withdrawal of calcineurin inhibitors with replacement
by immunosuppressants with anti-fibrotic properties (e.g. mammalian target of rapamycin inhibitors; mTORi have gained interest over the past years (Haller and Oberbauer, 2009). These weaning protocols, together with cellular therapy and tolerance induction protocols, are considered the cornerstone of future anti-rejection therapy.

### 2.5. Chronic allograft failure

Chronic allograft failure is characterized by the loss of graft function and scarring of glomeruli, tubulointerstitium and the vasculature by ongoing immune and nonimmune damage to the allograft (Solez et al., 2007; Zeisberg and Neilson, 2010). It is believed that any type of ongoing injury leads to a final common pathway of interstitial fibrosis and tubular atrophy with eventually complete loss of renal architecture (Halloran et al., 2010). The active player in this final common pathway of scarring is the myofibroblast that produces collagen fibers, the hallmark of fibrosis (Bechtel et al., 2010). To date, it is not completely clear where these myofibroblasts originate from (Cook, 2010). Possible sources are in situ activated resident fibroblasts, bone marrow-derived cells, dedifferentiated epithelial cells via epithelial-to-mesenchymal transition (EMT) (Kalluri, 2009; Kalluri and Weinberg, 2009; Zeisberg and Duffield, 2010), dedifferentiated endothelial cells via endothelial-to-mesenchymal transition (EndMT) (Kizu et al., 2009; Zeisberg et al., 2008) or the perivascular smooth muscle cells called the pericytes (Cooke et al., 2012; Humphreys et al., 2010). Besides myofibroblasts, it has been suggested that epithelial cells could also actively contribute to fibrogenesis via production of collagens. In fact, dedifferentiated epithelial cells have been shown to express chaperone proteins of the collagen synthesis, including heat shock protein-47 (HSP-47), connective tissue growth factor (CTGF) and laminin (Cheng et al., 2006; Dafforn et al., 2001; Hertig et al., 2006, 2008, 2010; Kers et al., 2010; Metalidis et al., 2013; Pallet et al., 2008; Xiao et al., 2012; Xu-Dubois et al., 2013). A more detailed introduction to chronic allograft failure is provided in Chapter 12.
3. Outline of the thesis

Part 1 focuses on post-transplant experimental and clinical ischemia-reperfusion injury. Necrosis and the release of danger-associated molecular patterns (DAMPs) from dying cells are crucial for priming of the adaptive immune system. It is therefore of importance to dissect both the intracellular signalling routes that lead to inflammatory cell death (necroinflammation) but also the early recognition of these cellular fragments by the innate immune system. In Chapter 2 we therefore reviewed the state-of-the-knowledge on regulated necrosis in the context of experimental renal diseases.

Chapter 3 is a detailed study on nicotinamide dinucleotide (NAD\(^+\)) and especially the NAD\(^+\)-sirtuin axis in a form of regulated necrosis called necroptosis.

Triggering receptor expressed on myeloid cells-1 (TREM-1) is an innate pattern recognition receptor that is believed to amplify the inflammatory responses that are elicited by Toll-like receptors (TLRs). In Chapter 4 we had the opportunity to investigate the potential role for TREM-1 in the context of experimental and clinical ischemia-reperfusion injury and renal allograft outcomes including DGF.

Various donor- and recipient-related factors have been suggested to predict the risk of delayed graft function after reperfusion and there exist statistical algorithms that claim to stratify patients in risk categories. In order to investigate the clinical applicability and generalizability of these algorithms, we performed external validation of these algorithms on data from the Dutch nation-wide Organ Transplant Registry in Chapter 5.

Part 2 encompasses those studies that had the aim to investigate the pathogenesis and prediction of different forms of acute renal allograft injury with a special interest on the differences between T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR) and BK polyomavirus-associated nephropathy (BKPyVAN). Donor-specific antibodies, when they attach to the graft endothelium, cause endothelial activation, complement activation, hemostasis and finally thrombosis (thrombotic microangiopathy). In Chapter 6 we investigated whether endothelial protein C
receptor (EPCR) expression on graft vessels could play a role during acute renal allograft rejection and whether its expression would relate to indices of antibody-mediated rejection in particular.

The hallmark of allograft rejection is an influx of leukocytes that are directly or indirectly harmful to the different compartments of the allograft (glomeruli, tubulointerstitium, vasculature). It has been observed in experimental models of allograft rejection that especially interleukin-17-producing T-helper cells or Th17 cells associate with a proinflammatory alloimmune response leading to graft rejection. Interleukin-17 is not solely produced by Th cells during adaptive immune responses, but also by cells of innate immunity, including neutrophils and mast cells. We therefore investigated in Chapter 7 whether interleukin-17 is expressed during renal allograft rejection, by which inflammatory cells and to what extent interleukin-17 is involved in a worse graft outcome.

Characteristic of antibody-mediated rejection is inflammation of the glomerular tuft by allo-antibody binding and/or complement activation. The study described in Chapter 8 investigated the consequence and predictive value of different forms of glomerulitis during episodes of acute renal allograft rejection. We evaluated whether different forms of glomerulitis are more related to indices of antibody-mediated rejection and whether these forms can better predict response to anti-rejection therapy and chronic allograft failure.

During renal damage, canonical inflammasome activation is able to recognize cellular damage, whereas non-canonical inflammasome signalling can lead to pyroptosis (inflammatory necrotic cell death). Both signalling routes can amplify alloimmune responses and we therefore questioned if non-synonymous single nucleotide variants in the \textit{NLRP3} gene in donors and/or recipients associated with post-transplant outcomes in Chapter 9.

TCMR and BKPyVAN have a very similar histological appearance during acute graft dysfunction, however their treatment is completely opposite. We designed two studies to dissect the differences between the alloimmune response during TCMR
on the one hand and the antiviral immune response during BKPyVAN on the other. Chapter 10 describes the differences in leukocyte influx by immunophenotyping of the inflammatory infiltrates during both diseases and Chapter 11 describes in both entities the relative mRNA transcription of a selected group of genes that are known to be of importance in antiviral- or allo-immunity.

Part 3 is a collection of studies that mainly focussed on the long-term effects of various post-transplant diseases. Ongoing injury to the graft orchestrates a final common pathway of fibrotic damage to glomeruli, the tubulointerstitium and the vasculature in a histological syndrome previously referred to as chronic allograft nephropathy. In Chapter 12, we introduce the readers to the (mis)concepts on chronic allograft nephropathy, risk factors for chronic allograft damage and its histological appearance in the allograft.

For many years it was believed that tubular epithelial cells are innocent bystanders during renal fibrogenesis, but recently, it has been suggested that they actively contribute to the development of interstitial fibrosis. An important concept for the development of fibrosis of the grafted kidney is epithelial-to-mesenchymal transition (EMT, also referred to as epithelial phenotypic changes or tubular dedifferentiation). In Chapter 13 we investigated whether EMT markers could predict the development of interstitial fibrosis, tubular atrophy and a decline in renal function at later time-points after biopsy assessment.

Toll-like receptors have been associated with acute as well as chronic renal injury. In Chapter 14, we address the association of non-synonymous single nucleotide variants in TLR genes in donors and recipients with the existence of chronic kidney diseases and acute and chronic outcomes following renal transplantation.

Since the introduction of calcineurin-inhibitors to the repertoire immunosuppressive medicine, rejection rates have declined significantly. These same calcineurin inhibitors, however, were shown to be nephrotoxic in models of renal and non-renal solid organ transplantation. To conclude this thesis, we describe in Chapter 15 the results from the randomized-controlled MECANO trial that aimed at preventing the
development of chronic allograft failure by the withdrawal of calcineurin-inhibitors via introduction of the mTOR inhibitor everolimus in a double immunosuppressive regimen with prednisolone in patients without rejection at randomization.

Overall, this thesis provides novel insights into the pathogenesis and prediction of acute and chronic renal allograft failure with the aim of improving the management of renal transplantation in the future.