Chapter 1

General introduction
CHRONIC KIDNEY DISEASE

With global prevalence ranging from 10.5 to 13.1%, chronic kidney disease (CKD) poses a substantial burden to society. In 2013, CKD-associated years lived with disability increased by 49.5% to 12.3 million. Between 1990 and 2013, CKD was the fastest growing non-communicable cause of death, ranking in the top tier of global disease [1, 2].

Improved quality of medical care leads to increased life span of renal patients and thus more patients reaching the final stage of CKD, that is end-stage renal disease (ESRD) [3]. Given that the prevalence of risk factors such as diabetes mellitus, hypertension and high body mass index (BMI) increases (e.g. 2.3% annual increase in years lived with disability for diabetes [4]), the burden of ESRD correspondingly increases steadily. This results in higher numbers of renal replacement therapy, which constitutes a substantial proportion of total health expenditure [5] and puts an increasing financial burden on the health insurance system of many countries [3].

RENAL TRANSPLANTATION

Due to its improved patient quality of life and survival, and the financial benefit compared to dialysis, the preferred treatment for patients with ESRD and far-progressed CKD is kidney transplantation. [6]. Progression in surgical techniques, the development of new and better immunosuppressive drugs, better screening and post-transplantation care have expanded the pool of potential transplant recipients [7] as well as donors [6]. Nevertheless, hundreds of renal patients waiting for a suitable donor kidney [8] underscore the necessity to further improve transplant and recipient survival, on top of preemptively preventing the need for renal replacement therapy to start with.

Already at the end of the last century, when the waiting list was expanding while shortage of donor kidneys was increasing, kidneys that used to be discarded were now considered for transplantation. These so-called expanded criteria donor (ECD) kidneys are transplantable deceased donor kidneys with an estimated risk of graft failure of ≥70%, inferior average patient survival and inferior renal function compared to standard criteria donor kidneys [9]. In practice, expanded donor criteria include donors with an old age (≥60 years), a history of hypertension, a serum creatinine level >1.5 mg/dL and/or a cerebrovascular cause of death. Patients who received kidneys from expanded criteria donors experienced a survival benefit compared to non-ECD recipients and patients on dialysis [6]. This emphasizes the importance and benefit of renal transplantation, even when performed using organs of sub-optimal quality.

CHRONIC TRANSPLANT DYSFUNCTION

One important complication after renal transplantation is the occurrence of chronic transplant dysfunction (CTD). CTD is clinically diagnosed through slowly rising serum creatinine levels, an increase in proteinuria and intensified hypertension [10]. It is a multifactorial process (with immunological as well as non-immunological
causes) resulting from a wide range of features implicated in poor long-term allograft outcome [11]. These include, but are not limited to, non-optimal donor organ quality, episodes of acute rejection and viral infections [12]. The most pronounced histopathological features include persistent inflammation, transplant arteriosclerosis [11] and interstitial fibrosis with tubular atrophy [13] (IF/TA). Inflammation is a vital stage during clearance of pathogens and initiation of wound healing that is self-limiting in healthy individuals through release of anti-inflammatory cytokines. However, if unresolved, it can result in persistent activation and an extensive range of immunity-related pathologies [14]. To prevent this, the immune response has to be tightly regulated.

Fibrosis results from a variety of factors, representing a final common pathway after various types of injury. These etiological causes are classified based on identifiable morphological features and include a number of subcategories; drug toxicity, bacterial or viral infection, hypertension, and acute and chronic cell- and/or antibody-mediated rejection [11]. When no specific etiology can be identified, it is labeled as “interstitial fibrosis and tubular atrophy without evidence of any specific etiology” according to the Banff classification [10].

IF/TA is correlated to reduced graft survival, particularly when combined with subclinical rejection, transplant glomerulopathy and transplant vasculopathy. Subjects with combined IF/TA and transplant vasculopathy demonstrated a 10-year graft survival of 41%, while for those with IF/TA without transplant vasculopathy it was 82%, and for those with normal histology it was 95% [15]. Transplant vasculopathy is often also classified as accelerated graft arteriosclerosis because of its shared features with arteriosclerosis. However, it only occurs in the graft and develops over a period of months to years [16]. Transplant arteriosclerosis describes vascular remodelling through generation of a neointima resulting from the migration and proliferation of vascular smooth muscle cells (VSMCs) [17]. This eventually results in hyperplastic lesions that cause ischemic graft failure because laminal flow is obstructed [18].

SMOOTH MUSCLE CELLS DURING ALLOGRAFT DYSFUNCTION

Vascular smooth muscle cells are major structural components of the arterial wall that play an essential role in maintaining vessel structure and facilitating its contractile function. These cells can exist on a continuum between two separate phenotypes on opposite sides of the spectrum; differentiated, contractile and quiescent versus dedifferentiated, secretory and proliferating [17]. Its specific embryonic origin, position in the arterial tree and the organ-dependent microenvironment generate substantial differences between individual smooth muscle cells [19]. The cells can adapt their phenotype in response to the microenvironment or pathophysiological conditions during a process called phenotype switching [17]. Expression of cytoskeleton and contractile proteins such as α-smooth muscle actin, smoothelin and SM22α is downregulated, while that of synthetic markers such as ICAM-1, syndecan-1 and collagen-1 is upregulated [20]. Switching from a contractile to a
secretory phenotype is accompanied by increased capacity to migrate to the intima [17]. Although the role of VSMCs in the generation of vasculopathy becomes more untangled, expanded understanding of the underlying mechanisms would provide additional insights for future therapeutic interventions.

REJECTION

Despite decades of research and evolving therapy, transplant rejection remains a challenge with complicated etiology and substantial clinical implications. Even though the rate of death-censored renal graft failure has substantially decreased over the last decades, it is still at 26% from deceased and 14% from living donors at five years post-transplantation [21]. It remains a challenge to prevent targeting of the transplanted graft by the host immune system, despite deeper understanding of host-transplant interaction, development of immunosuppression and the molecular mechanisms involved. Various stages of rejection can be distinguished after transplantation, all with their own etiology, complications and clinical implications. Hyperacute (within minutes, due to pre-existing anti-HLA antibodies in the recipient), acute (within days to weeks), late acute (after three months) and chronic rejection (six months to years post-transplantation) all constitute risk for loss of the transplanted kidney [22]. Even more, acute rejection is an important risk factor for chronic rejection, further complicating its development [23]. Alternatively, rejection can be classified according to the extent of histologic inflammation and injury, as well as according to the immunologic mechanisms involved [22].

IMMUNOSUPPRESSIVE REGIMENS

Successful application of immunosuppression aims at effectively preventing rejection without clinical signs of over-suppression, such as opportunistic infections and malignancy [24]. Physicians must constantly balance the benefits and drawbacks of immunosuppression through dosage adjustments and selection of specific antirejection agents. Ideally, the net immunosuppression is kept as low as possible, while limiting, and ideally excluding, rejection of the newly transplanted organ.

On the short-term this strategy proves successful judging from the low risk for acute rejection in the first year post-transplantation (10 – 20%), excellent short-term graft and patient survival (90 – 95%) and low rejection-induced graft loss in the first year (<5%) [24]. Nevertheless, rejection episodes that do occur tend to be more severe, and graft survival rates after five years have remained largely unaltered [22].

Multidrug immunosuppressive therapy is a means to effectively prevent rejection, given that each individual antirejection agent reinforces the action of additional agents in the regimen [24]. Drugs with a pharmacological activity against targets at various key steps in T cell replication are combined. These steps include; activation of T cell receptors by antigen-presenting cells, stimulation of co-stimulatory molecules and stimulation of the IL-2 receptor [25]. This synergistic approach allows for lower dosages of the individual agents, resulting in reduced drug toxicity and side-effects, while theoretically retaining optimal efficacy [24].
The application of immunosuppression is categorized into three clinical stages; induction therapy, maintenance therapy and treatment of established rejection [25]. Induction therapy is generally aimed at reducing the risk of acute rejection and is usually composed of an IL-2 receptor antagonist or agent to deplete T-lymphocytes [26]. It is followed by maintenance therapy aiming to prevent acute rejection alongside optimization of graft and patient survival on the long-term. Potential agents for maintenance therapy include calcineurin inhibitors (CNIs) (cyclosporine and tacrolimus), CD80 and CD86 costimulation blockers (belatacept), mammalian target of rapamycin inhibitors (sirolimus and everolimus), anti-proliferatives (azathioprine and mycophenolic acid derivatives) and corticosteroids. Maintenance regimens frequently include a CNI accompanied by an adjuvant agent, in presence or absence of corticosteroids, but specific combinations vary between transplantation institutions. It is generally recommended to adapt individual immunosuppressive therapy based on for instance clinical indications, pre-existing disease and drug-drug interactions with other medicine [25].

REGULATORY B CELLS AS IMMUNE REGULATORS

The capacity of B cells to produce antibodies, present antigens and produce cytokines generally classifies them as positive regulators of the immune response [14]. B cells can capture and present antigens through MHC-II molecules, albeit less efficient than dendritic cells and macrophages [22]. Next to that, they can produce antibodies directed against antigen on the transplanted graft, further exacerbating the rejection.

However, a specific population of immunosuppressive or regulatory B cells (Bregs) demonstrates broad potential in regulating the immunological responses after transplantation. This subpopulation of B cells display regulatory capacity in for instance systemic lupus erythematosus, autoimmunity, Graves’ disease, malignancy and immune thrombocytopenia [27]. Given the large impact of the immune system after transplantation, Bregs may fulfill an essential role as potential inducers of immunological tolerance and during prevention of rejection. To that end, renal transplant recipients with rejection demonstrated a lower number of Bregs, and an altered function of these Bregs, compared to healthy controls or recipients with stable allograft function [28]. Next to that, patients with chronic antibody-mediated rejection demonstrated impaired immunosuppressive activity associated with unique B cell phenotypes [29]. Besides, evidence is accumulating on the role of Bregs in clinical operational tolerance; sustained graft function in the absence of immunosuppressive drugs [30]. For instance, transitional B cells, presumed to contain a regulatory B cell population, were enriched in tolerant patients compared to those receiving immunosuppressive treatment [27].

Bregs suppress pro-inflammatory responses in vivo through suppressing pro-inflammatory cytokine production by dendritic cells, skewing T cell differentiation into a regulatory phenotype, expressing immunoregulatory cytokine IL-35, inducing apoptosis of effector T cells and regulating natural killer (NK) cell homeostasis.
Chapter 1

[14]. Cytokine IL-10 is assumed its most pronounced and distinguishing trait that provides inhibition of pro-inflammatory cytokines and supports regulatory T cell differentiation [14]. Nevertheless, regulatory functions can occur independent of IL-10, indicating the means for negative regulation resides not solely in IL-10 [31]. CMV may attempt to modulate immune regulation similar to regulatory B cells using its viral anti-inflammatory homologue of IL-10, cmvIL-10 [32].

Detection and isolation of Bregs remains challenging since no distinct combination of cell surface markers has uniquely identified this cell population. Detecting intracellular cytokine IL-10 requires fixed and permeabilized cells, which complicates subsequent functional characterization. Literature does not provide a conclusive specification of its phenotype but various markers (such as CD1d, CD24, CD27, CD138) have been proposed. Given that these markers are differentially expressed in immune activation, they vary based on developmental stage and experimental setting. It further cannot be excluded, and may even be likely, that multiple populations of regulatory B cells with a different cell surface profile exist [14, 33, 34]. In absence of distinct classification of Bregs, cell populations suspected to contain regulatory B cells are classified as a surrogate. Two of these distinct populations are consistently proposed in literature and will be the primary focus of our studies; transitional CD19⁺CD24hiCD38hi B cells [14, 35, 36] and memory CD19⁺CD24⁺CD27⁺ B cells [14, 37]. The highest percentage of IL-10-producing B cells were found in the CD24hiCD38hi B cell compartment after stimulation in vitro, and CD24hiCD27⁺ B cells produced high levels of IL-10 [14].

VIRAL INFECTIONS POST-TRANSPLANTATION

Application of immunosuppressive agents mitigates rejection on the short and longer term, but is accompanied by increased vulnerability for viral infection resulting from reduced immune surveillance. These viral infections can be community-acquired (e.g. influenza and adenovirus) or a reactivation from latent infection (i.e. herpesviruses such as HSV1 and 2, varicella zoster (VZV) and cytomegalovirus), but are also regularly transmitted via the transplanted allograft (cytomegalovirus, Epstein-Barr virus) [38]. Three periods of risk for infection can be distinguished after transplantation; early (<1 month), intermediate (1 – 6 months) and late (>6 months) [39]. Early infections are likely due to donor-derived infections or surgical complications, while intermediate infections are regularly opportunistic pathogens [40]. In the late stage mostly community-acquired infections occur with a risk comparable to that for the general population [39].

HUMAN CYTOMEGALOVIRUS

The most important viral infection after renal transplantation is human cytomegalovirus (CMV), also known as beta-human herpesvirus 5 (HHV-5) [41]. It is histologically characterized by cytomegalic cells (literally: large cell) containing the so-called “owl’s eye” phenotype, caused by intranuclear inclusions with nucleocapsid particles [42]. CMV is a large virus consisting of 165 genes encoded by a 235kb
General Introduction

long genome [42]. The linear double-stranded DNA is enveloped by consecutively an icosahedral nucleocapsid, tegument and a lipid bilayer envelope containing viral glycoproteins. The total size of one CMV particle ranges from 200 - 300 nm, making it the largest of all human herpesviruses [43]. Seroprevalence in the general population ranges from 50 - 90%, with prevalence increasing with higher age [44].

CMV infection results from exposure of mucosal epithelial cells of the respiratory and genital tract, followed by lytic replication and subsequent infection of adjacent non-epithelial cells [41]. Productive CMV infection results in the synthesis of proteins in three consecutive phases that can partially temporally overlap; immediate-early (IE) (between 0 and 2 hours post-infection), (delayed-)early (2 - 24 h), and late (> 24 h) antigens [43]. Completing one cycle of lytic infection generally takes 72 - 96 hours [32]. Studies suggest that the initial viremia is succeeded by infection of other internal organs and subsequently by more generalized infection in a secondary round of viremia. During the latter phase, infection disseminates towards tissues that support persistent infection in vivo, including salivary gland, breast secretory epithelium and renal tubular epithelium [41].

After clearance of active infection, latent infection occurs in the epithelium of exocrine glands, hematopoietic stem cells, monocytes and monocyte-derived cells [41]. CD14+ monocytes were established as a resident cell type for latent CMV infection, while reactivation from bone marrow-derived CD34+ hematopoietic progenitors has only been observed in vitro, not in vivo [45]. Additionally, ex vivo maturation of myeloid dendritic cell progenitors from healthy CMV carriers resulted in reactivation, which suggests these as an additional site of latency [46]. Since endothelial cells have been recognized as location of CMV replication, the interaction between endothelial and circulating mononuclear cells could synergize hematogenous viral spreading to distant sites of infection [41].

Even though infection is generally asymptomatic in immunocompetent individuals, it sometimes results in mononucleosis-like symptoms, and is suspected to account for around 8% of mononucleosis cases [42]. Symptoms include fever, myalgia and headache, but these are generally relatively mild [43]. However, CMV disease occurring in immunosuppressed individuals is characterized by CMV syndrome (fever, muscle pain, leukopenia and/or thrombocytopenia) or organ involvement (e.g. hepatitis, gastrointestinal ulceration, retinitis, myocarditis or pancreatitis) [47] with much more pronounced symptoms. Some of the earliest reports on clinical syndromes followed CMV infection after allograft transplantation, transfer of blood products and breast milk [41]. Since then, it has been found that CMV is readily transmitted through saliva, breast milk, urine, blood transfusion, hematopoietic stem cell transplantation and also solid-organ transplantation [43]. It is unknown whether CMV in epithelial cells periodically reactivates from latent infection or remains as a chronic persistent low-grade infection. No specific genetic background is associated with susceptibility or resistance to CMV infection. The good news is that transmission of the virus seems to be limited to close contact. The bad news, however, is that sustained virus shedding up to six months after active
infection can accommodate rapid spread over a susceptible population [41]. Next to that, coinfection with additional viruses is not uncommon (e.g. Epstein-Barr virus, herpes simplex virus, or human immunodeficiency virus), which further complicates the clinical development and treatment of CMV [48].

**CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION**

CMV is the most common viral infection after transplantation, with an estimated 90% of recipients experiencing CMV infection when receiving an organ from an CMV-infected donor [41]. Generally, 40 - 100% of renal transplant recipients undergo CMV infection in absence of preventive measures, and two-thirds develop CMV disease. Preventive measures lower these numbers to 17 - 92% and 0 - 37% respectively [49]. Clinical symptoms generally do not present until some weeks after transplantation. This is presumably due to minute amounts of virus in the transplanted graft and the time necessary for CMV to replicate to disease-inducing quantities and disseminate to distant sites. The immunosuppressive regimen allows accelerated and prolonged viral replication, and virus excretion can pertain even after resolution of active infection. Despite the suggestion that the initial CMV infection of mucosal surfaces can occur by cell-free virus, subsequent viral spreading is primarily, if not exclusively, cell-associated [41].

**IMMUNE RESPONSE AGAINST CMV**

The challenge during immunosuppression is to find the delicate balance between managing rejection and managing infections, including those of viral origin. The immunosuppressive regimen is therefore not constant, but it is adjusted regularly to allow necessary adaptations or alterations based on the status of the recipient. A complex function of dose, duration and sequence of immunosuppressive agents jointly determine the net state of immunosuppression, on which the risk for infection of the recipient is mainly based [39]. When time after transplantation progresses, the risk for acute rejection decreases, immunosuppression is tapered and recipients can develop an independent antiviral response to CMV, albeit delayed. The battle of the immune system against CMV is regularly supported by the application of anti-CMV therapy, mostly in the form of (val)ganciclovir (which will be further described later in this chapter).

Antiviral responses to CMV consist of an intricate interplay between partly temporally divided and partly overlapping processes. Toll-like receptor 2, an immunesensor molecule with the capacity to recognize foreign patterns, is essential in the detection of CMV surface glycoproteins gB and gH and activation of the innate immune system. The latter encompasses the recruitment of professional antigen presenting cells, phagocytes and NK-cells which provide rapid cytotoxic function and initiation of the adaptive immune system [51]. The adaptive immune response against CMV is extensive and consists of both humoral and cellular responses, with a large proportion of the B and T cells committed to CMV [52]. The comprehensive adaptive immune response is pivotal for sustained immune surveillance against
General Introduction

CMV so to prevent reactivation from latency and progression of lytic infection [51].

B cells are important players in the host immune response after infection. Primary infection with CMV results in antibody production targeted against a plethora of non-structural, tegument and surface glycoproteins. The latter includes gB, against which the majority of infected subjects directs a large proportion of antibodies [50]. Pre-existing antibodies against CMV provide protection for premature infants and the fetus during pregnancy, and are essential for minimizing clinical manifestations of disease and viral dissemination [51, 53].

CMV IMMUNE EVASION TECHNIQUES

CMV can establish a lifelong relationship with its host through cycles of lytic and latent infections, two fundamentally different stages in the host-virus interaction. Lytic infection describes the completion of a full replicative cycle, production of complete and infectious virions, with cytopathology (cytopathogenic effect) and cell lysis as a result [54]. Latency is defined as the maintenance of viral genomes in the absence of production of infectious virions with the ability to reinitiate a full replicative cycle under specific stimuli. It can be established after the initial CMV infection is resolved, and requires a specific set of strategies deployed by CMV. Latency is generally achieved with only few transcriptionally active viral genes and silencing of immediate early (IE) genes (for instance by type I interferon, which prevents lytic gene expression). Reactivation can occur in response to immunosuppression, inflammation or stress and serves as productive infection to disseminate virus [32].

CMV has to escape host defense mechanisms to sustain infection and survive. Its extensive collection of encoded proteins allows for many functions regarding replication, spread and persistence [41]. It additionally accommodates a wide variety of immune modulatory and evasions strategies to escape the host immune system and antiviral response [32]. These strategies interfere with activation of an effective innate immune response, efficient and high-quality adaptive immune response and contribute to subvert elimination by the host [53].

Immune evasion strategies vary between stages of infection, and a selection of key mechanisms of action will be discussed below. CMV interferes with apoptosis, a key mechanism in the host to prevent viral spreading, by restricting activation of the intrinsic and extrinsic apoptotic pathways [55]. Inhibition of NK-cell activation and cytotoxicity is achieved by interference with activating (such as UL16 and UL83) and inhibitory signals (such as UL18 and UL40) on the NK-cell [53, 56]. The presentation of cellular and extracellular peptides through the major histocompatibility complex (MHC) is an essential process during activation of the adaptive immune response. Antigens are presented by antigen presenting cells, which constantly scavenge the extracellular environment and present samples to the adaptive immune system. This leads to immunological memory for the virus, restraining its infection and limiting damage to the microenvironment [32]. CMV restricts processing and presentation of CMV antigens to CD8+ cytotoxic T cells by degrading synthesized MHC-I heavy chain or obstructing its processing route within the cell [53]. Similarly,
upregulation of MHC-II molecules signaling to CD4\(^+\)-helper T cells is inhibited by preventing its translocation to the cell surface or redirecting it for degradation [53]. Molecular mimicry has resulted in homologues of host cytokines (such as cmvIL-10), chemokines (such as UL146 and UL147) and receptors (such as UL144 and G-protein coupled receptors) [57, 58]. These could improve viral replication by interfering with immune system signaling, inducing cell proliferation or migration, redirecting the immune response or controlling cell homeostasis [57].

In spite of the wide range of CMV immune evasion strategies, the host immune system employs a strong CD4\(^+\) and CD8\(^+\) T cell response resulting in clearance of active primary infection and forcing CMV into latency [50]. On average, 10% of the CD4\(^+\) and CD8\(^+\) T cells are directed against CMV in seropositive subjects, which is paramount to the extensive CMV immunological response of the host [59]. The tightly controlled restriction of virus activity is commonly successful during immunocompetence, but regularly cannot be maintained while under immunosuppression [50].

**DETECTION CMV IN CLINICAL PRACTICE**

CMV infection is defined as the isolation of CMV or detection of viral proteins or nucleic acid in any body fluid or tissue specimen. Primary infection constitutes the initial infection; the detection of CMV infection in an individual previously found to be CMV seronegative. Recurrent infection is the renewed CMV detection in a subject that previously underwent primary infection, either with a strain that is indistinguishable (reactivation) or, less commonly, with a strain different from the subject’s original infection (reinfection) [60]. Interestingly, genetically distinct strains of CMV were detected at the same time in infected patients, which indicates that pre-existing immunity does not provide protection against an alternative strain of CMV [41, 61]. Given the large range of bodily fluids that CMV can reside in, it is important to mention both the diagnostic method and specific specimen used for diagnosis [60].

The reliable detection of CMV in renal transplant recipients, and the thresholds that define active infection and necessity for intervention are important in the clinic. Various methods have been developed, all with distinct and intrinsic advantages and disadvantages. During conventional cell culture with clinical samples, foci of flat, swollen cells are observed 2 – 21 days post-infection of human fibroblasts. This traditional method of CMV detection is rather slow, stimulating the development of alternative techniques. The pp65 antigenemia assay, in which immunofluorescent-labeled antibodies against the late protein pp65 are detected in leukocytes, generates quantitative results which correlate closely with viremia and severity of clinical disease. Unfortunately, this technique cannot be automated, is limited to CMV detection in leukocytes and is labor intensive. Tissue samples allow immunohistochemical detection of CMV antigens using fluorescent- or enzyme-labeled antibodies. This method is sensitive and specific, but requires specialized personnel and is also laborious. The detection of CMV DNA, which can be readily
isolated from whole blood, leucocytes, plasma, tissue biopsies and body fluids, allows for rapid and sensitive detection using Polymerase Chain Reaction (PCR). Despite a relative high price and necessity for careful calibration, its advantages including speed, automation, capacity for mRNA detection and quantitative reporting allow for continuous monitoring of immunocompromised subjects. Therefore, PCR is the most widely applied technique in clinical care [62].

Serology tests for detection of antibodies in blood or plasma are useful tools to determine the history of CMV infection and study the immune system of the patient. CMV IgM and IgG can be detected using a variety of methods, of which enzyme-linked immunosorbent assay (ELISA) is the most widely adopted. CMV antibodies are used to determine whether an infection, and an accompanying adequate immune response has occurred in the past [62].

**CMV TREATMENT**

Once detected, it is of vital importance to prevent long-term harm of CMV infection by applying appropriate treatment strategies. Viral infection and clinical complications require adapting the treatment in an attempt to reinforce antiviral immunity [38]. In renal transplant recipients this is generally complemented with one out of two major anti-CMV treatment strategies: universal prophylaxis or preemptive therapy. Universal prophylaxis is administered to all at-risk patients from early post-transplantation till three or six months later, regularly excluding the low-risk pairs of a seronegative donor to seronegative recipient. The exact duration of the prophylactic therapy is determined by donor and recipient serostatuses, as well as institution's guidelines. Disadvantages such as drug toxicities and occurrence of late-onset CMV disease after stopping prophylaxis are counterbalanced by its ease of administration, protection against infection with alternative herpes viruses and decreased incidence of indirect effects of CMV. The latter include allograft rejection, accelerated atherosclerosis, opportunistic infections and mortality [60] [63].

Alternatively, asymptomatic recipients are monitored at regular intervals with quantitative assays during preemptive therapy. Only recipients demonstrating a positive assay receive anti-CMV therapy, and infection is often detected early. Benefits include the low incidence of late-onset CMV and reduced drug toxicity as well as cost benefits. Both strategies could alternatively be combined, in which prophylaxis is applied during the high-risk first months after transplantation, when immunosuppression is intense, to be replaced by preemptive therapy in the following period [63].

Agents used during anti-CMV therapy are predominantly oral valganciclovir, oral ganciclovir or intravenous ganciclovir [63]. Valganciclovir is a prodrug of ganciclovir; an acyclic analog of the nucleoside guanosine that inhibits DNA polymerase resulting in reduced viral DNA synthesis [64, 65]. Enzymatic conversion results in ganciclovir triphosphate (ganciclovir TP), of which concentrations are ten-fold higher in CMV-infected compared to uninfected cells [66]. Ganciclovir TP competitively inhibits incorporation of deoxyguanosine triphosphate into DNA, and does this
more efficiently for viral DNA polymerases compared to their cellular counterparts. It additionally disrupts viral DNA synthesis because it is poor template for chain elongation [64]. However, sustained treatment with valganciclovir treatment can result in resistance of CMV, for which recipients with intensive immunosuppression are especially vulnerable [67].

Anti-CMV treatment complements a cautious reduction in immunosuppression, which is applied in attempt to restore natural antiviral immune responses. Reduction of immunosuppression will allow for the CMV-specific immunity to initiate or recover, and permit sustained control of the CMV infection [63]. Reduction of particularly MMF at the very beginning of the infection is a widely applied strategy to potentiate the host immune system to reinforce antiviral immunity. The exact strategy for reduction of the immunosuppressive regime varies between individual institutions, and ultimately also depends on the individual circumstances of the recipient.

G PROTEIN-COUPLED RECEPTOR AS TARGET FOR INTERVENTION

As described in a previous paragraph, CMV accommodates a wide variety of immune modulatory and evasions strategies to escape the host immune system and antiviral response [32]. One strategy is the expression of viral G protein-coupled receptor US28 (GPCR), which is homologue to cellular chemokine receptors [68]. The family of G protein-coupled receptors contains around 900 members, and provides a prominent target for pharmacological intervention [69]. The conformational change of GPCRs in response to a wide range of extracellular stimuli conveys external signals to the cell interior. Binding of hormones, neurotransmitters or chemokines induces the recruitment and activation of G proteins [70], which subsequently activates various intracellular signaling pathways and induces cellular changes [71]. Their localization on the cell surface separates the ligand interaction site from its effector site, allowing for manipulation of the downstream signaling pathway through a single target [72]. Although there is some variation between reports, an estimated 30 - 50% of all approved drugs target a GPCR. Of the 90 applications for new molecular entities approved by the Food and Drug Administration (FDA) between 2010 and 2012, 19% targeted GPCRs. The majority of these tend to more effectively target already known receptors through improvements in selectivity profiles, more than identifying a new receptor per se [73]. Only a small proportion of the available total variety of receptors is thus targeted, leaving a large pool of potential new therapeutic targets unaffected [71].

CMV-ENCODED G PROTEIN-COUPLED RECEPTOR US28

CMV encodes four viral G protein-coupled receptors: US27, US28, UL33 and UL78. In contrast to their cellular counterparts, viral GPCRs scavenge chemokines promiscuously (CCL2, CCL3, CCL5 and CX3CL1 [68] and constitutively activate signaling pathways [74]. US28 was identified as a homolog of human CCR1, CCR2 and CX3CR1 that can be modified using small molecules, albeit with low affinity.
It functions as a chemokine sink, in which it promiscuously binds and internalizes chemokines and thereby modifies the chemokine gradient. This affects the microenvironment of the infected cell and provides one mechanism through which US28 contributes to CMV immune evasion [68]. US28 has been demonstrated to affect tumorigenesis, for instance by inducing cell growth and cell cycle progression as well as upregulation of VEGF [75]. It additionally may ameliorate reactivation from latency, manipulate transcription and enhance cell-cell interaction through binding to cell surface ligands [76]. Interestingly, US28 mediates vascular smooth muscle cell migration which contributes to viral spreading and acceleration of vascular disease [68, 77]. US28 activates a variety of intracellular signaling pathways, ranging from nuclear factor-κB, cAMP response element binding protein, nuclear factor of activated T-cells and serum response factor [68]. It can hereby actively interfere with host defense mechanisms and substantially modulate immune activation after transplantation [78, 79, 80]. Elucidation of the expression, role and targeted intervention of viral GPCRs in the renal transplantation setting could therefore prove essential in preventing renal dysfunction.

**DESIGN AND AIM OF THE THESIS**

The developments described above have inspired us to focus on the impact of CMV after renal transplantation and characterize potential future targets. The overall aim is to better understand the impact and mechanisms underlying cytomegalovirus infection in renal transplant recipients, from the level of the patient to the cell. We wanted to describe the functional consequences of CMV infection on renal function and rejection, but also potential underlying mechanisms at the cellular level. These findings can shed additional light on the debate surrounding this highly interesting and clinically relevant virus. Clinical and experimental virology and nephrology as well as histopathological characterization will be combined to further understand the role of CMV. The insights obtained may be valuable for improving long-term kidney function and transplantation outcome resulting in the improved patient's quality of life.

**Chapter 2** starts by exploring the patient, studying the effect of pronounced post-transplantation CMV infection on renal function on the intermediate and long-term. The focus is on CMV DNAemia given that this is currently the most generally applied method for detection and diagnosis of CMV infection in the clinic. Since viral infections generally occur relatively soon after transplantation, and we expected that early infections to have the most pronounced effects, severity of infection was expressed as CMV peak viral load in the first three months post-transplantation. Our aim was to assess the effects of early human cytomegalovirus CMV DNAemia on long-term transplant renal function.
Chapter 1

In chapter 3 we studied the duration of CMV infection to quantify total viral burden, as an alternative method to the peak viral load in chapter 2. We studied the effect of the duration of CMV DNAemia on renal function, specifically in renal transplant recipients suffering from primary CMV infection. This allowed us to further zoom into the initial immune response after CMV infection, without the "interference" of a secondary immune response. We investigated whether time until initiation of a CMV IgM and IgG seroconversion was related to renal function. Since primary infection of renal transplant recipients can be medically dealt with by withdrawal of the immunosuppressive agent mycophenolate mofetil (MMF), we analyzed how the duration of MMF treatment was related to the progression of CMV and the immune response of the host. Our findings could serve as a model for primary infection after prophylactic treatment, when monitoring frequency is substantially reduced but primary infection occurs. This study aimed to characterize the effect of the duration of CMV viremia and the time-to-CMV seroconversion on renal outcome during primary CMV infection, and the impact of MMF withdrawal.

In addition to studying the effect of withdrawing immunosuppressive medicine, we were interested to explore immunoregulatory potential of the host itself, specifically through regulatory B cells. Downregulation of these cells could mimic withdrawal of immunosuppressive therapy, which we studied in chapter 3. Therefore, in chapter 4 we zoomed in a step further by specifically assessing B cell subpopulations presumed to contain B cells with regulatory potential. The precise identity and immunological effects of these cells remain elusive, but increasing evidence implicates them as regulators of immunological tolerance and as potential protector from rejection. The number and relative contribution of different subpopulations of B cells were determined before transplantation, and associated to graft rejection. Our first aim was to evaluate B cell phenotyping as a measure of predicting transplant rejection in renal transplant recipients. Immune regulation by Bregs may concomitantly suppress protective host responses against pathogens, decrease the effectiveness of the anti-CMV response and increase susceptibility to infections. Our second aim was therefore to determine the relation between pre-transplant B cell phenotypes and the incidence of CMV infection after transplantation.

One of the characteristic properties of CMV is its immunomodulatory capacity, which allows it to modulate the host immune response. An alternative strategy to limit CMV infection, would be to restrict its ability to modulate the host immune response, and make it more vulnerable to clearance by the immune system. The focus of chapter 5 is on one of the immune evasion strategies employed by CMV: G protein-coupled receptor US28. The localization of US28 on the virion and the surface of infected cells could make it an accessible potential target for therapeutic intervention. First we determined the localization of US28 in kidney biopsies from renal transplant recipients, and the extent of its expression in various compartments of the kidney. The pronounced and mostly segmental expression of US28 in vascular smooth muscle cells suggested partial spreading of CMV and a potential role for US28 in this process. We therefore examined whether US28 could facilitate viral
spreading in vascular smooth muscle cells in vitro, and studied viral spreading for US28-deficient CMV. Our aim was to identify the expression and localization of US28 in renal allograft biopsies by immunohistochemistry, and determine its role in viral spreading in vitro.

Based on the localization of US28 in vascular smooth muscle cells in transplant biopsies and its role during in vitro viral spreading experiments, we were interested to further characterize the functional effects of US28 in chapter 6. To study US28 in detail, an in vitro inducible US28 model system of vascular smooth muscle cells was developed. The US28 expression levels in iUS28-VSMCs resembled those of WT CMV-infected VSMCs, suggesting it could provide a suitable model system for US28 after CMV infection. First the functional effects of US28 expression were determined, after which its role in viral spreading was assessed.

In Chapter 7 all the experimental data collected are contextualized and integrated into the current setting on CMV infection in renal transplantation. Additionally, perspectives for future studies as well as considerations for developments in the field are provided.

REFERENCES

[22] Nankivil BJ, Alexander SL. Rejection of the Kidney Allograft. 
Chapter 1

[41] Brit W. Virus entry into host, establishment of infection, spread in mechanism of tissue damage 2007.
[57] Brit W. Virus entry into host, establishment of infection, spread in mechanism of tissue damage 2007.
1

General Introduction

71.