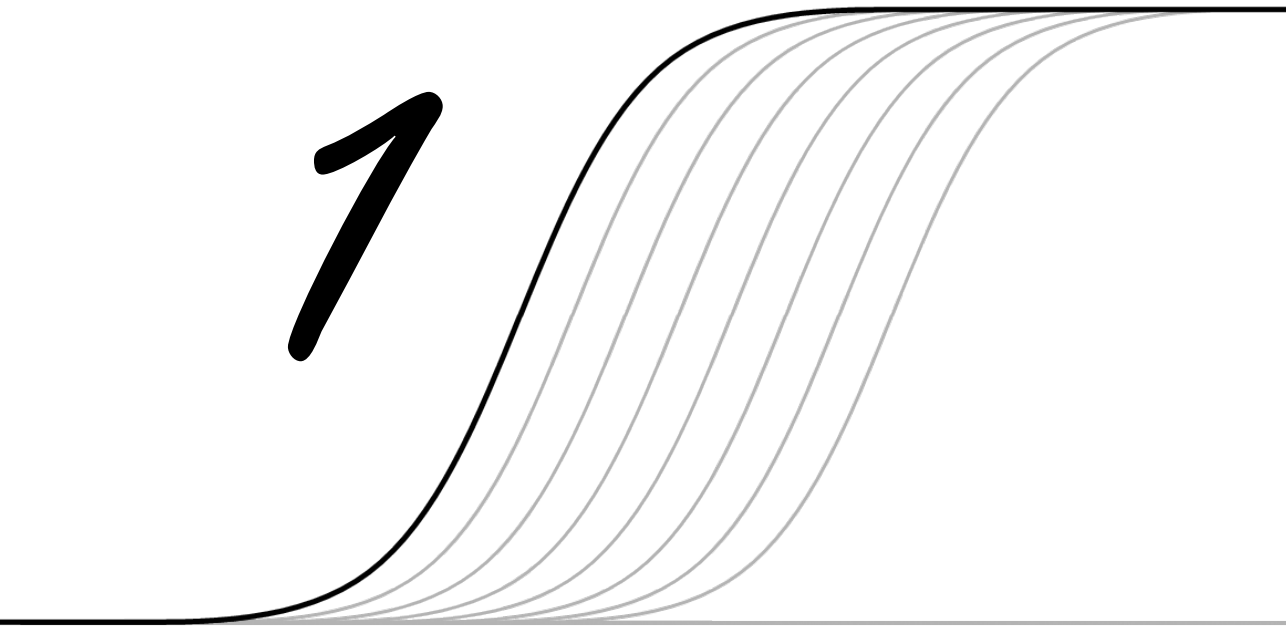
General introduction



General introduction

Kidney transplantation

Patients with chronic kidney disease experience a progressive loss of renal function over a period of months or years. The chronic decline in kidney function can progress to end-stage renal disease, a condition where the kidneys are no longer able to filter enough blood and the body retains fluids and harmful waste products [1;2]. This complete or almost complete failure of kidney function is permanent and usually requires renal replacement therapy in the form of either dialysis or transplantation. The preferred treatment for patients suffering from end-stage renal disease is kidney transplantation.

The first successful kidney transplantation was performed in 1954 in Boston, when Joseph Murray, John Merrill and Hartwell Harrison transplanted a kidney from one identical twin to another [3]. However, vigorous immune responses directed against the donor graft remained a major barrier for successful kidney transplantation between genetically non-identical humans, eventually leading to rejection of the allograft. This boosted the interest in research into organ transplantation, resulting in increased knowledge on transplantation related immunology. Since the 1960s, improvement in surgical techniques, tissue matching, and immunosuppressive medication has led to a significant reduction in the incidence of acute allograft rejection and to a substantial improvement in graft survival rates [4;5]. Nowadays, kidney transplantation has become a routine procedure. However, renal allograft rejection remains an important problem that affects long-term graft outcome.

Immunity and allograft rejection

The first important studies for organ and tissue transplantation were performed in the early 1940s by Peter Medawar [6;7]. His studies on the transplantation of skin grafts revealed that the immune system plays a major role in allograft rejection. The human immune system can be divided into the innate and the acquired immune system. The innate immune system is the first line of defense against infectious agents. It provides an immediate, but non-specific immune response to invading pathogens, but does not confer long lasting or protective immunity. The innate immune system acts via both the complement system and cellular responses. The complement system consists of a set of soluble factors, which can opsonize and kill pathogens [8].

The innate immune cells include natural killer (NK) cells, which respond to cells missing 'self' markers, and phagocytic cells, such as macrophages and dendritic cells, which internalize and kill pathogens [9-11]. Innate immune cells also play a role in the activation of the acquired immune system [12]. Transplantation procedures can lead to activation of the innate immune system through heat and cold shock, and ischemia and reperfusion insult [8].

The acquired immune system is composed of a repertoire of antigen-specific cells that become activated upon an antigenic challenge, aimed at protecting the body against foreign pathogens. In contrast to non-specific immune cells of the innate immune system, cells of the acquired immune system express antigen-specific receptors on their cell surface and retain long-term immunological memory after the encounter of the antigen. Upon activation, naïve immune cells start to expand, and they differentiate into effector cells for the elimination of the pathogen. Part of the naïve cells differentiate into memory cells, which enables a rapid response in case of a second encounter of the body with the same pathogen [13]. Acquired immune responses involve both cellular and humoral components. These include T cells involved in cellular immunity and B cells involved in humoral immunity. The renal allograft contains many foreign antigens that can activate the recipient's acquired immune system.

Human leukocyte antigens

An important hallmark in the transplantation field was the discovery of the antigens of the major histocompatibility complex (MHC) [14-18]. MHC antigens are cell surface molecules that present peptides to T cells, thereby initiating the acquired immune response. The glycoproteins encoded by the MHC are present on virtually all cells of vertebrates. In humans, the MHC molecules are known as human leukocyte antigens (HLA). The HLA system is the most polymorphic system in humans [19;20]. This high degree of polymorphisms is an evolutionary feature providing the human population as a whole with optimal protection against the wide range of pathogens it can encounter.

Classes of HLA molecules

The HLA molecules are divided into two classes, based on their structure and function in the immune response: HLA class I and HLA class II (Figure 1). The HLA

class I molecules (HLA-A, -B, and -C) are constitutively expressed on all nucleated cells and are involved in the protection against intracellular infections. They present endogenously generated peptides, such as self-peptides or virus-induced peptides [21;22]. These peptides have a length of 8-13 amino acids [22-24]. The HLA class II molecules (HLA-DR, -DQ, and -DP) are less widely expressed than class I molecules and present peptides derived from extracellular proteins. They are mainly expressed on professional antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages (MΦ), and B cells, but also on activated T cells. The peptides presented by HLA class II molecules are primarily of exogenous origin and have a typical length of 12-25 amino acids [21;22;25].

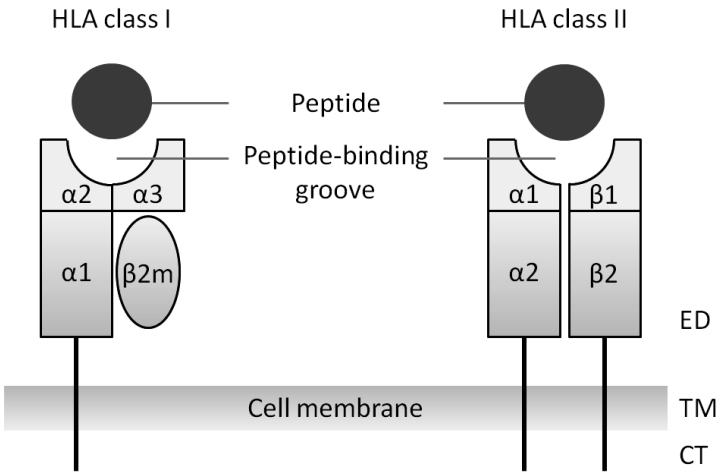


Figure 1. The structure of HLA class I and HLA class II molecules. HLA class I (left) consists of a heavy α-chain linked to a light chain β2-microglobulin (β2m). HLA class II (right) is a heterodimer consisting of an α-chain and a β-chain. ED, extracellular domain; TM, transmembrane region; CT, cytoplasmic tail.

HLA matching

Incompatibility of HLA molecules between the donor and the recipient may impede successful graft outcome after transplantation. T cells detect the presence of foreign antigens through their polymorphic T cell receptor (TCR), which can recognize foreign peptides bound by HLA molecules. All T cells are antigen specific and recognize an antigenic peptide presented only by one self-HLA molecule. This HLA restriction presents a big advantage for coping with pathogens, but represents a hurdle in transplantation. T cells can recognize both donor immune cells bearing mismatched

HLA molecules as well as peptides derived from donor antigens presented by self-HLA on APCs. Both mechanisms may contribute to transplant rejection [26]. Matching for HLA molecules between donor and recipient lowers the chance for a patient to develop acute rejection. A higher degree of HLA matching, especially at the HLA-DR locus, is associated with better graft outcome [27-31]. The high degree of polymorphism in HLA makes it difficult to find a matched unrelated donor in most cases [19].

Immunosuppression

Immunosuppression has become a cornerstone of the transplantation field for the prevention of allograft rejection. Investigation into the use of immunosuppression to prevent transplant rejection started in the early 1950s. Medawar's demonstration that allograft rejection is an immunological process increased the interest into methods to suppress the recipient's immune system and protect the allograft from rejection [6;7]. The first tested therapies were total body irradiation [32;33] and adrenal cortical steroids [34-36]. Both therapies led to prolonged skin graft survival. These early findings set the stage for the development of the current immunosuppressive drug therapies. Nowadays, almost all transplant recipients are treated with immunosuppressive drugs to minimize the chance of acute rejection, which act by inhibiting the activation and/or effector functions of T cells.

Immunosuppression is used as induction, maintenance and anti-rejection therapy. Induction therapy is a conditioning treatment given at the time of transplantation, which leads to a short-term depletion of lymphocytes. This conditioning is achieved by administering depleting antibody treatment, such as the interleukin-2 receptor blocker Daclizumab or anti-thymocyte globulin (ATG) [37;38]. The aim of induction therapy is to prevent acute rejection during the first weeks after transplantation. After transplantation, patients receive lifelong maintenance therapy. This therapy consists of a combination of corticosteroids and a calcineurin inhibitor, with or without the addition of a cytostatic drug.

Corticosteroids

Synthetic corticosteroids, such as prednisone and methylprednisolone, were first used as maintenance therapy in transplantation during the early 1960s [39;40]. They modulate the gene transcription of immune cells, resulting in a strong anti-inflammatory effect [41]. Besides their use in maintenance therapy, corticosteroids are also used as anti-

rejection therapy. More detailed information on corticosteroid treatment of allograft rejection can be found in the "treatment of acute rejection" section.

Calcineurin inhibitors

The calcineurin inhibitors Cyclosporine and Tacrolimus were introduced in the 1980s and marked a great improvement in maintenance therapy for solid organ transplantation [42-44]. Cyclosporine and Tacrolimus reduce the activation of T cells by inhibiting the protein phosphatase calcineurin. Activation of T cells via their TCR induces an increase in intracellular calcium. This increase in calcium activates calcineurin, which subsequently activates members of the nuclear factors of activated T cells (NFAT) family. The activated NFAT translocate to the nucleus and upregulate the expression of IL-2, which in turn stimulates the growth and differentiation of T cells [45;46].

Cytostatic drugs

The first proliferation inhibitors, 6-mercaptopurine and azathioprine, were introduced in the 1960s in an attempt to mimic the immunosuppressive effects of total body irradiation [33;47;48]. This first generation of cytostatic drugs inhibited DNA synthesis, thereby preventing proliferation of lymphocytes. Currently, the commonly used cytostatic drug for maintenance therapy is Mycophenolate Mofetil (MMF). MMF is a reversible inhibitor of the enzyme inosine-5'-monophosphate dehydrogenase (IMPDH). This enzyme is involved in purine synthesis, which is an essential substrate for DNA and RNA synthesis in lymphocytes [49;50]. By inhibiting IMPDH, MMF inhibits DNA synthesis of lymphocytes and prevents their proliferation.

Renal allograft rejection

Renal allograft rejection can be classified into three phases based on the time of occurrence: hyperacute rejection, acute rejection, and the development of chronic allograft injury. The main focus of this thesis is acute rejection of kidney allografts.

Hyperacute rejection

In hyperacute rejection, the transplanted organ is rejected within minutes or hours after vascularization of the graft. Hyperacute rejection results from preexisting antibodies that are either directed towards foreign HLA on the donor allograft or ABO

blood group antigens [51-53]. These antibodies may have developed in recipients due to a previous transplantation, blood transfusion or pregnancy [54]. Alloantibodies bind to vascular endothelium of the graft and activate the complement system, which causes thrombotic occlusion and loss of the allograft [53]. To determine if a patient has preformed donor-specific antibodies, a serological crossmatch test is performed prior to the transplantation [55]. Since the introduction of this test, the incidence of hyperacute rejection has fortunately been reduced dramatically.

Acute rejection

The most common form of rejection in the early post-transplant period is acute rejection. This type of rejection generally occurs within the first 6 months after transplantation, with the highest risk in the first 3 months. It is primarily a cellular immune response mediated by T cells directed against mismatched donor HLA antigens present on the cells of the allograft [56;57]. APCs express donor antigens and activate antigen-specific T cells, which infiltrate the allograft. The activated T cells cause lysis of graft cells and produce cytokines that recruit other inflammatory cells, such as monocytes, macrophages, and dendritic cells [58]. The infiltrating immune cells accumulate in the renal interstitium, and may penetrate the tubules (tubulitis) and/or the vessels (endovasculitis) [56].

A second form of acute rejection is antibody-mediated rejection (AMR), which is a humoral immune response mediated by donor-specific antibodies (DSA) [56]. The DSA are most often directed towards foreign HLA antigens, but may also target other antigens including minor histocompatibility antigens, endothelial cell specific antigens or other transplanted antigens [56;59]. AMR can occur in patients with *de novo* DSA or in sensitized patients with undetectable DSA levels at time of transplantation [60]. Memory B cells of the recipient can become activated by the allograft and start the production of DSA which, in turn, can interact with antigens in the graft. This interaction may lead to complement-mediated allograft damage through cellular lysis and recruitment of inflammatory cells and/or antibody-dependent cellular cytotoxicity [59;61].

T cell mediated rejection (TCMR) and AMR can occur individually or may coincide. Besides acute rejection during the first months after transplantation, both TCMR and AMR can also occur at later time points after transplantation [56]. These late acute rejection episodes are mostly due to noncompliance to medication by the patient [62].

Chronic allograft injury

During the years after transplantation, the renal allograft can be subjected to a process of slow deterioration. This development of chronic allograft injury is characterized by renal interstitial fibrosis and tubular atrophy (IFTA), which was formerly known as chronic allograft nephropathy (CAN) [63-65]. It is manifested clinically by a progressive decline in renal transplant function and, in many cases, by loss of the renal transplant. Chronic allograft injury may result from both non-immunological and immunological factors. Non-immunological factors include delayed graft function, donor-related factors (such as old age and hypertension), post-transplant infections, and nephrotoxic effects by immunosuppressive medication [65;66]. The process of chronic immune activity towards the graft is clinically reflected by the presence of chronic transplant dysfunction (CTD) within the graft. CTD develops over a period of years and involves both cellular and humoral immune responses leading to a variety of fibrosing and sclerosing changes in the allograft [67-69]. CTD may be the result of HLA incompatibility between the donor and the recipient, immunologic sensitization of the patient, and the occurrence of acute rejection episodes [65;70]. In addition, alloimmune responses may lead to exposure of self-antigens, which may induce autoimmune responses involved in the pathogenesis of CTD [71]. The underlying mechanisms of CTD have not been completely elucidated, making treatment difficult. Current therapeutic strategies focus on minimizing risk factors for CTD [64].

Diagnosis of allograft rejection

Serum creatinine

Reliable and timely detection of acute renal allograft rejection is important for the prevention of adverse graft outcome. Most patients who develop an acute rejection episode are asymptomatic and present only with an increase in serum levels of creatinine, a waste molecule that is generated during normal muscle metabolism. Phosphocreatine, an energy-storing molecule in muscles, is catalyzed by creatine kinases into creatine and adenosine triphosphate (ATP) molecules, which provide the phosphates needed for muscle contraction [72-74]. This reversible reaction causes the spontaneous by-product creatinine. This production of creatinine is continuous and proportional to muscle mass. Approximately 2% of the body's creatine is converted to creatinine each day, which is excreted from the body by the kidneys [72;74]. The serum creatinine levels are used as an important indicator of renal health. Creatinine

is filtered out of the blood by the kidneys, and therefore the serum creatinine level depends on the glomerular filtration rate. In renal transplant recipients, an increase in serum creatinine levels reflects a decline in graft function [75]. Significant histologic damage to the graft leads to a diminished ability to filter creatinine, resulting in a rise of the creatinine concentration in the serum.

Banff classification

A decline in renal function may result from a rejection episode, but may also be caused by other conditions such as medication toxicity or a viral infection [75;76]. The cause of graft dysfunction is determined on the basis of nephropathologic criteria and histological assessment of a renal allograft biopsy. Due to the associated risk of procedural complications, renal biopsies are mainly performed after indication of functional graft impairment [77]. To limit subjectivity of histological assessment, renal allografts are interpreted according to the Banff classification. This classification system originates from a meeting held in Banff, Canada in 1991, and was first published in 1993 [78]. The Banff scoring scheme was developed for the standardization of the histomorphologic criteria used for the diagnosis of graft rejection. In subsequent years, the histomorphologic grading scheme of the Banff classification has been updated and refined at regular Banff conferences on allograft pathology [63;79-81]. Nowadays, the Banff classification system is universally applied for interpretation of renal graft biopsies, in relation to renal allograft dysfunction.

Histological parameters of acute rejection

The Banff classification is used to designate the rejection severity on the basis of the site and degree of inflammation in the renal allograft biopsy. Three important lesions are used for the diagnosis of acute T cell-mediated rejection [81]. Interstitial inflammation (i-score) describes the infiltration of leukocytes in the interstitium of the kidney. Because focal or mild diffuse infiltrates of mononuclear cells can be present in biopsies from patients with well-functioning grafts, the i-score is not by itself indicative of acute rejection. The principal lesions indicative of acute renal allograft rejection are tubulitis and intimal arteritis [79;81]. Tubulitis (t-score) indicates the presence of mononuclear cells within the tubular epithelium. The infiltrated leukocytes can recognize and lyse epithelial cells, resulting in tubular damage and a decline in graft function. This form of TCMR is known as acute tubulointerstitial

rejection (Banff grade I) [81-83]. An additional lesion which may be present during acute rejection is intimal arteritis (v-score), which is defined as infiltration of lymphocytes and monocytes beneath the endothelium of arteries in the renal cortex. TCMR with intimal arteritis is indicated as acute vascular rejection (Banff grade II) [81;84;85].

Impact of acute rejection on graft outcome

In the 1960s, acute rejection was the most important cause of graft loss. Only 40% of the renal allograft recipients had a functioning graft at one year after transplantation [86;87]. The introduction of more potent immunosuppressive medications and refinement in treatment regimens have led to a reduction in the incidence of acute rejection from over 80% in the 1960s to below 15% nowadays [87;88]. Over the same period, the short-term survival of kidney grafts has substantially improved, with one-year graft survival rates in excess of 90% in current daily practice [5;89;90]. Despite these advances in short-term outcome, long-term graft outcome improved only marginally over the past two decades [5;70;90]. Approximately 50% of grafts from deceased donors and 30% of grafts from living donors fail within ten years after kidney transplantation [91]. The graft attrition rates after the first year are between 3% and 5% annually. This is mainly due to death with a functioning graft and chronic allograft failure [90;92;93].

Although most acute rejection episodes can be reversed with the currently available immunosuppressive therapies, it continues to be a primary cause of renal allograft failure. Approximately 10% of all graft losses are due to acute rejection [93]. Furthermore, several studies have shown that the occurrence of acute rejection correlates with a significant reduction in long-term allograft survival [89;94-96]. Besides the association with risk of graft loss, acute rejection is also associated with the development of chronic allograft failure. IFTA is the most prevalent cause of chronic allograft failure after the first post-transplant year [87;93]. Analyses of factors related with the development of IFTA revealed acute rejection as one of the most important risk factors [92;97-99]. In addition, it was shown that the acute rejection associated risk for chronic transplant failure has increased during the last decades [99]. Although the incidence has decreased during this time period, the negative impact of acute rejection on the subsequent development of IFTA has become more prominent [99].

Important aspects of acute rejection associated with increased risk of adverse graft outcome include the timing, recurrence, severity, and therapy sensitivity of the acute rejection episode [96]. The occurrence of both early (within first 3 months

of engraftment) and late (after 3 months) acute rejection episodes associate with a higher risk of graft failure. The risk increases as the time to acute rejection increases and was most pronounced with late acute rejection episodes [96;100-105]. Similarly, patients experiencing repeated acute rejection episodes are at greater risk of adverse graft outcome than those with no or only one episode [106-109]. In addition, patients with acute vascular rejection (Banff grade II) have a higher risk of graft failure compared to patients with acute tubulointerstitial rejection (Banff grade I) [110-112]. Acute rejection episodes unresponsive to anti-rejection treatment have been associated with increased risk of allograft failure [112-114].

Treatment of acute rejection

Despite the combination of HLA matching and maintenance immunotherapy, renal transplant recipients can still develop acute allograft rejection. Several therapeutic options are available for the reversal of acute rejection episodes, including pulse corticosteroid therapy and polyclonal and monoclonal antibody therapy.

High-dose corticosteroids

The first report on the use of immunosuppressive drugs for the treatment of acute renal allograft rejection was in 1960 [115]. A young female recipient of her mother's kidney developed multiple rejection episodes, which were temporarily reversed with prednisone. This case sparked the interest in corticosteroid therapy for both the prevention and the treatment of acute rejection episodes. In 1963, Starzl and colleagues demonstrated in ten renal allograft recipients that acute rejection could readily be reversed by temporarily adding high doses of prednisone to the patients maintenance therapy [116]. All ten patients showed an essentially complete recovery of their renal function. Based on these early findings, increasing the daily dose of oral prednisone became the main therapy for acute rejection [117;118]. The treatment of acute rejection with high doses of oral prednisone was found to potentially induce toxic side effects, such as gastrointestinal hemorrhage and increased susceptibility to infection. To prevent these complications, the treatment was switched from oral prednisone to intravenous application of methylprednisolone during the early 1970s [117;118]. Comparison of the two regimens revealed that both forms of corticosteroids were equally successful in reversing acute rejection [119;120]. However, pulse therapy with intravenous methylprednisolone is associated with fewer side effects than

oral prednisone therapy [117;120]. Since these early developments, pulse therapy with high-dose steroids has remained the typical approach to treat acute renal allograft rejection.

Polyclonal and monoclonal antibodies

Other therapy regimens for the treatment of acute rejection episodes imply the use of anti-lymphocyte antibodies. The first report on antibody-based immunosuppression was by Metchnikoff in 1899 [121]. His observations on the lymphocyte-depleting activity of heterologous anti-lymphocyte serum were validated in the 1960s [122-124]. These findings resulted in the introduction of ATG as a treatment of allograft rejection [125-127]. ATG is the purified polyclonal antibody fraction of sera from horses or rabbits that have been immunized with human thymocytes or T cell lines [128;129]. ATG contains antibodies specific for many common leukocyte antigens, including co-stimulation, adhesion, and cell trafficking molecules [130]. ATG therapy causes the depletion of circulating T cells and other leukocytes through various mechanisms, including antibody- and complement-dependent lysis and the induction of apoptosis [130]. ATG is an effective treatment of acute renal allograft rejection with high graft survival rates [131-133]. However, ATG can induce complications, such as leukopenia, cytokine release syndrome, and viral infections [129;132]. ATG is mainly used for the treatment of steroid-resistant acute rejection and recurrent acute rejection.

The development of cell-hybridization techniques provided the possibility to produce monospecific antibodies [134]. The first monoclonal antibody used for the treatment of acute renal allograft rejection was OKT3 [135;136]. The murine-derived OKT3 is directed against the CD3 molecule, which is closely associated with the TCR. OKT3 treatment modulates the TCR, resulting in the depletion of circulating T cells. OKT3 has been used as primary treatment of acute rejection and as rescue therapy of steroid-resistant rejection [137;138]. The use of OKT3 is associated with serious side effects, which include cytokine release syndrome, pulmonary edema, nephropathy, and infections. Due to its lower efficacy and higher incidence of side effects compared with ATG treatment, OKT3 has been withdrawn from the market and is no longer in clinical use [131;132;139;140].

Immunoregulatory effects of corticosteroids

A temporary treatment with high doses of corticosteroid is used to combat acute renal allograft rejection. A protective effect on the allograft is obtained by inhibiting T-cell proliferation and cytokine gene transcription (see Figure 2). Glucocorticoids (GC) act via the intracellular glucocorticoid receptor (GR), which is expressed by almost every cell in the body [141;142]. Steroids diffuse across the cell membrane and bind to the GR in the cytoplasm. Upon ligand binding, the GR becomes activated and the GC-GR complex translocates to the nucleus, where it directly or indirectly regulates the transcription of target genes [143;144]. Corticosteroids regulate approximately 20% of all genes expressed in leukocytes [145]. The estimated number of genes directly regulated by corticosteroids lies between 10 and 100 depending on the cell type [146]. Many inflammatory genes are indirectly regulated through GR interference with activating transcription factors and their co-activators. The major action of corticosteroids is the suppression of inflammatory genes that are activated during acute rejection [142;143;146;147]. These include genes encoding for cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors [146]. Besides the downregulation of pro-inflammatory genes, high-dose corticosteroid therapy also upregulates the expression of anti-inflammatory genes, which include interleukin-10, mitogen-activated protein kinase phosphatase-1 (MKP-1), secretory leukoprotease inhibitor, and annexin-1 [146;148]. In addition, glucocorticoid therapy can suppress acute rejection via its potential to prevent migration of leukocytes, induce cell death in lymphocytes, and influence the growth and lineage commitment of T cells [141;144;149;150].

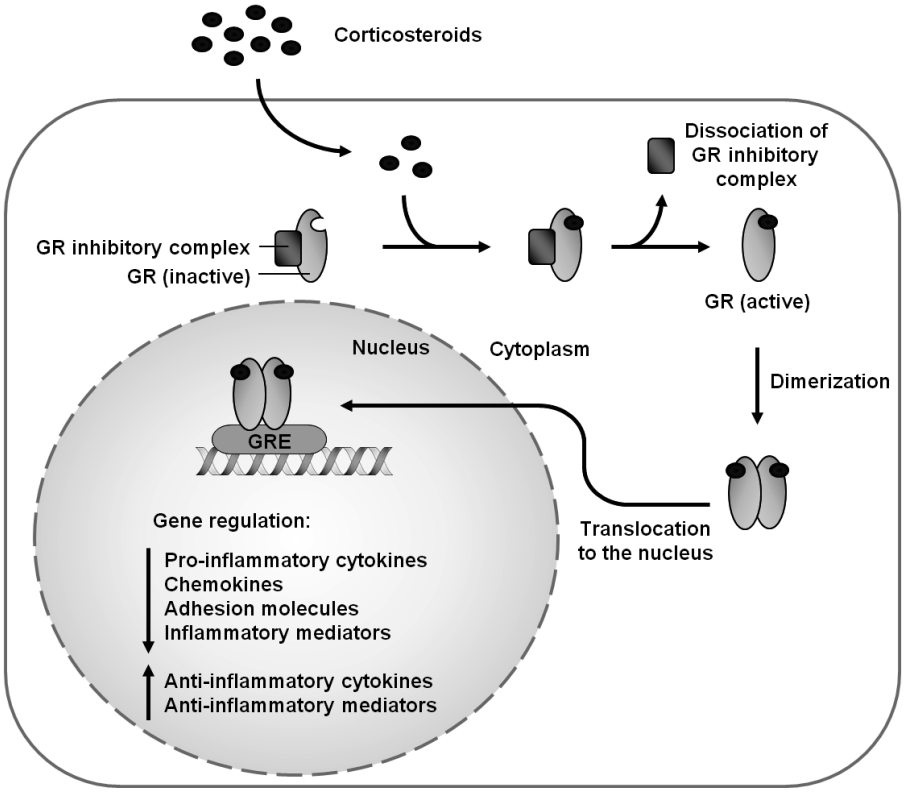


Figure 2. Mechanism of glucocorticoid signaling. GR, glucocorticoid receptor; GRE, glucocorticoid responsive elements.

Steroid resistance

One of the main parameters determining graft outcome is therapy sensitivity of the acute rejection episode [112-114]. Nowadays, the first-line therapy for acute rejection in most centers is pulse therapy with intravenous methylprednisolone. The majority of acute rejection episodes can be adequately treated with high-dose corticosteroids. However, in approximately 25 to 30% of the patients the rejection episode cannot be reversed with corticosteroid therapy alone [114;151-156]. Similarly, poor or no response to steroid therapy for acute rejection reversal also occurs in a proportion of the recipients of other solid organ transplants, including liver, lung and cardiac allografts [157-159]. In case of steroid resistance, the patient requires more rigorous therapy with anti-lymphocyte antibodies to reverse the acute rejection episode. Renal allograft recipients with steroid-refractory rejection are generally treated with ATG, which results in a salvage rate of 70 to 90% [140;160-162].

An acute rejection episode is considered steroid resistant when the patient's serum creatinine levels do not return to within 120% of the pre-rejection baseline value after pulse therapy with high-dose steroids, and ATG treatment is required within 14 days after the start of the steroid therapy [57;162-164]. The first few days after the start of the steroid treatment are crucial. Analysis of creatinine courses of steroid-resistant and steroid-responsive cases revealed that the minimal time period for assessment of the response to steroids is five days after the beginning of the treatment [151]. The change in serum creatinine levels was similar between patients with steroid responsive and steroid resistant acute rejection until day 5, at which time the responders showed a significant decrease in serum creatinine, while the creatinine level of non-responders remained high. This 5 day period is also the average time delay used by clinicians before considering a rejection as being steroid resistant [165]. The incomplete restoration of graft function in steroid resistant rejection may lead to progression of chronic damage to the graft and has a detrimental effect on graft outcome [66;112-114;153].

Assessment of risk for steroid resistance

Prediction of steroid resistance at the time of biopsy could prevent unnecessary exposure to high-dose corticosteroid therapy. More importantly, the development and progression of irreversible nephron loss during the period that steroid resistant acute rejection is undertreated with steroids alone could be avoided. This impact of steroid-refractory rejection on graft integrity stresses the need for tools to assess the response to anti-rejection treatment in an early stage.

Clinical and pathologic indicators of steroid resistant rejection

At present, clinical parameters and histopathologic assessment of kidney biopsies remain the golden standard for evaluating short- and long-term graft outcome. Several parameters have been associated with response to steroid treatment. Acute vascular rejection is related to resistance to high-dose steroid therapy and a subsequent higher chance of graft failure [163;166;167]. In addition, unresponsiveness to steroid therapy has been associated with the presence of mononuclear cells at endothelial cells of large and small vessels in the graft [163]. Another aspect associated with steroid resistance is the presence of an immune response directed against the microvasculature. Patients with moderate to severe microvascular destruction

respond less adequately to steroid therapy compared to patients with only mild destruction of the microvascular endothelium [167]. Steroid-refractory acute rejection has been associated with more extensive leukocyte infiltration into the peritubular capillaries (PTC) [167]. Circulating leukocytes target HLA molecules expressed on the PTC, which results into cellular rejection. In addition, the HLA molecules can also be targeted by donor-specific antibodies, leading to local complement activation and humoral rejection. The activation of the complement cascade leads to the formation of complement degradation factor C4d, which can covalently bind to the PTC endothelium [79]. C4d deposition in PTC has been associated with steroid resistance [168-171], although in a recent study this association was not found [172].

Cellular and molecular markers of steroid resistant rejection

It remains difficult to predict the risk of graft loss and the response to anti-rejection treatment on basis of histopathologic assessment and clinical parameters. Biomarkers for molecular and cellular mechanisms involved in graft survival and medication responsiveness could provide complementary parameters for assessing the risk of adverse graft outcome. Indeed, expression of various markers, particularly those of inflammatory cell types, was found to be informative with respect to therapy response. Analysis of gene expression patterns in acute rejection biopsies of pediatric renal transplant recipients revealed an association between the presence of distinct lymphocyte populations and poor graft outcome [154]. Steroid resistance correlated with increased expression of B cell-, cytotoxic T cell-, and natural killer cell signatures compared to steroid responsiveness. The extent of B cell infiltration, investigated through immunostainings for B cell marker CD20, was associated with steroid resistant acute rejection [154;173;174]. However, more recent studies failed to confirm that the presence of intragraft B cells is related to therapy response and/or graft function after rejection [155;164;175;176]. High expression of cytotoxic T cell markers [177], high FasL mRNA expression [156], and dense granulysin staining [178] in renal allograft biopsies, as well as low FoxP3 expression in urinary sediments [179], have all been described to be associated with steroid resistant rejection. The infiltration of macrophages into the interstitium and glomeruli of the renal allograft was also found to be associated with steroid-refractory acute rejection [180-183]. Although various markers for graft outcome have been proposed, the heterogeneity in transcriptional regulation observed among acute rejection biopsies makes interpretation of the findings difficult.

Aim and outline of this thesis

The aim of this thesis was to identify cellular and molecular markers associated with and/or predictive for outcome of acute renal allograft rejection. The main focus was on the response to pulse therapy with high-dose steroids for the treatment of first acute rejection episodes. For this purpose, we also optimized molecular techniques required to define these biomarkers.

The optimal protocols for storage of clinical samples, RNA extraction, and cDNA synthesis were defined in studies described in **chapter 2**.

To identify cellular and molecular markers associated with risk of allograft failure or resistance to steroid treatment of acute rejection, we performed retrospective studies in a large cohort of renal transplant patients with a first acute rejection episode. In **chapter 3**, we evaluated a broad panel of immunological markers at the RNA level within the renal grafts, including previously reported biomarkers, and their relevance for the prediction of the response to corticosteroid treatment. In **chapter 4**, we used microarray analysis to identify novel molecular markers associated with steroid-refractory acute rejection, in order to gain further insight into the mechanisms underlying steroid resistance. Investigation of intragraft gene expression profiles revealed that the expression of metallothioneins in renal allografts is associated with response to steroid treatment. C**hapter 5** describes the impact of DNA polymorphisms in genes involved in glucocorticoid signaling and drug metabolism as predisposing factors on the response to high-dose steroid therapy for acute renal allograft rejection.

In **chapter 6**, the association between the expression of S100A9 and S100A8 during acute rejection and graft survival was studied. The study presents evidence that intragraft S100A9 and S100A8 expression levels are indeed predictive markers of graft outcome in renal allograft recipients.

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