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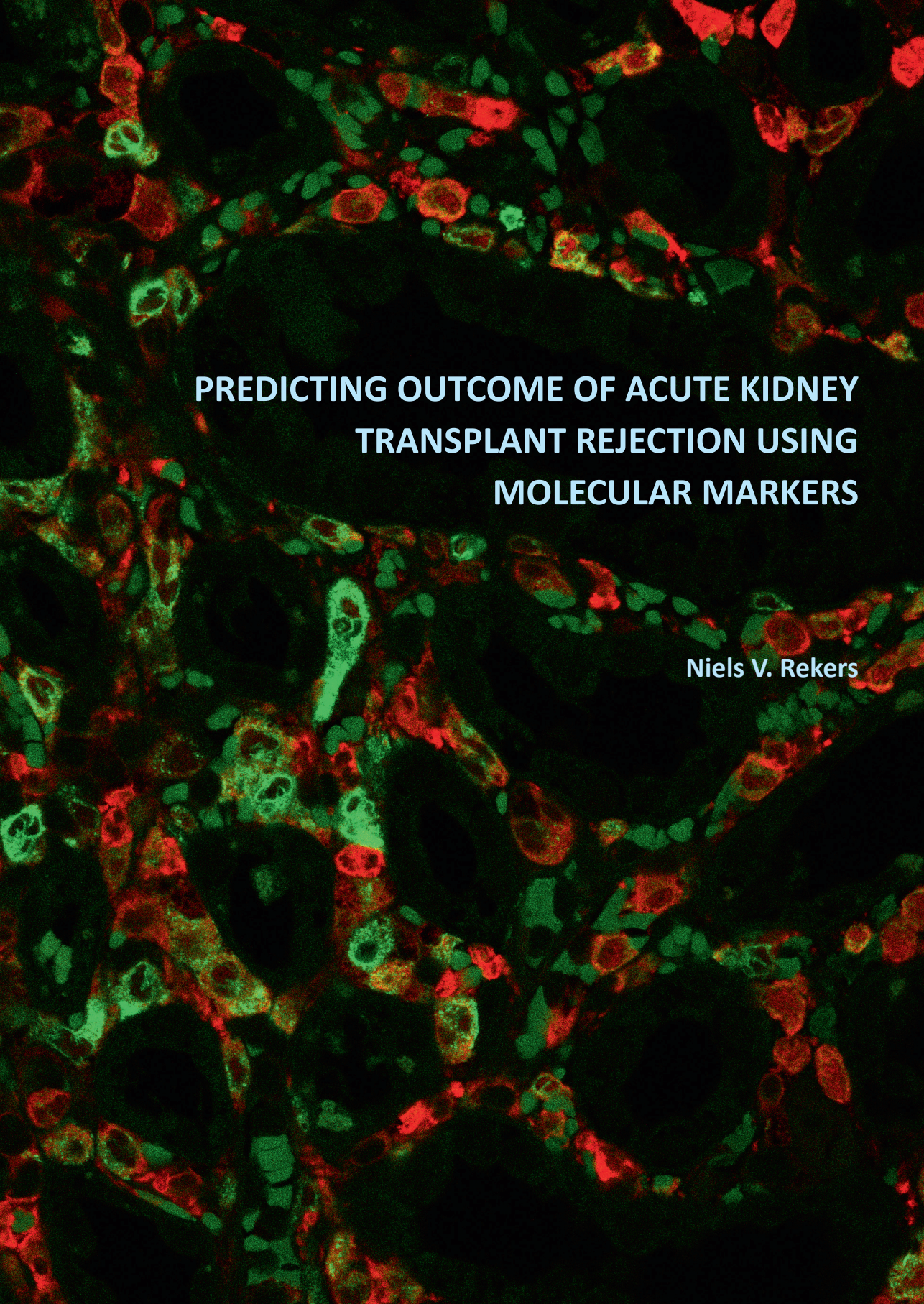


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PREDICTING OUTCOME OF ACUTE KIDNEY TRANSPLANT REJECTION USING MOLECULAR MARKERS

Niels V. Rekers

PREDICTING OUTCOME OF ACUTE KIDNEY TRANSPLANT REJECTION USING MOLECULAR MARKERS

Niels Vincent Rekers

Predicting outcome of acute kidney transplant rejection using molecular markers
Dissertation, University of Leiden, Leiden, the Netherlands

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PREDICTING OUTCOME OF ACUTE KIDNEY TRANSPLANT REJECTION USING MOLECULAR MARKERS

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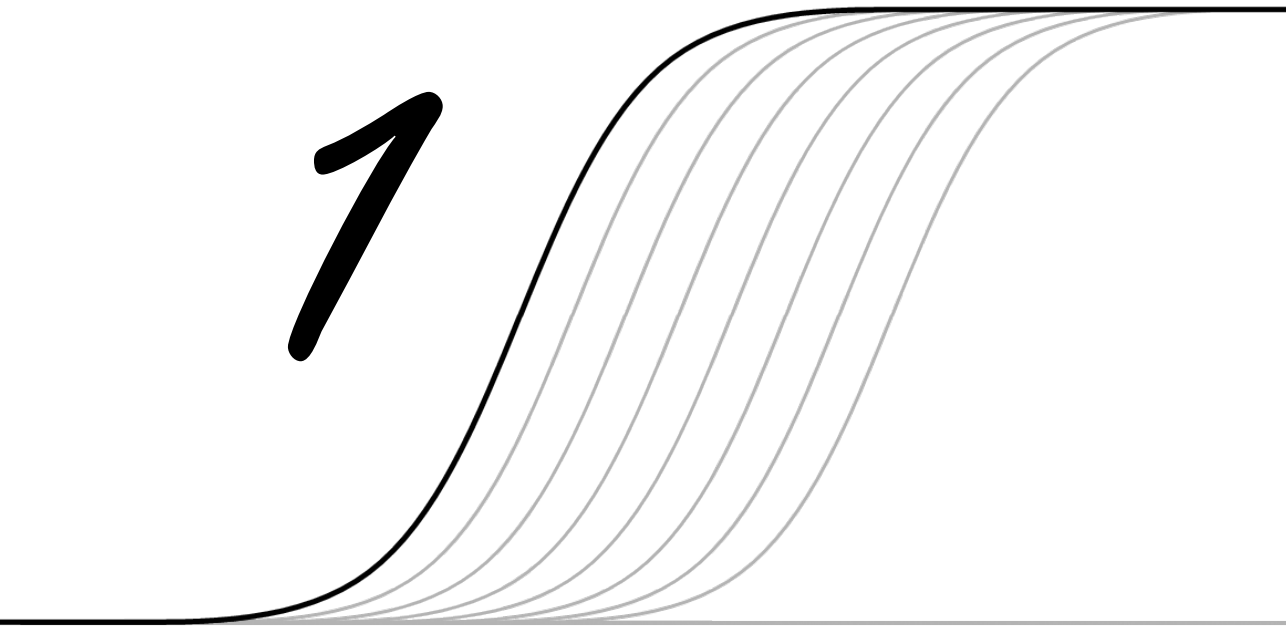
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Voor Opa

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].

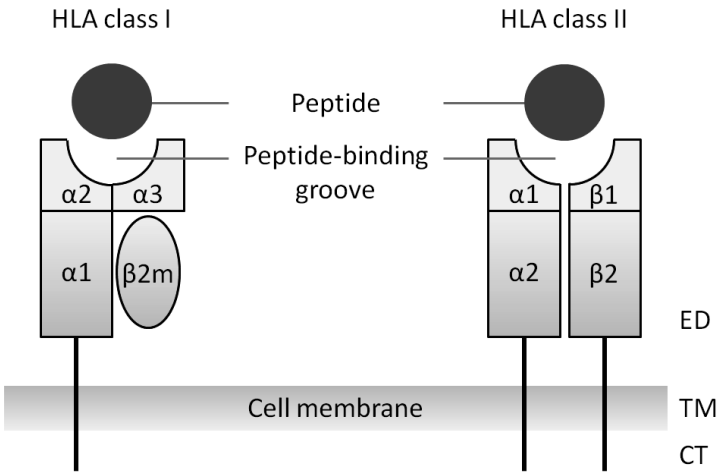


Figure 1. The structure of HLA class I and HLA class II molecules. HLA class I (left) consists of a heavy α-chain linked to a light chain β2-microglobulin (β2m). HLA class II (right) is a heterodimer consisting of an α-chain and a β-chain. ED, extracellular domain; TM, transmembrane region; CT, cytoplasmic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

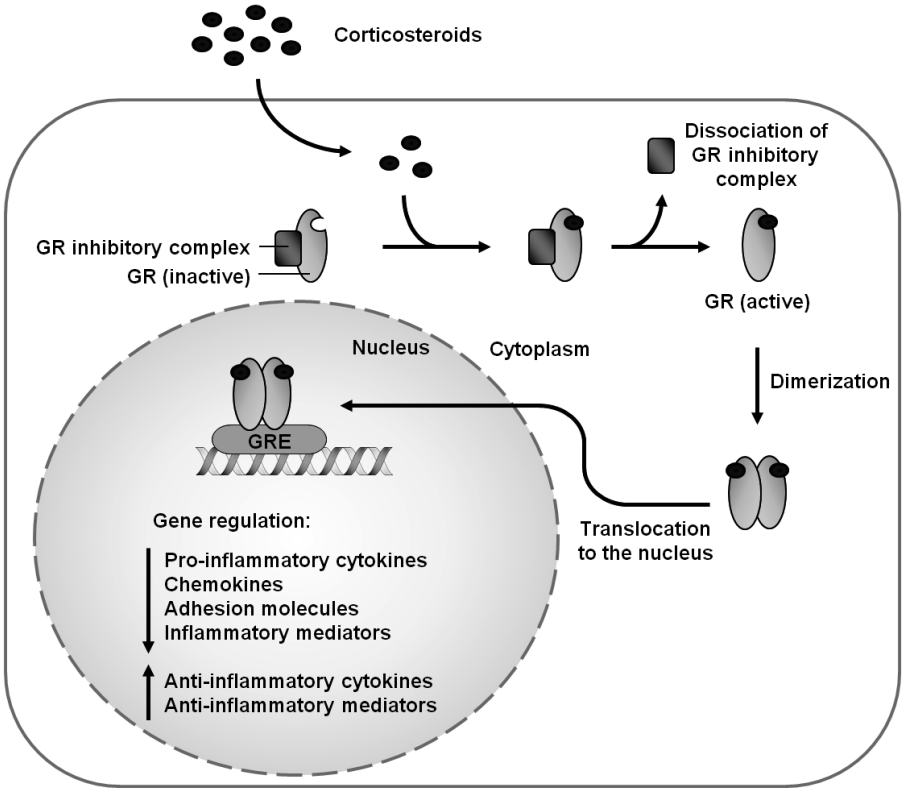


Figure 2. Mechanism of glucocorticoid signaling. GR, glucocorticoid receptor; GRE, glucocorticoid responsive elements.

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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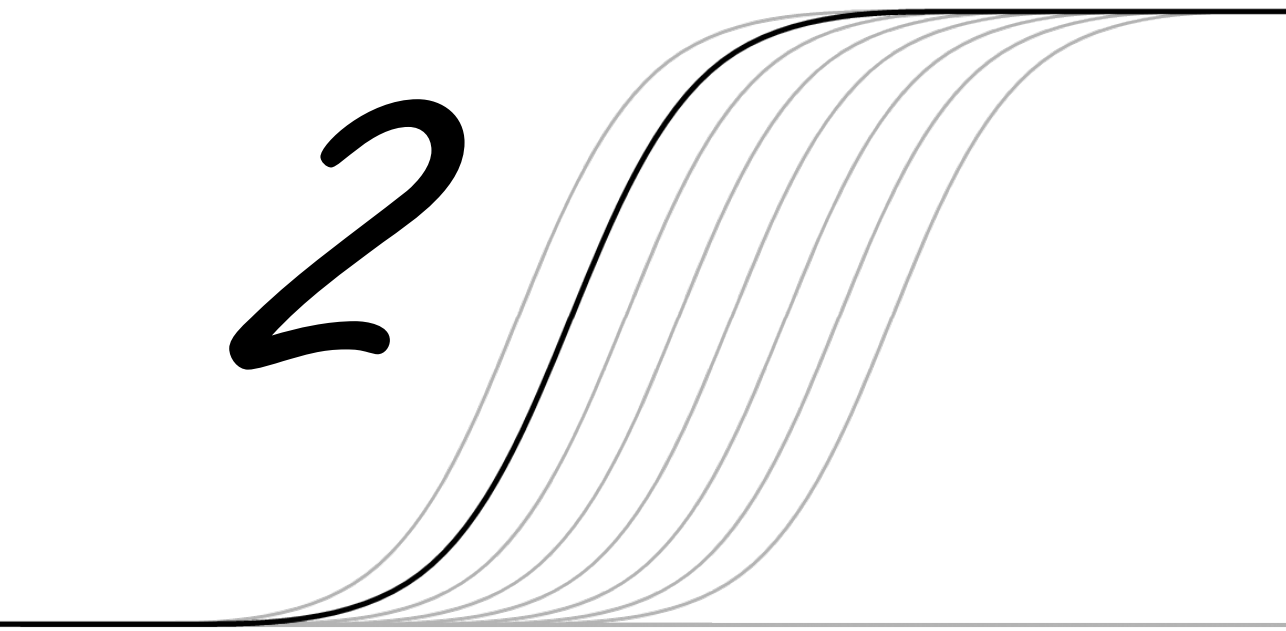
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Blood cell mRNAs and microRNAs:

optimized protocols for extraction and preservation



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Abstract

Assessing mRNA and microRNA levels in peripheral blood cells may complement conventional parameters in clinical practice. Working with small, precious samples requires optimal yields of RNA and minimal extent of RNA degradation. Several procedures for RNA extraction and cDNA synthesis were compared for their efficiency. The effect of freeze-thawing peripheral blood cells and storage in preserving reagents on RNA quality was investigated. In terms of RNA yield, quality, qPCR signals per ng of total RNA, and convenience, the use of NucleoSpin and *mir*Vana columns is preferable. The SuperScript III protocol results in the highest yields of cDNA. During conventional procedures of storing peripheral blood cells at -180°C and thawing them thereafter, RNA integrity is maintained. TRIzol preserves RNA in cells stored at -20°C. Detection of mRNA levels significantly decreases in degraded RNA samples, whereas microRNA molecules remain relatively stable. When standardized to reference targets, mRNA transcripts and microRNAs can be reliably quantified in moderately degraded (quality index 4-7) and severely degraded (quality index <4) RNA samples, respectively. We describe a strategy for obtaining high-quality and quantity RNA from fresh and stored cells from blood. The results serve as a guideline for sensitive mRNA and microRNA expression assessment in clinical material.

Introduction

Data generated with molecular techniques, when combined with clinical parameters and biopsy derived histopathology data, are instrumental for improved diagnostic and prognostic assessment.

In cancer biology, RNA derived gene expression signatures of peripheral blood cells or the tumor itself allowed the classification of breast carcinomas [1-3], skin melanomas [4], lung adenocarcinomas [5], and hematologic malignancies [6-8]. These signatures were also useful to establish their relationship to prognosis. In solid organ transplantation, gene expression profiling of RNA from biopsied tissues has aided in distinguishing subtypes of acute rejection which respond differently to therapy [9]. Using peripheral blood cells as a source of RNA is less invasive for the patient, and it enables frequent immunologic monitoring after transplantation [10-13]. Messenger RNA biomarkers have been identified in peripheral blood from patients who show operational tolerance toward their graft [14-17].

MicroRNAs are small, non-coding RNA molecules that negatively regulate mRNA expression by degradation or translational repression [18;19]. In recent years, microRNAs have gained interest for their usefulness in classification of (blood) cancer [20-24], for their role in hematopoiesis and immune cell function [25-28] and for their role in rejection of solid organ transplants [29-31].

Reliable quantification of mRNA and microRNA levels for diagnostic and prognostic purposes requires optimal RNA quality and quantity. In the current study we compared the efficiency of various procedures for obtaining RNA and cDNA from peripheral blood cells. We examined the impact of storage of peripheral blood cells on RNA degradation. We also investigated the effect of RNA degradation on mRNA and microRNA signals.

Materials and methods

Cells and cell culture

Blood, anticoagulated with heparin, was obtained from anonymous donors (Sanquin blood bank, Leiden, the Netherlands) after informed consent, and mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. Peripheral

blood lymphocyte (PBL) blasts were studied, since they are a readily available source of RNA for study. Lymphocyte blasts (PBL blasts) were generated by culturing PBMC from 4 donors at $1x10^6$/mL for 7 days in RPMI1640 (Gibco, Grand Island NY, US), containing 15% human serum, and penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco, cat.no. 15070-063). The medium was further supplemented with 10 U/mL IL-2 and 2 µg/mL phytohaemagglutinin (both from Remel Inc, Lenexa KS, US).

Performance of molecular methods was also tested on PBMC without any culturing, for which separate donors were used (n=3).

Cell storage and thawing

Freshly obtained PBMC were processed for RNA extraction, either directly or after storage for 6 days at -20°C in 25 µL of RNA*later*™ (RNA Stabilization Buffer, Qiagen) or 800 µL of TRIzol (see Figure 1). Alternatively, cells were cryopreserved in culture medium (RPMI1640) with 20% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO) prior to RNA extraction. Storage of vials, containing 1 ml cell suspension each, was at -80°C for at least 24h, and at -180°C for 3 days.

Cells were thawed by two different methods (Figure 1): using a standard protocol or a protocol that includes benzonase-nuclease (Sigma-Aldrich, Zwijndrecht, the Netherlands, cat.no. E1014; henceforth called benzonase) [32]. In the standard thawing protocol, cells are thawed in a 37°C water bath and are transferred dropwise to 3 mL of FCS at room temperature. After adding 12 mL of culture medium, cells were pelleted and resuspended in 10 mL of culture medium with 10% FCS. In the benzonase thawing protocol, cells are thawed at 37°C and transferred dropwise to 2 mL of a 1:1 FCS / culture medium mixture supplemented with 0.6 µL of benzonase. Cells were pelleted and resuspended in 5 mL of culture medium with 1 µL of benzonase. After washing steps, cells were resuspended in culture medium. After application of any thawing protocol, $1x10^6$ PBMC were processed for RNA extraction either directly or after storage for 6 days at -20°C in 25 µL of RNA*later* or 800 µL of TRIzol. RNA quality and qPCR signals were determined, as described below.

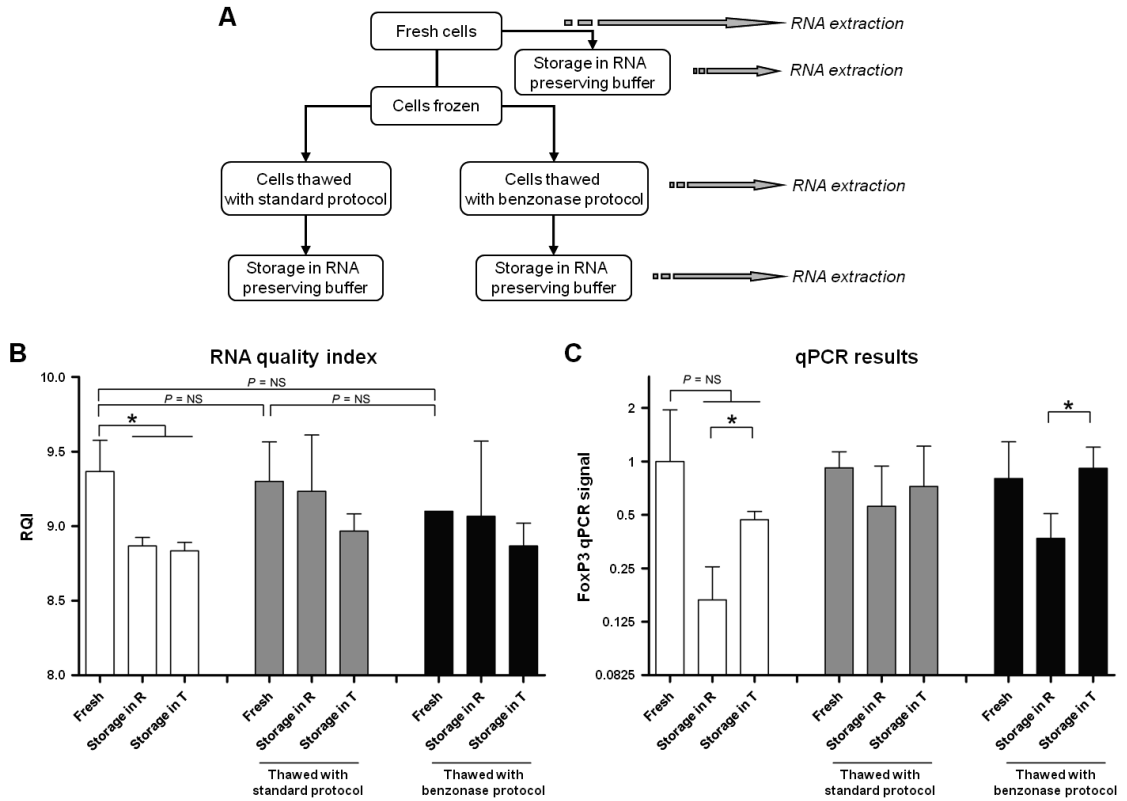


Figure 1. Cell storage and thawing. (A) The scheme shows manipulations of PBMC prior to RNA extraction. Where appropriate, RNA was preserved in both RNA*later* and TRIzol. (B) The effect of freeze-thawing PBMC, combined with the effect of storage in various RNA-preserving reagents, on RNA quality is shown. RNA samples were analyzed on StdSense gels to assess integrity expressed as RNA quality index (RQI). (C) For the same conditions, PCR signals in cDNA that was synthesized from the RNA samples were determined. R, RNA*later*; T, TRIzol. *, P<0.05.

RNA extraction protocols

RNA was extracted using RNeasy spin columns (Qiagen, Venlo, the Netherlands), TRIzol® Reagent (Life Technologies™, Grand Island NY, US), RNA-Bee™ (Tel-Test, Friendswood TX, US), the Ambion® *mir*Vana™ PARIS™ kit (Life Technologies), and the NucleoSpin® miRNA kit (Macherey-Nagel, Düren, Germany). Protocols for the column-based kits (RNeasy, *mir*Vana, NucleoSpin) were followed as described by the manufacturers. With *mir*Vana and NucleoSpin, small and large RNAs were purified in one fraction, without separate enrichment of small RNAs. Since the RNeasy procedure does not allow efficient extraction of RNA molecules of less than 200 bases, it was not included in the comparison of microRNA yields. The TRIzol and RNA-Bee reagents were supplemented with 0.2 mL of chloroform per 1 mL of reagent. After rigorous mixing and centrifugation (15 min, 12,000g, 4°C), the upper layer was isolated. To pellet the RNA, 0.5 mL of isopropanol (per 1 mL of reagent) was added, followed by 10 min incubation at room temperature and centrifugation (15 min, 12,000g, 4°C). The pellet was washed once in 0.5 mL of 75% ethanol.

RNA quantity and quality assessment

RNA quantity was determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville NC, US). Results are expressed as micrograms RNA per million cells. Furthermore, signals obtained from qPCR on cDNA that was synthesized (with SuperScript III; see below) from a fixed amount of RNA (i.e., 200 ng) per sample served as an additional criterion of suitability of the RNA extraction methods.

RNA quality was assessed using the StdSense Analysis kit and the Experion™ RNA analyzer (Bio-Rad, Hercules CA, US). The dynamic range of StdSense chips is 5 to 500 ng/µL. The RNA quality index (RQI), also known as RNA integrity number [33], was used as an indicator of RNA integrity, and it ranges from 10 (intact) to 0 (fully degraded). For RQI calculations the 28S ribosomal (r)RNA, 18S rRNA, and pre-18S region (below the 18S rRNA band) on electropherograms are taken into account. A high 28S:18S ratio (i.e., around 2:1) and a low number of shadow bands below and above the 18S band are indicative of a high RQI value.

cDNA synthesis protocols

mRNA

Complementary DNA synthesis from total RNA (extracted with *mir*Vana) with an input of 100 ng, 500 ng, and 1500 ng was carried out with four different methods, following the manufacturers' manuals: AMV-RT (Promega, Madison WI, US) (10 U of RT, 0.5 mM dNTP, 20 U of rRNasin); the RevertAid™ kit (Fermentas, St.Leon-Rot, Germany; 200 U of M-MuLV RT, 1 mM dNTP, 20 U of RiboLock™ RNAse inhibitor); BioScript™ RT (Bioline, London, UK; 200 U of MMLV RT, 0.5 mM dNTP); and SuperScript™ III RT (Invitrogen; 200 U of RT, 0.5 mM dNTP, 40 U of RNAse OUT™ rRNAse inhibitor, 5 mM DTT). RNA was combined with oligo dT (Invitrogen; 0.25 µg) and random nucleotide hexamers (Invitrogen; 0.25 µg) and incubated at 65°C (70°C for BioScript) for 5 min. Subsequently, the remaining constituents supplied by each manufacturer were added on ice, and the reactions were allowed to proceed at 25°C for 5 min and at 42°C (50°C for SuperScript III) for 1 h. Reactions were terminated by increasing the temperature to 70°C for 5 min.

microRNA

Complementary DNA synthesis of microRNAs (100 ng total RNA as input) was carried out with the miRCURY LNA™ Universal cDNA kit from Exiqon (Vedbaek, Denmark; cat.no. 203300). The reverse transcription reaction was carried out according to the manufacturer's manual. Reactions were allowed to proceed for 60 min at 42°C and were terminated by increasing the temperature to 95°C for 5 min.

Quantitative PCR assays

Specific mRNA targets (Table 1) and miRNA targets (Table 2) with various abundance in expression were analyzed. Signals for mRNA transcripts were determined by qPCR on 1:10 diluted cDNA. Optimal primers pairs were selected using Primer 3 (v. 0.4.0), which is an open source project hosted by SourceForge. To prevent amplification of genomic DNA, forward and reverse primers for each transcript were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. To ensure high specificity of the primers without amplification of genomic DNA, all primer sets were tested before use on control cDNA (from Human Reference Total RNA; Clontech, Mountain View CA, US) and genomic DNA. All PCR efficiencies were between 90% and 110%. Signals for microRNAs were determined by qPCR on

1:40 diluted cDNA. Primer sequences were obtained from Exiqon (see Table 2 for catalogue numbers), and contained built-in locked nucleic acids (LNA) for enhanced sensitivity and specificity. Further details concerning the qPCR assays have been described elsewhere [34-37].

PCR assays were carried out using iQ™ SYBR® Green Supermix and a MyiQ Real-Time PCR detection system (Bio-Rad). The PCR program consisted of 10 min hotstart at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Upon completion of each run, a melting curve analysis was done to check specificity of the primers.

In real-time PCR the Cq (quantification cycle) value is a measure of the number of cDNA copies. Cq indicates the number of PCR cycles where fluorescence increases above the threshold. In experiments for evaluation of the effect of RNA degradation, Cq ratios of individual targets and reference targets (for mRNA: β-actin, GAPDH [38]; for microRNA: miR-103, miR-191, SNORD38B, SNORD49A) were calculated using the $2^{-\Delta\Delta Cq}$ method [39].

Table 1. Characteristics of primers for detection of mRNA transcripts

Transcript	Forward sequence	Reverse sequence	Amplicon size	Expression level (Cq) [1]
High expression				
β-actin	5'-accacaccttctacaatgag-3'	5'-tagcacagcctggatagc-3'	161 bp	13.9 ± 1.4
GAPDH	5'-acccactcctccacctttgac-3'	5'-tccaccaccctgttgctgtag-3'	110 bp	15.1 ± 1.4
Intermediate expression				
TGF-β1	5'-cccagcatctgcaaagctc-3'	5'-gtcaatgtacagctgccgca-3'	101 bp	18.6 ± 0.8
Low expression				
CD25	5'-gactgctcacgttcatcatggt-3'	5'-aatgtggcgtgtgggatctc-3'	82 bp	20.8 ± 2.8
HPRT-1	5'-agatggtcaaggtcgcaagc-3'	5'-tcaagggcatatcctacaacaaac-3'	165 bp	21.7 ± 2.6
FoxP3	5'-acagcacattcccagagttc-3'	5'-caggtggcaggatggtttc-3'	190 bp	21.9 ± 1.4

[1] Values represent mean Cq values (with standard deviation) in cells from four donors.

Table 2. Characteristics of primers for detection of microRNAs and small RNAs.

microRNA	Cat. Nr. [1]	NCBI Accession	Target size [2]	Expression level (Cq) [3]
High expression (Cq<20)				
SNORD49A	203904	NR_002744	71 bp	19.0 ± 0.6
SNORD38B	203901	NR_001457	69 bp	19.1 ± 1.0
miR-155	204308	HC_040174	23 bp	19.6 ± 0.8
Intermediate expression (Cq: 20-25)				
miR-191	204306	HC_040210	23 bp	21.9 ± 0.9
miR-103	204063	HC_885819	23 bp	22.2 ± 0.9
miR-142-5p	204722	HC_040142	21 bp	22.6 ± 1.6
Low expression (Cq>25)				
miR-223	204256	HC_040280	22 bp	25.9 ± 1.5
miR-26b	204172	HC_885768	21 bp	26.5 ± 2.7

[1] Catalogue numbers from Exiqon.
[2] PCR amplicon size for all targets is between 60 and 70 bp on agarose gel.
[3] Values represent mean Cq values (with standard deviation) in cells from three donors.

Effect of RNA degradation

RNA (extracted with *mir*Vana) was diluted to 100 ng/µL and exposed to 90°C. At t=0 and after 15 min, 30 min, 45 min, 1 h, 1.25 h, 1.5 h, 2 h, 2.5 h, and 3 h of heat exposure, RNA quality was determined (StdSense chips). Complementary DNA was synthesized on mRNA (SuperScript III RT) and microRNA (Universal cDNA kit, Exiqon). As read-out for yields, PCR signals of six mRNA transcripts (Table 1) and eight microRNAs (Table 2) were assessed by qPCR. For each marker the PCR signal per time point was statistically compared to the mean signal of the first two time points (t=0, t=15 min). In addition, the ratios of individual mRNA transcripts and the mean signal of two reference transcripts were calculated. Similarly, ratios for individual microRNAs and the mean signal of four reference microRNAs were calculated.

We investigated the effect of RNA degradation on PCR signals for four different mRNA transcripts, while varying the amplicon size for each transcript. The reverse primer was selected to target a fixed sequence within the coding sequence of GAPDH (5'-TGC TGT AGC CAA ATT CGT TG-3'), TGF-β1 (5'-GTC CTT GCG GAA GTC AAT GT-3'), CD25 (5'-GAC GAG GCA GGA AGT CTC AC-3'), and FoxP3 (5'-CAG GTG GCA GGA TGG TTT C-3'). The location of the forward primer was varied to obtain primer pairs that generated PCR product of around 50 bp, 100 bp, 200 bp or 400 bp.

Statistics

Experiments were performed with PBL blasts from three or more donors, and with uncultured PBMC from three additional donors. Results are presented as means ± standard deviation, unless stated otherwise. Graphs were made in GraphPad Prism 5 (La Jolla, CA). Differences between groups were tested by independent t-tests using SPSS Inc software version 20 (IBM Corp, Armonk NY, US). $P<0.05$ was considered a statistically significant difference.

Results

Cell storage and thawing

The effect on RNA quality of freezing and thawing cells with various protocols, combined with the effect of storage of cells in several RNA-preserving reagents, was investigated. Freshly obtained cells were directly processed for RNA extraction or stored at -20°C in various RNA-preserving reagents (Figure 1A). RNA quality decreased with 0.6 points on the RQI scale ($P<0.05$) after storage in RNA*later* (R) or TRIzol (T) (Figure 1B, open bars). Accordingly, qPCR signal with cDNA synthesized from the RNA samples was at least two-fold lower after storage in RNA*later* or TRIzol, although this difference did not reach statistical significance (Figure 1C).

Freezing the cells in DMSO and thawing them with either a standard protocol (Figure 1B-C, first grey bar) or with the benzonase protocol (first black bar) did not negatively affect RNA quality compared to freshly-processed cells (first open bar; P=NS). Benzonase is reported to prevent cell clumping when PBMC are thawed [32]. No adverse effect on RNA quality was observed when benzonase was included in the thawing procedure (Figure 1B-C: first black bar), compared with the standard thawing protocol (first grey bar; P=NS).

Storage of thawed cells for 6 days in the freezer in TRIzol (T) (Figure 1C: third grey and third black bar) had a slightly higher RNA-preserving effect for downstream qPCR application than storage in RNA*later* (R) (second grey and second black bar).

RNA extraction protocols

Various methods for extraction of RNA from PBL blasts were compared. The average RNA yield for all 5 methods tested was 2.8 ± 0.8 µg per $1x10^6$ PBL blasts, and the average RQI was 9.7 ± 0.3. Yield and quality of RNA did not differ between extraction procedures (Figure 2A). Use of the TRIzol and RNA-Bee procedures in our hands led to suboptimal RNA yields in 50% of cases due to complete or partial loss (0.03 to 0.7 µg per $1x10^6$ cells) of the RNA pellet after precipitation. As an additional criterion for considering suitability of the procedures, qPCR signals of high-abundance (β-actin, green bars in Figure 2B), medium-abundance (TGF-β1, red bars), and low-abundance transcripts (CD25 and FoxP3, blue bars) were determined on a fixed amount of RNA (200 ng). Signals with NucleoSpin columns were similar to signals obtained with *mir*Vana columns, but were on average 1.5 to 1.7 times higher than those obtained with RNeasy, TRIzol, and RNA-Bee (Figure 2B, P=NS). PCR signals for high-abundance (miR-155, green bars in Figure 2C), medium-abundance (miR-142-5p, red bars), and low-abundance (miR-223, blue bars) microRNAs were analyzed after RNA extraction. On average, *mir*Vana and RNA-Bee gave highest signals per ng of RNA, followed closely by NucleoSpin (1.4-1.5 fold lower). We found the NucleoSpin columns the most convenient procedure to work with.

Efficiency of RNA extraction protocols was also tested on mononuclear cells obtained directly from peripheral blood (n=3), without subsequent culturing. The average RNA yield for the RNA extraction procedures was 0.9 ± 0.4 µg per $1x10^6$ cells and the average RQI was 8.8 ± 0.8. Use of *mir*Vana gave the highest yield of RNA per million PBMC, but this was not significantly different from yield obtained with the other extraction procedures (Figure 2D, open bars). Although all procedures gave high-quality RNA with an RQI>8, the use of RNeasy and *mir*Vana gave RNA that on average had the lowest RQI (Figure 2D, black bars). Results of qPCR signals on a fixed amount of RNA (200 ng) are shown for the column-based extraction protocols (RNeasy, *mir*Vana, NucleoSpin) (Figure 2E). Signals obtained for the mRNA transcripts were higher for NucleoSpin compared with *mir*Vana. Signals for microRNAs were highest with *mir*Vana.

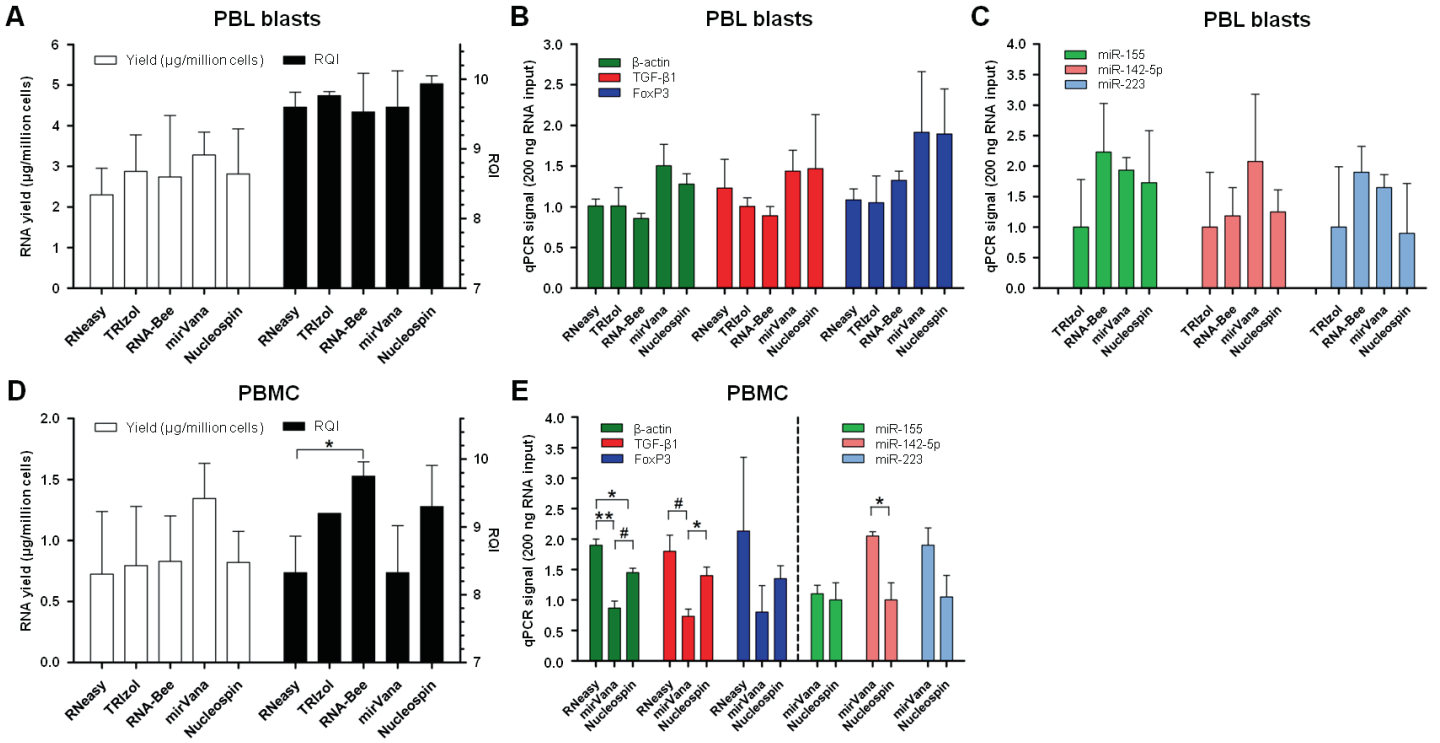


Figure 2. RNA extraction protocols. (A) Yield per million PBL blasts (open bars) and integrity (black bars) of RNA obtained with five different extraction protocols. (B) PCR signals in cDNA synthesized from 200 ng total RNA of PBL blasts on high-abundance (green bars, β-actin), medium-abundance (red bars, TGF-β1), and low-abundance (blue bars, FoxP3) mRNA transcripts, following five RNA extraction protocols. (C) PCR signals for high-abundance (green bars, miR-155), medium-abundance (red bars, miR-142-5p), and low-abundance (blue bars, miR-223) microRNAs. Signals for TRIzol were set to 1. (D) Yield per million PBMC (open bars) and integrity (black bars) of RNA obtained with five different extraction protocols. (E) PCR signals in cDNA synthesized from 200 ng total RNA of PBMC obtained directly from blood. *, P<0.05. #, P<0.01. **, P<0.001.

cDNA synthesis protocols

Four protocols for synthesizing cDNA from RNA (extracted with *mir*Vana) were tested. The same mRNA transcripts were analyzed as those in the RNA extraction experiment above. With RNA from PBL blasts, the order of cDNA efficiency from highest to lowest was SuperScript III > BioScript > RevertAid > AMV-RT (Figure 3, upper panel). This was observed for high-, medium-, and low-abundance transcripts. With both 100 ng and 1500 ng of RNA as input for cDNA synthesis, the use of the SuperScript III protocol led to 2- to 32-fold higher yields of cDNA, as measured by qPCR, compared to the other protocols (Figure 3, upper panel).

Experiments were repeated with RNA from PBMC obtained directly from blood (Figure 3, lower panel). Results were comparable with the results obtained with PBL blasts. The use of SuperScript III resulted into the highest yields of cDNA, whereas AMV gave the lowest cDNA yields. BioScript and RevertAid did not significantly differ in the amount of cDNA yield that they rendered.

Effect of RNA degradation

RNA degradation was induced by exposing total RNA from PBL blasts to 90°C for various lengths of time. Over a period of 3 h the RQI decreased from 10.0 to 1.9, whereas the ratio of 28S rRNA (upper band) to 18S rRNA (lower band) decreased from 1.8 to 0.18 (Figure 4A). When RNA had reached an RQI of 7.0, the signal for all mRNA transcripts tested, irrespective of transcript abundance, was already at least two-fold lower than the mean signal of the first two time points (Figure 4B; $P<0.05$). At an RQI of 5.4 or lower, PCR signals for mRNA had 3- to 5-fold decreased ($P<0.01$). Thus, with decreasing RNA quality the amplification signals declined rapidly. In contrast, all microRNAs tested remained relatively stable (Figure 4C), except small nucleolar RNA SNORD49 which showed a decline of PCR signals at an RQI of 4.7 or lower (2- to 3-fold, $P<0.01$).

The ratio of each of three mRNA transcripts (TGF-β1, CD25, FoxP3) to reference gene signals was not significantly altered at an RQI as low as 4.2 (Figure 4D). Below this point, CD25 to reference ratios significantly deviated (>2-fold increase, $P<0.05$) from the ratio observed at the first time point (RQI=10.0). In contrast, ratio of each of four microRNAs tested (miR-155, miR-142-5p, miR-223, miR-26b) and the mean signal of reference microRNAs remained stable even in severely degraded RNA with an RQI as low as 1.9 (Figure 4E).

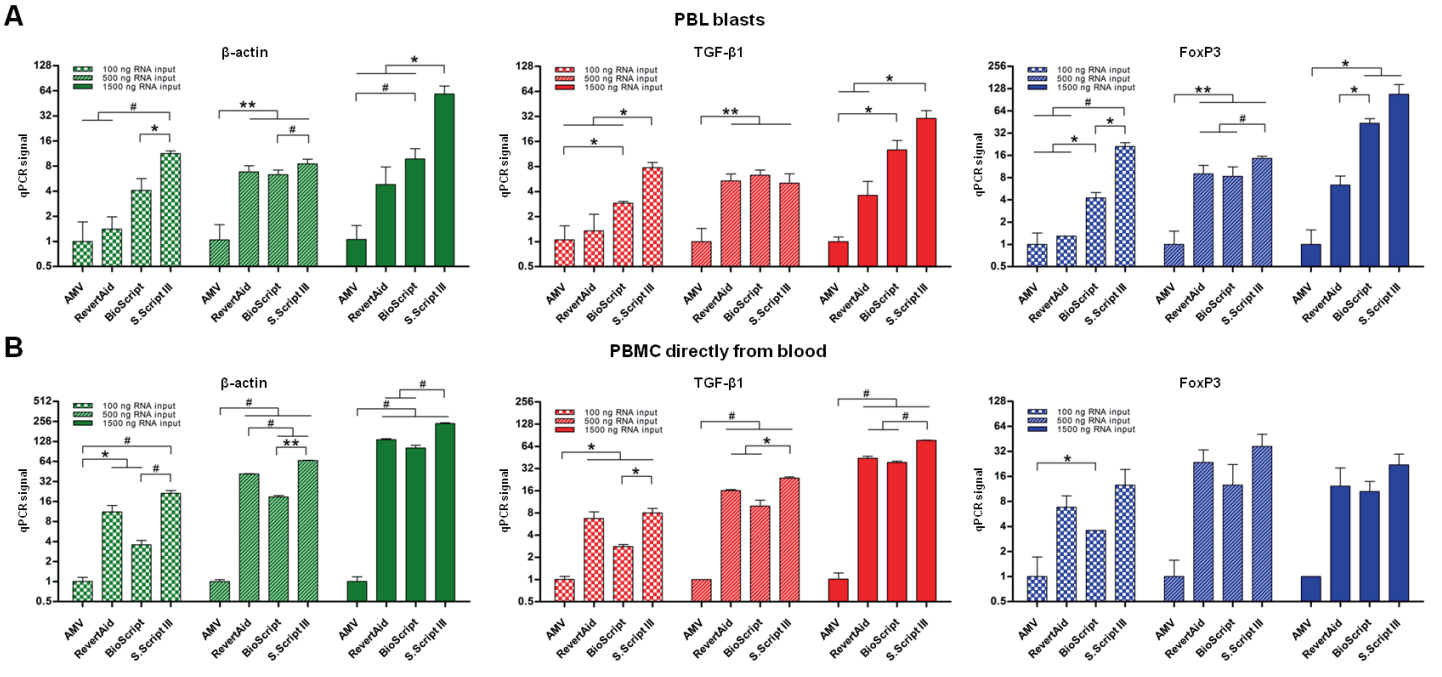


Figure 3. cDNA synthesis protocols. Relative yields of cDNA, following four cDNA synthesis protocols (synthesized from *mir*Vana-extracted RNA), were calculated from PCR signals obtained for high-abundance (green bars, β-actin), medium-abundance (red bars, TGF-β1), and low-abundance (blue bars, FoxP3) mRNA transcripts. This was investigated with RNA from PBL blasts (upper panel) and from PBMC obtained directly from blood (lower panel). Signals for AMV were set to 1. *, P<0.05. #, P<0.01. **, P<0.001.

The effect of RNA degradation on qPCR signals for mRNAs and microRNAs was also tested with mononuclear cells obtained directly from peripheral blood. Similar results were seen as with the RNA from the PBL blasts. Below an RQI of 5, amplification signals for all mRNA transcripts tested had significantly declined (Figure 4F; $P<0.05$). Signals for almost all microRNAs tested, except small nucleolar RNAs 38B and 49A, remained stable at an RQI as low as 3.5 (Figure 4G).

RNA degradation negatively affects PCR signals irrespective of mRNA amplicon size

It is expected that in degraded RNA samples, larger PCR amplicons cease amplification sooner than smaller amplicons. We examined the possibility that amplicon size differences account for the sustained stability of microRNA molecules vis-à-vis increasing degradation of RNA. Amplicon size of each of the miRNA targets tested was between 60 and 70 bp (examined on agarose gel, data not shown). For high, medium, and low-abundance mRNA transcripts, we varied the size of the PCR amplicon between 50 and 400 bp, and investigated the effect of RNA degradation on PCR signals. For the four transcripts tested, larger amplicon sizes resulted in quicker loss of PCR signal upon RNA degradation (Figure 5). However, with an mRNA amplicon size of between 45 and 60 bp (Figure 5, open triangles) the extent of loss of PCR signals remained significantly higher compared to that of the microRNA targets (Figure 4C,G).

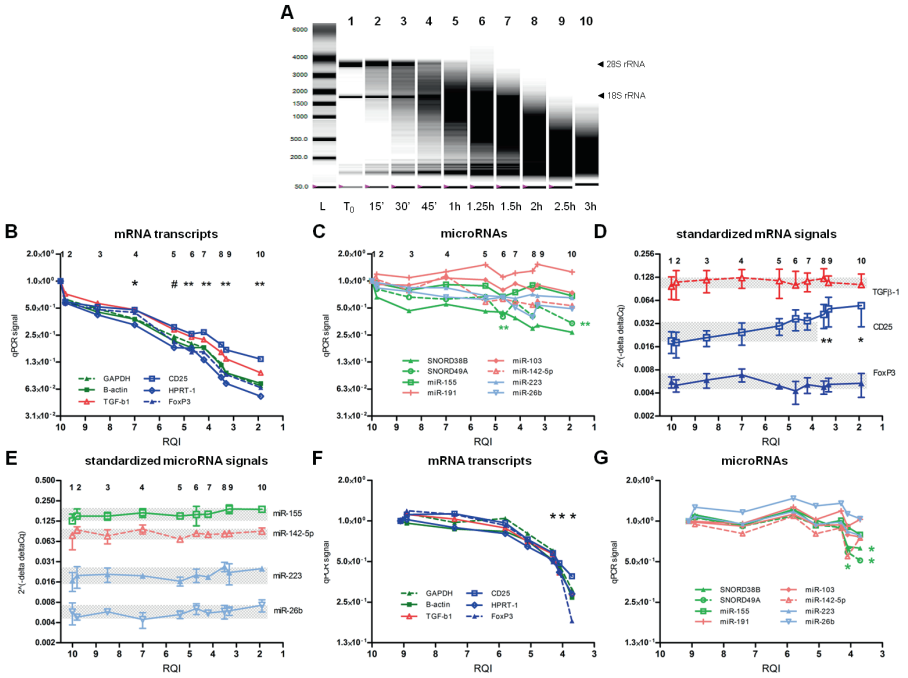


Figure 4. Effect of RNA degradation. RNA was extracted from PBL blasts, and RNA degradation was induced by exposure to 90°C for times indicated. (A) Representative picture of RNA electropherogram showing impact of heat exposure on RNA integrity. (B) RQI, as an indicator of RNA quality, was analyzed (x-axis). The numbers 1 to 10 at the top of the graph indicate time points corresponding with the numbers in the RNA electropherogram. Messenger RNA levels were assessed by qPCR, targeting high-abundance (green), medium-abundance (red), and low-abundance (blue) markers. For each marker, the mean PCR signal at t=0 was set to 1. The graph shows means of four experiments. *, P<0.05; #, P<0.01; **, P<0.005 versus mean of the first two time points. (C) MicroRNA levels were assessed by qPCR, targeting high-abundance (green), medium-abundance (red), and low-abundance (blue) markers. The graph shows means of three experiments. **, P<0.005 versus mean of first two time points for SNORD49A. (D-E) PCR signals for individual mRNA transcripts and microRNAs were corrected for reference markers using the 2-ΔΔCq method. The upper and lower boundaries of the shaded areas indicate maximum and minimum values, respectively. *, P<0.05 versus first time point. (F-G) Experiments investigating the effect of RNA degradation on PCR signals were repeated with RNA from PBMC obtained directly from blood. Messenger RNA transcripts (F) and microRNAs (G) were analyzed. *, P<0.05 versus mean of the first two time points (F: each mRNA transcript; G: only for SNORD38B and SNORD49A).

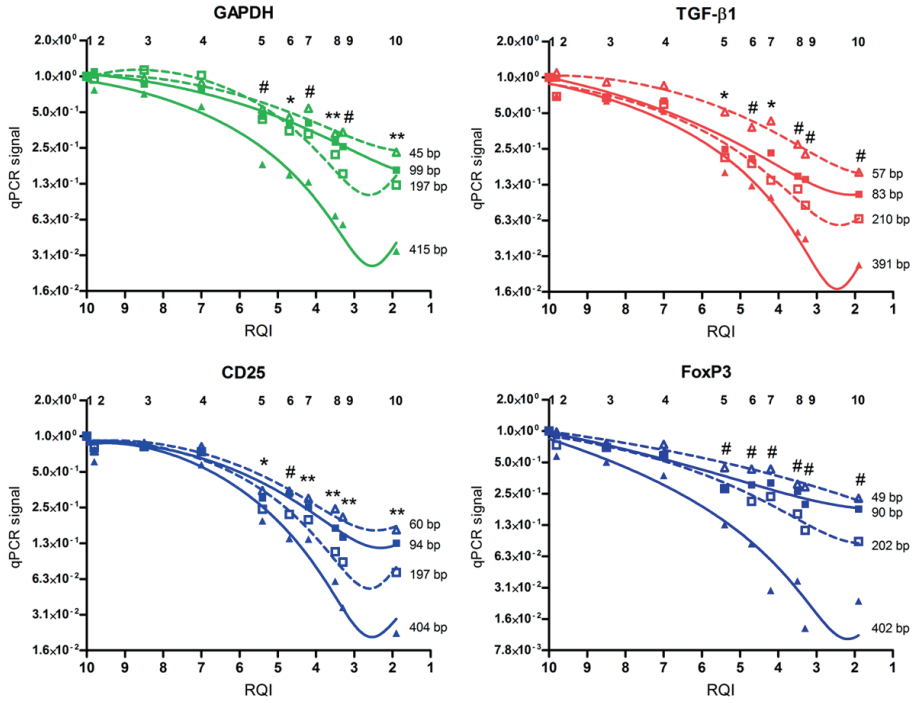


Figure 5. RNA degradation negatively affects PCR signals irrespective of mRNA amplicon size. RNA from PBL blasts was degraded by exposure to 90°C. PCR signals for high-abundance (green), medium-abundance (red), and low-abundance (blue) mRNA transcripts were assessed. For each transcript, size of the amplicon was varied (50 bp, 100 bp, 200 bp or 400 bp). For each condition, the mean PCR signal at t=0 was set to 1. Graph represents means of four experiments, with third-order polynomial lines drawn through the data points. *, $P<0.05$; #, $P<0.01$; **, $P<0.001$ versus mean of first two time points for the smallest amplicon.

Discussion

Molecular techniques are vital for identification and validation of markers at the RNA level. Novel molecular markers are needed for improving current conventional diagnostic practice. Faced with the challenge of small sizes of clinical samples, researchers optimize their protocols for isolation of mRNA and microRNA. Here we describe an optimized protocol for RNA extraction, cDNA synthesis, and storage of peripheral blood cells for obtaining high yields of high quality mRNA and microRNA.

RNA integrity was maintained upon freezing and thawing cells, following conventional procedures, when compared to RNA from freshly isolated cells. This finding shows that RNA preserving agents are not mandatory when workup

needs to be postponed, and enables retrospective RNA analysis of cells that were cryopreserved for cellular assays. We used the RNA quality index from nanochips to determine RNA quality. This is a suitable means to reliably assess RNA integrity, as was also reported by others [33;40]. In addition, we compared two commonly used protocols for thawing cells, one of which incorporates benzonase-nuclease. Benzonase has no negative effect on the preservation of high-quality RNA, and has the additional benefit of preventing clumping of PBMC during thawing [32]. In daily laboratory practice, functional cellular tests are often performed with PBMC recovered from cryopreservation. With the additional goal of molecular analyses, one may obviously proceed with RNA extraction from thawed cells immediately. Alternatively, the thawed cells may be stored again for RNA extraction on a later day, for instance when functional tests need to be performed and interpreted first. In that case, TRIzol (at -20°C) is recommended, since its use leads to preservation of RNA integrity, as reflected by RNA quality index and qPCR signal. Since storage time of cell pellets at -20°C in our experiments did not exceed 6 days, a negative impact on RNA quality of extended cell storage cannot be ruled out.

In terms of RNA yield, quality, qPCR signals per ng of total RNA, and convenience, the use of NucleoSpin and *mir*Vana columns is preferable. TRIzol and RNA-Bee, which have been used in many studies for RNA extraction, offer rapid procedures but showed a relatively high failure rate in our laboratory, due to (partial) loss of the RNA pellet in the precipitation step. Four of the 5 RNA extraction procedures tested were comparable in the amount of PCR signal generated for microRNA targets per ng of total RNA. RNeasy spin columns are not suitable for efficient microRNAs extraction (data not shown and [41]). The miRNeasy kit may better suit this goal, but it was not available at the start of our tests.

Of the cDNA synthesis protocols that we tested, SuperScript III resulted in the highest yield of cDNA. This is in line with the recommendation of using a thermostable reverse transcriptase enzyme for cDNA synthesis [42]. Of note, SuperScript III was considerably superior to the first generation of SuperScript (data not shown) that we reported on in a previous study [43].

Finally, we showed that RNA degradation has a major negative impact on mRNA expression levels, which were determined by qPCR. This finding is in line with the highly negative correlation found between the RNA integrity number (RNA quality index or RQI in our study) and the Cq value from the RT-PCR assay in earlier studies

[44-46]. After correction of individual mRNA transcripts to reference genes, the ratio was not significantly altered with an RQI as low as 4.2. Below this value, the signal to reference ratio was significantly altered for one of the three transcripts studied, indicating different rates of degradation for different mRNA molecules. We recommend that the RQI of RNA samples should be 4.2 or higher for reliable quantification of standardized transcript levels. In another study, an index of 5 or higher was regarded as suitable for downstream qPCR application [46]. In contrast, we observed that individual microRNAs, and especially microRNA signals standardized to reference microRNA signals, remained stable even in severely degraded RNA samples (having an RQI as low as 1.9). In an earlier study, microRNAs did not suffer from degradation during storage of lymphocytes at -80°C [41], but the quality of the RNA was not investigated in that study. Even with mRNA amplicons of approximately 50 bp we observed significant loss of PCR signals at an RQI of 5.4 or lower. This shows that the increased stability of microRNAs, compared to that of mRNAs, cannot be explained by smaller amplicon sizes. MicroRNAs remain stable in different human bodily fluids [47;48]. Our observations and those from others hold promise for reliable quantification of microRNA expression in specimens that are prone to RNA degradation such as blood plasma, serum, and urine.

In summary, we described an optimized protocol for extracting and preserving RNA molecules from blood cells. The results serve to enhance sensitivity of mRNA and microRNA expression assessment for clinical purposes.

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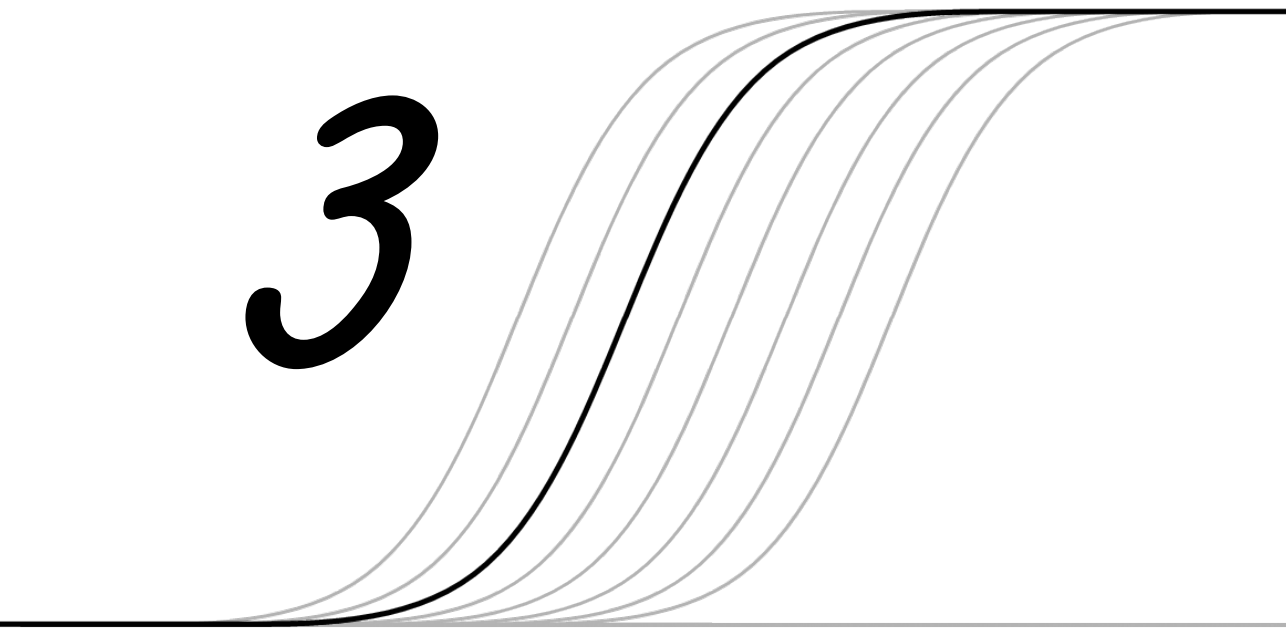
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Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment

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Abstract

Background. Steroid resistant acute rejection is a risk factor for inferior renal allograft outcome.

Methods. From 873 kidney transplant recipients (1995-2005) 108 patients with a first rejection episode were selected for study using strict inclusion criteria and clinical end point definition. We aimed to predict response to corticosteroid treatment using gene expression of 65 transcripts. These reflect cytokines, chemokines, and surface and activation markers of various cell types including T cells, macrophages, B cells, and granulocytes. Steroid resistance (40% of the patients) was defined as requirement for anti-thymocyte globulin treatment within two weeks after corticosteroid treatment.

Results. None of the clinical and histomorphologic parameters showed a significant association with response to treatment. Univariate logistic regression analysis resulted in eleven mRNA markers, including T cell-related transcripts CD25, LAG-3, Granzyme B, and IL-10, and macrophage-specific transcripts MannoseR and S100A9, which significantly discriminated steroid resistant from steroid responsive rejections ($P<0.05$). In multivariate logistic regression the combination of T cell activation markers CD25:CD3e ratio (OR=8.7, CI: 2.4-31.2) and LAG-3 (OR=3.3, CI: 1.4-7.7) represented the best predictive model for steroid response ($P<0.0001$). Specificity and sensitivity were 78% and 60%, respectively. After internal stratified ten-fold cross-validation the model remained significant. Inclusion of clinical variables into the model with molecular variables did not enhance prediction.

Conclusions. Differences in intragraft expression profiles reflect variability in the response to anti-rejection treatment. In acute rejection, molecular markers, particularly those reflecting T cell activation, offer superior prognostic value compared to conventional parameters.

Introduction

After kidney transplantation, the occurrence of acute rejection is one of the most important risk factors for adverse graft outcome [1-3]. Acute rejection is a complex process in which infiltrating cells of the host immune system cause injury to the allograft. The Banff classification [4-7] represents histomorphologic criteria to determine the severity of the acute rejection based on the site and degree of inflammation in the renal allograft biopsy.

A main parameter determining graft outcome is therapy sensitivity of the acute rejection episode [3;8;9]. In most cases a first rejection episode can be adequately treated with high-dose corticosteroids. However, in approximately 30% of the patients the rejection episode cannot be reversed with corticosteroid therapy alone [2;8;10]. In these cases of steroid resistance the patient requires more rigorous therapy with anti-thymocyte globulin (ATG). Acute rejection episodes leading to incomplete restoration of graft function upon treatment may lead to progression of chronic damage to the graft, and have a detrimental effect on graft outcome [8;9;11].

Once the diagnosis acute rejection has been made, it is difficult to predict the response to anti-rejection treatment using clinical parameters and histopathologic assessment of the biopsy. Availability of molecular markers could help to assess which patients will respond to steroid treatment and which patients are in need of immediate ATG treatment. Indeed, several studies have described cellular and molecular markers in the graft tissue, of which the expression was found to be associated with therapy resistance (reviewed in [11]). Sarwal and colleagues found increased expression of B cell-, cytotoxic T cell-, and natural killer cell signatures in steroid resistant acute rejection in renal allografts of children [12]. In more recent studies investigators have not been able to confirm that the presence of intragraft B cells, on basis of CD20 expression, is associated with therapy response and/or graft function after rejection [13-16]. It has further been shown that high FasL mRNA expression [17] and dense granulysin staining [18] in renal allograft biopsies, as well as low FoxP3 expression in urinary sediments [19], were associates of steroid resistance. The presence of macrophages during acute rejection within the graft was also found to be associated with steroid resistance [20-23]. Together, these results give an indication of the complexity in accurately predicting the response of kidney transplant patients to anti-rejection treatment with steroids.

In the current study we investigated a broad panel of immunological markers, measured by quantitative PCR (qPCR), in a large cohort of patients suffering from a first acute rejection episode. We aimed to predict response to anti-rejection treatment on the basis of molecular markers in the allograft biopsy.

Materials and methods

Patients

We reviewed all 873 patients who received a renal allograft in our center between 1995 and 2005. All patients who had suffered from histologically proven acute rejection were included in the study. Further criteria for this study were: 1) only first rejection episode biopsy specimens were included; 2) only patients who received corticosteroids as anti-rejection treatment were included; 3) patients who received ATG as induction therapy were excluded; 4) all studied biopsy cores had been obtained prior to the start of anti-rejection treatment. Frozen material, for RNA extraction and quantitative PCR (qPCR), was available from 108 patients who met the criteria. Nine samples were excluded from analysis: 1 sample due to RNA degradation and 8 samples due to too low RNA yield from the biopsy. In total, 99 patients were included in the statistical analysis (Figure 1).

Seventy patients (70.7%) received no induction therapy and 29 patients (29.3%) received induction therapy with an IL-2 receptor blocker (Daclizumab). Maintenance immunosuppressive medication consisted of a calcineurin inhibitor (Cyclosporine A or Tacrolimus) and prednisone. Fifty-six patients (56.6%) also received an antimetabolite (Mycophenolate Mofetil) as part of the maintenance immunosuppression. Immunosuppression for the treatment of acute rejection consisted of pulse therapy for 3 days with a 1-g bolus of intravenous methylprednisolone daily.

Definition of primary clinical endpoint

The primary clinical endpoint was response to anti-rejection treatment with methylprednisolone. Steroid resistant acute rejection was defined as lack of clinical response (serum creatinine level did not return to within 120% of the pre-rejection baseline value [16;24;25]) to steroid pulse therapy, and a requirement for anti-thymocyte globulin treatment within 14 days after the start of the steroid therapy. In the steroid

resistant group, the time between initiation of steroid treatment and ATG treatment was on average 7.3 ± 3.5 days (in 82% of the patients an interval of ≥ 5 days [25;26]). In seven cases, ATG was given within 5 days after initiation of steroid treatment. Reason was insufficient decline in serum creatinine, stagnation of the decline above the 120% level, or a rising serum creatinine level despite the steroid treatment. A total of 59 patients with steroid responsive acute rejection and 40 patients with steroid resistant acute rejection were evaluated in the study (Figure 1).

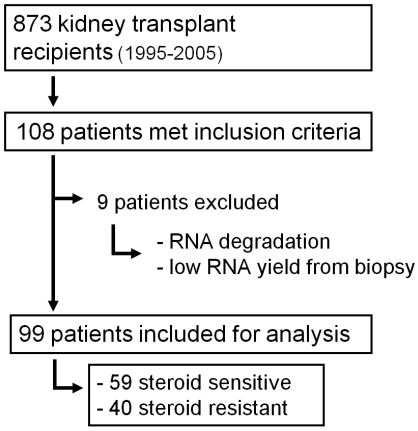


Figure 1. Flow diagram of the sample selection. All patients who received a renal allograft in our center between 1995 and 2005 were reviewed (n=873). All patients who had suffered from histologically proven acute rejection were considered for study. Further selection criteria led to 59 patients with steroid responsive acute rejection and 40 patients with steroid resistant acute rejection for investigation in the study.

Biopsy samples

At least two biopsy cores were collected from each patient. One core was formalin fixed, embedded in paraffin, and used for histochemical stainings. Three independent pathologists (I.B., N.G. and M.G.) blinded to the clinical data of the patients and outcome of the acute rejection revised individual Banff lesions on all biopsies according to Banff 2011 criteria [7]. The pathologists highly agreed on acute and chronic changes and reached a satisfactory inter-observer agreement. The second biopsy core was immediately snap frozen in liquid nitrogen and stored at -80°C. Immunofluorescent staining for C4d was performed on frozen sections from 93 of the 99 (94%) patients with mouse anti-human C4d antibody, as described previously [13;27]. Biopsies with diffuse C4d+ staining were assessed for histomorphologic characteristics indicative of a possible antibody-mediated rejection.

RNA extraction and quality assessment

Eight to ten 10-µm sections were cut with a cryomicrotome from each snap frozen biopsy core. Total RNA from the renal tissue was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA quality was determined on Nano LabChips with the Aligent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA concentration was 2.75 ± 1.13 µg, mean 28S:18S ratio was 2.1 ± 0.05, and mean RIN was 8.00 ± 0.98.

Real-time quantitative PCR analysis

An average of 0.89 ± 0.19 µg of RNA from each frozen biopsy core was transcribed into cDNA, according to a previously described protocol [28]. To prevent amplification of genomic DNA, forward and reverse primers for each transcript targeted separate exons, spanning at least one intron with a size of 800 bp or more. Primer sequences can be provided on request. All primer sets were tested before use on control cDNA and genomic DNA to ensure optimal performance and no amplification of genomic DNA. Reactions and PCR conditions have been described previously [28]. All PCR efficiencies were between 90% - 110%. Relative gene expression levels were determined on the basis of a standard curve (in duplicate) of five serial dilution points of reference cDNA (qPCR Human Reference Total RNA, Clontech, France). Differences in mRNA expression levels were normalized either to the geometric mean signal of the reference genes GAPDH, 18S rRNA and β-actin (inter-correlation ranged between 0.88 and 0.95), or normalized to the signal of CD3e. For each marker reference-gene-normalized mRNA levels and CD3e-normalized mRNA levels were calculated.

Statistical analysis

Comparison of categorical data between patient groups was evaluated using Pearson chi-square tests. With respect to steroid response of the acute rejection, the predictive value of individual mRNA markers and of clinical and histomorphologic parameters was analyzed using univariate logistic regression analysis. Multivariate logistic regression analysis was used to test if multiple markers combined could provide a model with a higher predictive value. Markers were included stepwise in the model based on the likelihood ratio statistics. The obtained candidate marker set was validated using stratified ten-fold cross-validation. All logistic regression analyses were performed using a classification cutoff of 0.5, and logistic regression data is presented as odds ratios (OR) with 95% confidence interval (CI). Receiver operating

characteristics (ROC) analysis was performed to evaluate the predictive value of multivariate parameters.

All statistical tests were two-sided and P values less than 0.05 were considered as significant. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., version 17.0.2, Chicago, IL, US).

Results

Demographics and clinical data

Demographic and clinical characteristics were not significantly different between the steroid responsive (n=59) and steroid resistant (n=40) patients (Table 1). No significant differences were found in rejection severity (P=0.64, Table 2) and C4d positivity (P=0.40, Table 1). In the 13 biopsies showing C4d positivity we investigated histomorphologic characteristics (granulocytic infiltrate, microthrombi, peritubular capillaritis, and necrotizing vascular rejection) indicative of a possible antibody-mediated rejection. None of these biopsies showed such features.

The steroid resistant group had significantly higher serum creatinine levels at 6 months (P=0.008) and 12 months (P=0.008) after the transplantation compared with the steroid responsive group. The graft survival after 12 years was 60.4% for the steroid resistant group and 81.9% for the steroid responsive group.

Table 1. Demographic and clinical data of patients with steroid responsive and steroid resistant acute rejection.

Variable	Steroid responsive (N=59)	Steroid resistant (N=40)	P value
Patient age			0.69
≥ 50 years	26 (44.1%)	16 (40.0%)	
Patient gender			0.89
Female	20 (33.9%)	13 (32.5%)	
Donor age			0.35
≥ 50 years	21 (35.6%)	18 (45.0%)	
Donor gender			0.66
Female	38 (64.4%)	24 (60.0%)	
Year of transplantation			0.15
1995 through 1999	36 (61.0%)	30 (75.0%)	
2000 through 2005	23 (39.0%)	10 (25.0%)	
Donor type			0.30
Living	11 (18.6%)	11 (27.5%)	
Post mortal	48 (81.4%)	29 (72.5%)	
HLA-A matching			0.75
≥ 1 mismatch	38 (64.4%)	27 (67.5%)	
HLA-B matching			0.89
≥ 1 mismatch	45 (76.3%)	31 (77.5%)	
HLA-DR matching			0.36
≥ 1 mismatch	36 (61.0%)	28 (70.0%)	
Virtual PRA			0.74
Immunized (6-100%)	17 (29.3%)	13 (32.5%)	
Cold ischemia time			0.46
> 18 hours	32 (66.7%)	26 (74.3%)	
Induction therapy			0.44
Daclizumab	19 (32.2%)	10 (25.0%)	
None	40 (67.8%)	30 (75.0%)	
Maintenance therapy			0.28
Double therapy	23 (39.0%)	20 (50.0%)	
Triple therapy	36 (61.0%)	20 (50.0%)	
Delayed graft function			0.18
Yes	17 (29.3%)	7 (17.5%)	
Rejection time			0.80
<3 months post Tx	57 (96.6%)	39 (97.5%)	
3-6 months post Tx	2 (3.4%)	1 (2.5%)	
C4d staining			0.40
Positive	6 (11.3%)	7 (17.5%)	
Vascular rejection			0.25
Yes	17 (28.8%)	16 (40.0%)	

PRA, panel reactive antibodies; Tx, transplantation

Table 2. Diagnostic categories according to Banff 2011 criteria of patients with steroid responsive and steroid resistant acute rejection [1].

Diagnostic category	Steroid responsive (N=54)	Steroid resistant (N=36)
Borderline changes	25 (46.3%)	12 (33.3%)
TCMR grade IA	6 (11.1%)	2 (5.6%)
TCMR grade IB	6 (11.1%)	6 (16.7%)
TCMR grade IIA	12 (22.2%)	10 (27.8%)
TCMR grade IIB	2 (3.7%)	3 (8.3%)
TCMR grade III	3 (5.6%)	3 (8.3%)

[1] No significant differences were found between groups (P=0.64). In each group, three biopsies did not contain sufficient cortex to determine a Banff score. Three additional patients had a clinical rejection (with i- and t-scores of 0). TCMR, T-cell mediated rejection

Evaluation of predictive value of clinical and molecular variables with respect to steroid response

Univariate analysis

We questioned whether differences in the expression of immunomarkers between the treatment response groups could predict steroid-refractory acute rejection. No significant differences in the RNA concentration, purity, and quality between the groups were found (data not shown). Expression of 73 markers was quantified by qPCR (see Table S1). Eight markers were excluded from further analysis due to an inferior melt curve in the PCR assay or due to low expression in all biopsies.

We tested whether clinical and histomorphologic parameters influenced the response to steroid treatment. None of these parameters had a significant relationship with steroid response in univariate logistic regression analysis (Table 3). We tested whether mRNA expression of molecular markers can predict response to corticosteroid treatment by using univariate logistic regression analysis. Eleven markers had an odds ratio (OR) below 0.5 or above 2.0 (Table 4).

Table 3. Univariate analysis of predictive value of clinical and histomorphologic parameters for steroid response.

Variable	Steroid responsive (N=59)	Steroid resistant (N=40)	OR	95% CI	P value
Clinical parameters					
Patient age (years)	47.4 (12.81)	45.8 (12.88)	0.99	0.96 – 1.02	0.54
Donor age (years)	44.1 (14.59)	46.7 (13.14)	1.01	0.98 – 1.04	0.38
HLA-AB mismatches	1.71 (1.05)	1.73 (1.04)	1.01	0.69 – 1.49	0.95
HLA-DR mismatches	0.68 (0.60)	0.73 (0.51)	1.16	0.57 – 2.39	0.68
Virtual PRA (%)	11.3 (22.29)	17.1 (29.27)	1.01	0.99 – 1.03	0.27
Cold ischemia time (hours)	20.4 (8.23)	21.9 (11.12)	1.02	0.97 – 1.07	0.47
Rejection time (days)	28.4 (32.24)	21.7 (21.97)	0.99	0.98 – 1.01	0.28
Year of transplantation (2000-2005)	39.0%	25.0%	0.52	0.22 – 1.27	0.15
Delayed graft function (yes)	29.3%	17.5%	0.51	0.19 – 1.38	0.19
Histomorphologic parameters					
Interstitial inflammation	51.8%	51.4%	0.98	0.43 – 2.26	0.97
Tubulitis	44.6%	59.5%	1.82	0.78 – 4.22	0.16
Intimal arteritis	32.1%	48.5%	1.99	0.82 – 4.87	0.13
Interstitial fibrosis	39.3%	37.8%	0.94	0.40 – 2.21	0.89
Tubular atrophy	44.6%	40.5%	0.85	0.36 – 1.96	0.70
C4d positivity	11.3%	17.5%	1.66	0.51 – 5.40	0.40

All clinical parameters were included in the univariate logistic regression analysis as continuous variables, except for year of transplantation (dichotomized for early period [1995-1999] and late period [2000-2005]) and delayed graft function (dichotomized for presence and absence). All histomorphologic parameters were dichotomized with a 1/0 parameter indicating presence or absence of the feature; except for interstitial inflammation and tubulitis, which were dichotomized for mild (0, 1) and severe (2, 3). Values are expressed as mean ± SD, or as percentage (presence or severe). OR, odds ratio; CI, confidence interval; PRA, panel reactive antibodies

Table 4. Univariate and multivariate logistic regression analysis of predictive value of molecular markers for steroid response.

Variable	Specificity	Sensitivity	Positive PV	Negative PV	OR	95% CI	P value
Univariate analysis							
CD25:CD3e	86.4%	37.5%	65.5%	67.1%	5.76	1.76 – 18.80	0.004
CD25	83.1%	40.0%	61.5%	67.1%	3.70	1.41 – 9.70	0.008
MannoseR	81.4%	30.0%	52.2%	63.2%	4.67	1.42 – 15.36	0.011
S100A9	83.1%	42.5%	63.0%	68.1%	2.33	1.22 – 4.44	0.011
LAG-3:CD3e	81.4%	22.5%	45.0%	60.8%	3.80	1.30 – 11.15	0.015
CXCL13:CD3e	81.4%	37.5%	57.7%	65.8%	2.06	1.10 – 3.83	0.023
LAG-3	84.7%	25.0%	52.6%	62.5%	2.43	1.12 – 5.26	0.025
RORγT:CD3e	84.7%	25.0%	52.6%	62.5%	0.48	0.24 – 0.94	0.032
IL-10	83.1%	25.0%	50.0%	62.0%	3.02	1.07 – 8.63	0.038
STAT6:CD3e	88.1%	20.0%	53.3%	61.9%	0.28	0.09 – 0.93	0.038
Granzyme B	83.1%	17.5%	41.2%	59.8%	2.05	1.01 – 4.17	0.048
Multivariate analysis							
Multivariate model	78.0%	60.0%	64.9%	74.2%			0.00008
CD25:CD3e					8.66	2.40 – 31.21	0.001
LAG-3					3.29	1.41 – 7.69	0.006

All molecular markers with a significant P value in the univariate logistic regression analysis, and the clinical and histomorphological parameters were included in the multivariate logistic regression analysis. Only markers and parameters that reached significance and represent independent variables in the multivariate analysis are shown in the table. PV, predictive value; OR, odds ratio; CI, confidence interval

Multivariate analysis

Multivariate analysis with logistic regression resulted in a predictive model (P=0.00008) that contained the CD25:CD3e ratio (OR=8.7; P=0.001) and LAG-3 (OR=3.3; P=0.006) as independent covariates (Table 4). The specificity and sensitivity of this multivariate predictive model were 78% and 60%, respectively. The receiver operating characteristics (ROC) analysis showed that the predictive value of the multivariate model was significant (P=0.00002). Area under the curve (AUC) was 0.76 with a 95% CI of 0.66 – 0.85 (Figure 2). Inclusion of the clinical and histomorphologic parameters in the multivariate logistic regression model containing the molecular predictors did not enhance predictive power.

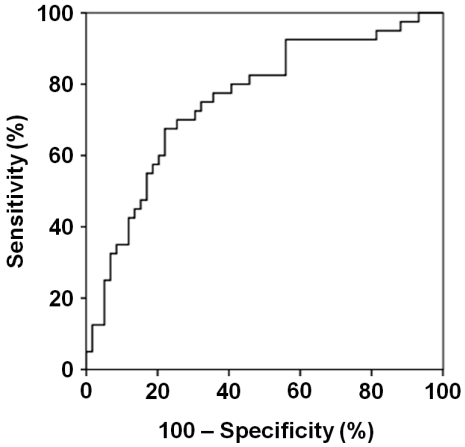


Figure 2. Predictive value of the multivariate logistic regression model for steroid resistant acute rejection. The receiver operating-characteristic (ROC) curve shows the percentage of true-positive results (sensitivity) and false-positive results (100 – specificity) for various cut-off levels of the multivariate logistic regression model, containing the molecular markers CD25:CD3e and LAG-3. The area under the curve of the ROC curve is 0.76 (P<0.001) with a 95% CI of 0.66 – 0.85.

Cross-validation model

For internal validation of the findings, a stratified ten-fold cross-validation was performed. The multivariate logistic regression analysis with cross-validation resulted in the same significant predictive model as before, containing the CD25:CD3e ratio and LAG-3 as independent covariates (mean P-value of 0.0004 ± 0.0004). The specificity and sensitivity of the cross-validated multivariate predictive model were 76% ± 20% and 43% ± 22%, respectively. ROC analysis for the model was significant (P=0.037).

Discussion

Steroid-refractory acute rejection is a risk factor for adverse renal allograft outcome [8;9]. To identify molecular associates of steroid resistant acute rejection, we performed a retrospective cohort study in a large cohort of renal transplant patients with a first rejection episode. None of the clinical and histomorphologic parameters tested showed significant association with treatment response. Eleven transcripts significantly discriminated steroid resistant rejections and steroid

responsive rejections in univariate analysis. In multivariate logistic regression, the combination of T cell activation markers CD25:CD3e ratio and LAG-3 represented the best predictive model for steroid response. The specificity and sensitivity of this multivariate predictive model were 78% and 60%, respectively. After internal cross-validation the predictive model remained significant.

For the current study, we selected a panel of immune-related genes, which included markers previously reported in association with steroid response [12;17-23;29-33], and which together reflected the full immune repertoire that may be present in the grafts (Table S1). These included cytokines, chemokines, and surface and activation markers of various cell types including cytotoxic T cells, T helper cells, regulatory T cells, classically and alternatively activated macrophages, B cells, plasma cells, natural killer cells, granulocytes, and mast cells. We found that the combination of the CD25:CD3e ratio and LAG-3 represented the best predictive model for steroid response. These markers are mainly expressed by activated T cells and regulatory T cells and represent cell surface proteins involved in signal transduction [34-38]. CD25 is the α-subunit of the IL-2 receptor (IL-2R), which is important in the regulation of the survival and proliferation of T cells [35;36]. Lymphocyte activation gene-3 (LAG-3 or CD223) is an activation-induced marker involved in the negative regulation of homeostasis and T cell function [34;38]. It is for the first time that these markers are linked to steroid resistance in renal transplantation. Interestingly, several studies have demonstrated that T cell characteristics, through disparities in IL-2 responses, play a role in steroid resistance [30;31]. Lee and colleagues identified in patients with steroid refractory ulcerative colitis a characteristic subgroup of peripheral $CD25^{int}$ T helper cells, which continued to proliferate *in vitro* upon activation, despite exposure to glucocorticoids [32]. This finding together with observations from the current study may suggest that steroid resistance resides in specific activated T cell populations and is not a feature of all lymphocytes. Hueso and colleagues have shown that low pretransplant levels of $CD3^{+}CD25^{high}$ lymphocytes were associated with an increased risk for early acute renal allograft rejection [39], which is likely a reflection of low levels of regulatory T cells [40;41]. The CD25:CD3e ratio at the mRNA level, associating with steroid resistant acute rejection, is probably more a reflection of activated T cells, comparable to the $CD25^{int}$ cells described by Lee and colleagues, rather than of regulatory T cells. A potential clinical implication of current findings is the identification of patients who do not respond to steroid treatment and may benefit from immediate ATG treatment after the diagnosis of acute renal allograft rejection.

In other studies, the presence of B cells (CD20) [12;29;33], macrophages (CD68) [20-23], and cytotoxic T cells (Granzyme B, FasL) [17] was associated with steroid resistant acute rejection. With did find by univariate analyses association between expression of Granzyme B and macrophage-specific transcripts (Mannose receptor, S100A9) with steroid resistant rejection. We could not confirm, however, the predictive value of CD20 expression. Complementary B cell specific transcripts, including CD19, CD22 and CD252, gave similar results. Besides the set of immunomarkers, transcripts of glycine amidinotransferase (GATM), phytanoyl-CoA hydroxylase (PHYH), and epidermal growth factor (EGF), of which relatively high levels were related to steroid responsive acute rejection in a previous study [12], were not of prognostic relevance in the current study. One aspect that may have influenced the discrepancy between findings from the current study and some findings from others could be differences in the age of the patients (for example, adults versus adolescents and children), type of immune suppression, the number of HLA mismatches, the sensitization grade of the patients, and the time between transplantation and the rejection episode. Of note, several other investigators also have not been able to confirm the association of CD20 expression with steroid response of the acute rejection [13-16]. Although not significant, in our study a higher percentage of vascular rejection was seen in the steroid resistant group (40%) compared to the steroid sensitive group (29%) (Table 1). Vascular rejection was found to be associated with steroid resistance in previous studies [24;42;43], and its presence may have had an influence on therapy sensitivity in the current study.

We handled strict inclusion criteria, clinical end point definition, and quality controls for RNA processing and PCR assays. We decided to use qPCR rather than microarray analysis to measure intragraft mRNA expression, since the former technique is the best tool to quantify expression. Furthermore, expression of most of the interleukins and chemokines studied cannot be detected by microarray analysis. To have defined clinical criteria for response to anti-rejection therapy, steroid resistance of the acute rejection was adopted when the patient required ATG within two weeks after initiation of the steroid administration.

Some comments need to be made with regard to the current findings. In this retrospective study, none of the C4d-positive biopsies showed histomorphologic characteristics indicative of a possible antibody-mediated rejection. However, we had no information on the presence of donor specific antibodies at the time

of rejection. Therefore, we were unable to firmly conclude on the contribution of antibody-mediated rejection. Furthermore, we observed, as pointed out previously [12], a considerable heterogeneity in transcriptional regulation among the acute rejection biopsies. Presence of multiple mechanisms underlying steroid resistance probably accounts for the restricted predictive power. Firstly, the outcome of acute rejection may be determined by the response of the renal parenchyma to inflammation besides the composition of the infiltrate itself. Secondly, interaction of the glucocorticoid receptor (GR) with its ligand might in part determine therapy response. Polymorphisms in *NR3C1*, the gene encoding for the GR, may affect the ability of this receptor to bind glucocorticoids [44;45]. Further research needs to be performed to test the impact of polymorphisms in the *NR3C1* gene on therapy response during acute rejection.

In conclusion, we evaluated a broad panel of immunological markers by qPCR profiling in a large cohort of renal allografts with acute rejection. Differences in intragraft expression profiles reflect variability in the response to anti-rejection treatment. With respect to therapy response of the rejection episode, molecular markers offer superior prognostic value compared to conventional parameters. The combination of the CD25:CD3e ratio and LAG-3 expression represents the best model for risk assessment of steroid resistance in patients suffering from acute rejection. This is of interest in terms of the pathogenic mechanism of steroid resistance. In the future, the results could be used to identify which patients will not respond to steroid treatment and may benefit from immediate ATG treatment. Our findings indicate that steroid resistance resides in specific T cell populations and is not a feature of all lymphocytes.

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Supplemental data

Table S1: Molecular markers investigated in the study by qPCR.

Marker:	Full gene name:	Sources / function	Reference
CD markers			
CCR5	Chemokine (C-C motif) receptor 5; CD195	Mo, MΦ, DC, Th1, NK	
CD3e	CD3e molecule, epsilon (CD3-TCR complex)	T	
CD4	CD4 molecule; OKT4	T subset	
CD8	CD8 molecule; OKT8	T subset	
CD11b	CD11 antigen-like family member B; integrin M alpha, Mac-1	Myeloid cells, NK, Mo	
CD19	CD19 molecule; B4	B	
CD20	CD20 molecule; membrane spanning 4 domains (MS4A1)	B	12
CD22	CD22 molecule; B lymphocyte cell adhesion molecule (BL-CAM)	B	
CD25	CD25 molecule; IL-2 receptor alpha chain	Tact, Bact, Treg	
CD28	CD28 molecule	T, plasma cells	
CD56	CD56 molecule; neural cell adhesion molecule (N-CAM)	NK	
CD66b	CD66b molecule	Granulocytes	
CD68*	CD68 molecule	Mo, MΦ	21-23
CD69	CD69 molecule	Tact, B, NK, granulocytes	
CD86	CD86 molecule; B7-2	Mo, DC, Bact, Tact	
CD103	CD103 molecule; integrin αE	Lymph	
CD115	CD115 molecule; c-fms; CSF-1 receptor	Mo differentiation / proliferation	
CD117	CD117 molecule; c-kit	HSC, F, MC	
CD127	CD127 molecule; interleukin 7 receptor (IL-7R)	T, preB, thymocytes	
CD138*	CD138 molecule; Syndecan 1; SDC1	Plasma cells, preB, epithelium	
CD163	CD163 molecule	Mo, MΦ (MΦ2 in vitro)	
CD205	CD205 molecule; DEC-205	DC	
CD252	CD252 molecule; OX40-ligand	B, DC, MC, EC	
CTLA4*	Cytotoxic T-lymphocyte-associated protein 4; CD152	Tact, B	
ICOS	Inducible T-cell co-stimulator; CD278	Tact, Th2	

Table S1: Continued

Marker:	Full gene name:	Sources / function	Reference
LAG-3	Lymphocyte-activation gene 3; CD223	Tact, NK	
MannoseR	Mannose receptor, C type 1 (MRC1); CD206	MΦ (alternative activation)	
PD-1	Programmed cell death 1; CD279	Tact, Bact, thymocytes	
Activation markers			
AIF-1	Allograft inflammatory factor 1	MΦ	
Blimp-1	PR domain containing 1, with ZNF domain; PRDM1	T, B, NK	
EBI3	Epstein-Barr virus induced 3	Treg, regulation of Th1 and Th2	
FasL	Fas ligand; TNF receptor superfamily, member 6; CD178	Tact, N, Mo, NK	[17]
FoxP3	Forkhead box P3	Treg, T	[19]
Granulysin	Granulysin (GNLY); lymphocyte-activation gene (LAG-2)	CTL	[18]
Granzyme B	Granzyme B (Grzb)	CTL, NK	[17]
HLA-DR	Major histocompatibility complex, class II, DR	Tact, Bact, DC, MΦ	[22]
IgG	Immunoglobulin G	B, plasma cells	
IgM	Immunoglobulin M	B, plasma cells	
NOS2A*	Nitric oxide synthase 2A	Produced by MΦ1	
RORγT	RAR-related orphan receptor C; RORC	T, Th17 pathway	
S100A8	S100 calcium binding protein A8; MRP-8	Mo, MΦ	
S100A9	S100 calcium binding protein A9; MRP-14	Mo, MΦ	
Cytokines			
IFN-γ	Interferon gamma	Regulation of immune cells	
IL-1β	Interleukin 1 beta	MΦact	
IL-2*	Interleukin 2; T cell growth factor (TCGF)	T, L proliferation	
IL-4*	Interleukin 4; B-cell stimulatory factor 1 (BSF-1)	T, Th2 pathway	
IL-5*	Interleukin 5; eosinophil differentiation factor (EDF)	T, Th2, E, MC	
IL-6	Interleukin 6; interferon beta 2	T, MΦ, B	
IL-8	Interleukin 8; chemokine (C-X-C motif) ligand 8 (CXCL-8)	Mo, MΦ, EP	
IL-10	Interleukin 10; cytokine synthesis inhibitory factor (CSIF)	Mo, MΦ, T	
IL-15	Interleukin 15	Mo, MΦ	

IL-16	Interleukin 16; lymphocyte chemoattractant factor (LCF)	L (CD8+), E	
IL-18	Interleukin 18; interferon-gamma inducing factor (IGIF)	MΦ	
IL-21	Interleukin 21	Tact, NKT, MC	
TGF-β_1	Transforming growth factor beta 1	Regulation of immune cells	
TNFα	Tumor necrosis factor alpha	Regulation of immune cells	
Chemokines			
CCL-2	Chemokine (C-C motif) ligand 2; MCP-1	MΦ, L, EC, EP	
CCL-3	Chemokine (C-C motif) ligand 3; MIP-1a	MΦ, L, N, E, F, MC	
CCL-4	Chemokine (C-C motif) ligand 4; MIP-1b	MΦ, L, N, F, MC	
CCL-13	Chemokine (C-C motif) ligand 13; MCP-4	Lung, colon, intestine	
CCL-18*	Chemokine (C-C motif) ligand 18; AMAC-1	Lung, lymph, thymus	
CCL-20	Chemokine (C-C motif) ligand 20; MIP-3α	Liver, lung	
CCL-22	Chemokine (C-C motif) ligand 22; ABCD-1	DC, MΦ, T	
CXCL-9	Chemokine (C-X-C motif) ligand 9; MIG	MΦ, N	
CXCL-12	Chemokine (C-X-C motif) ligand 12; SDF-1	Stromal cells	
CXCL-13	Chemokine (C-X-C motif) ligand 13; BCA-1	Liver, spleen, lymph	
Others			
EGF	Epidermal growth factor		[12]
GATA3	GATA binding protein 3	Th2 pathway	
GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)		[12]
PHYH	Phytanoyl-CoA 2-hydroxylase; PAXH		[12]
STAT4	Signal transducer and activator of transcription 4	Th1 pathway	
STAT6	Signal transducer and activator of transcription 6	Th2 pathway	
T-bet	T-cell-specific T-box transcription factor	Th1 pathway	

* Gene expression results excluded from study due to inferior melt curve.

Abbreviations:
B, B cells; Bact, activated B cells; CTL, cytotoxic T lymphocytes; DC, dendritic cells; E, eosinophils; EC, endothelial cells; EP, epithelial cells; F, fibroblasts; HSC, hematopoietic stem cells; iDC, immature dendritic cells; L, lymphocytes; MC, mast cells; Mo, monocytes; MΦ, macrophages; MΦact, activated macrophages; N, neutrophils; NK, natural killer cells; NKT, NKT cells; preB, B cell precursor; T, T cells; Tact, activated T cells; Th1, T helper cells; Th2, T helper 2 cells; Treg, regulatory T cells

Increased metallothionein expression reflects steroid resistance in renal allograft recipients

4

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Abstract

Steroid-refractory acute rejection is a risk factor for inferior renal allograft outcome. We aimed to gain insight into the mechanisms underlying steroid resistance by identifying novel molecular markers of steroid-refractory acute rejection. Eighty-three kidney transplant recipients (1995-2005), who were treated with methylprednisolone during a first acute rejection episode, were included in this study. Gene expression patterns were investigated in a discovery cohort of 36 acute rejection biopsies, and verified in a validation cohort of 47 acute rejection biopsies. In the discovery set, expression of metallothioneins (MT) was significantly ($P<0.000001$) associated with decreased response to steroid treatment. Multivariate analysis resulted in a predictive model containing MT-1 as an independent covariate (AUC=0.88, $P<0.0000001$). In the validation set, MT-1 expression was also significantly associated with steroid resistance ($P=0.029$). Metallothionein expression was detected in macrophages and tubular epithelial cells. Parallel to the findings in patients, *in vitro* experiments of peripheral blood mononuclear cells from 11 donors showed that non-response to methylprednisolone treatment is related to highly elevated MT levels. High expression of metallothioneins in renal allografts is associated with resistance to steroid treatment. Metallothioneins regulate intracellular concentrations of zinc, through which they may diminish the zinc-requiring anti-inflammatory effect of the glucocorticoid receptor.

Introduction

The occurrence of steroid-refractory acute rejection is a risk factor for adverse outcome in renal allograft recipients [1-5]. First rejection episodes are most of the time treated by high-dose steroids, leading to reversal of the acute rejection. However, approximately 30% of patients have no or an inadequate response to corticosteroid therapy alone and require therapy with anti-thymocyte globulin (ATG) [2;5-7]. Such steroid resistant patients may show progression of chronic damage to the graft, which has a detrimental effect on graft outcome [4;5;8].

Cellular and molecular markers in the graft tissue may complement clinical parameters and histomorphology when assessing steroid resistance of the acute rejection episode. Indeed, expression of markers, particularly those of inflammatory cell types, was found to be associated with therapy response (reviewed in [8]). Recently, we showed that the combination of T-cell activation markers CD25:CD3 ratio and LAG-3 offers a superior prognostic value for assessing steroid response, compared with conventional parameters [9]. We observed considerable molecular heterogeneity among the biopsy samples with acute rejection, underlining the complexity of the mechanisms involved in response to steroid therapy.

In the current study we aimed to gain further insight into the mechanisms underlying steroid resistance by identifying novel molecular markers of steroid-refractory acute rejection using genome wide expression profiling. Our results reveal that relatively high intragraft expression of metallothioneins (MT), a group of small cysteine-rich molecules that regulate intracellular zinc concentrations, during acute rejection is associated with resistance to steroid treatment. These proteins were further studied in biopsies and steroid-treated leukocyte cultures.

Materials and methods

Patient information

We reviewed all 873 patients who received a renal allograft in our center between 1995 and 2005. We selected patients suffering from a histologically proven, first rejection episode, from whom a renal biopsy had been taken prior to the start of anti-rejection treatment. Only patients receiving pulse therapy with methylprednisolone

(3 days with 1 g bolus) as anti-rejection treatment were included. The primary clinical endpoint was response to anti-rejection treatment with methylprednisolone, as described previously [9]. Steroid resistant acute rejection was defined as lack of clinical response to methylprednisolone, and a requirement for anti-thymocyte globulin treatment within 14 days after the start of the steroid therapy. The response to steroid therapy was monitored by creatinine concentration measurements. Maintenance immunosuppression consisted of prednisone and a calcineurin inhibitor (Cyclosporine A or Tacrolimus), with or without Mycophenolate Mofetil.

In total, 83 patients were included in the study. For genome-wide expression profiling, a discovery set (n=36) was studied: 18 patients with steroid responsive acute rejection and 18 patients with steroid resistant acute rejection. As microarray analysis requires high-quality RNA [10-13], biopsy samples that had generated the highest quality of RNA were included in the discovery set. Four biopsies, taken during acute decrease of graft function but with no histomorphologic indication of rejection, were included as controls. Results from the discovery set were verified in a validation set (n=47): 31 patients with steroid responsive acute rejection and 16 patients with steroid resistant acute rejection.

Biopsy samples

Rejection severity of all biopsy specimens was assessed on paraffin-embedded biopsy tissue according to Banff 2011 criteria [14]. A second biopsy core was snap frozen in liquid nitrogen and stored at -80°C. In addition to the biopsies taken on clinical indication, paired pretransplant biopsy samples (stored at -80°C) were available from 14 steroid resistant and nine steroid responsive patients.

RNA extraction and quality assessment

Eight to ten 10-µm sections were cut with a cryomicrotome from each snap frozen biopsy core. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA quality was determined on Nano LabChips with the Aligent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The mean RNA concentration was 2.76 ± 1.11 µg, mean 28S:18S ratio was 2.1 ± 0.04, and mean RNA integrity number (RIN) was 8.11 ± 0.92.

Microarray Analysis

Gene expression profiling was performed with Illumina HumanRef-8 v3.0 BeadChips (Illumina, San Diego, CA), as previously described [15]. Only samples with a RNA quality index > 8 were included in the microarray analysis. Illumina GenomeStudio software was used to verify the adequacy of signal-background ratio and hybridization controls. All data have been deposited in the Gene Expression Omnibus public database, accession number: GSE47097.

Real-time quantitative PCR analysis

Synthesis of cDNA, primer design, and quantitative PCR (qPCR) analysis were performed according to previously described protocols [9;16]. An average of 0.92 ± 0.15 µg of RNA from each frozen biopsy core was transcribed into cDNA. Primer sequences can be provided on request. PCR efficiencies were between 90% - 110%. Differences in mRNA expression levels were normalized to the geometric mean signal of the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin.

Immunohistochemistry

Immunohistochemical analyses were performed on 32 paraffin-embedded biopsy samples: 17 from steroid responsive patients and 15 from steroid resistant patients. Protein staining for metallothionein, CD68, and CD163 was performed on 4-µm thick sections. Primary monoclonal mouse anti-human antibodies against metallothionein (Abcam, Cambridge, UK; clone UC1MT), CD163 (Abcam; clone 10D6, prediluted), and CD68 (Dako, Glostrup, Denmark; clone KP1) were used for immunohistochemistry. Standardized protocols [17] were applied. Details concerning antigen retrieval and staining procedure are provided in Table 1. Photos were taken with a Leitz DMRD microscope (Leica Microsystems, Rijswijk, the Netherlands).

Table 1. Characteristics of antibodies.

Protein	Antigen retrieval	Type	Dilution	Blocking	Pre-incubation	Incubation time	Secondary antibody	Detection
MT	Citrate (pH 6.0)	Monoclonal	1:300	0.1% H_2O_2	5% NGS, 1 h [1]	1 h	Mouse-EnVision+	DAB
CD68	Tris/EDTA (pH 9.0)	Monoclonal	1:1000	0.03% H_2O_2		1 h	Mouse-EnVision+	DAB
CD163	Citrate (pH 6.0)	Monoclonal	1:10	0.03% H_2O_2		1 h	Mouse-EnVision+	DAB

[1] In the MT staining the slides were pre-incubated for 1 h with 5% normal goat serum (NGS), diluted in PBS.

***In situ** hybridization for Y chromosome*

The presence of male, Y-chromosome-expressing cells was determined by *in situ* hybridization, as described previously [18]. Briefly, 4-µm sections were hybridized overnight with digoxigenin (DIG) labeled, Y-chromosome-specific DNA probe, followed by consecutive incubations with mouse anti-DIG monoclonal antibody (Sigma-Aldrich, St. Louis, MO) and rabbit anti-mouse Ig-horseradish peroxidase (anti-mouse Envision-HRP, Dako). Sections were developed with Nova Red and counter-stained with hematoxylin. Photos were taken with a Leitz DMRD microscope (Leica Microsystems).

Investigation of metallothionein expression in various cell types

Metallothionein expression was investigated in RNA from a panel of various cell types [19]. This panel included peripheral blood mononuclear cell (PBMC) fractions (n=9), cultured dendritic cells (n=6), monocytes (n=3), differentiated macrophages (n=7), stimulated macrophages (n=6), T cells (n=5), stimulated T cells (n=5), B cells (n=6), proximal tubular epithelial cells (n=2), human aorta endothelial cells (n=5), and human umbilical vascular endothelial cells (n=5). RNA from pretransplant biopsy samples (n=65) was included as a reference.

***In vitro** methylprednisolone response experiments*

Blood was obtained from healthy blood bank donors (n=11; Sanquin, Leiden, the Netherlands) after informed consent. PBMC were isolated by Ficoll Hypaque density gradient centrifugation, cultured in 96-well plates (10^5/well) in RPMI 1640 medium with 10% fetal calf serum (Gibco), and stimulated for 50 h with PHA (2 µg/ml). This was done either in the absence or presence of 10^{-4} M methylprednisolone (Solu-Medrol® from Pfizer, Capelle a/d IJssel, the Netherlands; powder for intravenous infusion). After the culture, the cells were harvested and stored in RNA*later* (Qiagen) until RNA extraction and qPCR analysis.

Statistical analysis

Comparison of categorical data between groups was evaluated using the Fisher's Exact test. Microarray data were normalized using the Quantile Normalization method. Only genes showing expression above background ($P<0.05$) in at least 20 of the 40 samples were included. Hierarchical clustering was performed with the

gplots R package (version 2.10.1). The Mann-Whitney U-test was performed to select differentially expressed genes between the steroid response groups. Multiple testing correction was performed with the Benjamini-Hochberg method [20].

Lasso regression analysis, performed with the *penalized* R package (version 0.9-33) [21], was used to analyze the discriminative value between groups of genes analyzed in the microarrays. Leave-one-out cross-validated receiver operating characteristics (ROC) analysis, using the R package *ROCR* (version 1.0-2) [22], was applied to evaluate the robustness of the multivariate model from the lasso regression analysis. The enrichment of specific functional groups of genes was analyzed on the basis of hypergeometric tests using the online gene set analysis toolkit WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) [23;24].

PCR data are expressed as mean ± standard error of the mean. Differences in mean mRNA and protein expression between groups were tested with independent samples t-tests and Mann-Whitney U-tests. Logistic regression analysis and ROC analysis were performed for multivariate analysis of qPCR data. All statistical tests were two-sided, and P values less than 0.05 were considered as significant. Statistical analyses were performed using the IBM SPSS statistical package (version 20.0.0, Amsterdam, the Netherlands) and the R statistical package (version 2.12.0, http://www.r-project.org/).

Results

Demographics and clinical data

No significant differences in the demographic and clinical characteristics were found between steroid responsive and steroid resistant patient groups in the discovery cohort (n=36) and validation cohort (n=47) (Table 2 and Table S1). There was no significant difference in the RNA concentration, purity, and quality of the samples from the patient groups (data not shown).

Table 2. Demographic and clinical data of patients with steroid responsive and steroid resistant acute rejection in the discovery set and the validation set.

Variable	Discovery set			Validation set		
	Steroid responsive (n=18), n (%)	Steroid resistant (n=18), n (%)	P value	Steroid responsive (n=31), n (%)	Steroid resistant (n=16), n (%)	P value
Patient age			1.00			1.00
≥ 50 years	8 (44.4%)	8 (44.4%)		14 (45.2%)	7 (43.8%)	
Patient gender			0.71			0.54
Female	4 (22.2%)	6 (33.3%)		13 (41.9%)	5 (31.2%)	
Donor age			0.15			1.00
≥ 50 years	3 (17.6%)	8 (44.4%)		13 (41.9%)	7 (43.8%)	
Donor gender			0.73			0.32
Female	11 (64.7%)	10 (55.6%)		20 (64.5%)	13 (81.2%)	
Year of transplantation			0.71			0.73
1995 through 1999	12 (66.7%)	14 (77.8%)		23 (74.2%)	13 (81.2%)	
2000 through 2005	6 (33.3%)	4 (22.2%)		8 (25.8%)	3 (18.8%)	
Donor type			1.00			0.07
Living	5 (27.8%)	4 (22.2%)		4 (12.9%)	6 (37.5%)	
Post mortal	13 (72.2%)	14 (77.8%)		27 (87.1%)	10 (62.5%)	
HLA-A matching			0.29			1.00
≥ 1 mismatch	10 (58.8%)	14 (77.8%)		18 (58.1%)	9 (56.2%)	
HLA-B matching			1.00			1.00
≥ 1 mismatch	13 (76.5%)	14 (77.8%)		22 (71.0%)	12 (75.0%)	
HLA-DR matching			0.31			0.75
≥ 1 mismatch	9 (52.9%)	13 (72.2%)		19 (61.3%)	11 (68.8%)	
Virtual PRA			1.00			0.32
Immunized (6-100%)	4 (23.5%)	5 (27.8%)		7 (22.6%)	6 (37.5%)	
Cold ischemia time			0.71			1.00
> 18 hours	8 (57.1%)	11 (68.8%)		20 (76.9%)	10 (76.9%)	
Induction therapy			1.00			0.73
Daclizumab	4 (22.2%)	4 (22.2%)		8 (25.8%)	3 (18.8%)	
None	14 (77.8%)	14 (77.8%)		23 (74.2%)	13 (81.2%)	
Maintenance therapy			1.00			0.36
Double therapy	8 (44.4%)	8 (44.4%)		14 (45.2%)	10 (62.5%)	
Triple therapy	10 (55.6%)	10 (55.6%)		17 (54.8%)	6 (37.5%)	
Delayed graft function			1.00			0.49
Yes	3 (16.7%)	2 (11.1%)		10 (32.3%)	2 (18.8%)	
Rejection time			1.00			1.00
<3 months post Tx	18 (100.0%)	17 (94.4%)		30 (96.8%)	16 (100.0%)	
3-6 months post Tx	0 (0.0%)	1 (5.6%)		1 (3.2%)	0 (0.0%)	
C4d staining			1.00			0.17
Positive	3 (17.6%)	3 (16.7%)		2 (7.1%)	4 (25.0%)	
Vascular rejection			1.00			1.00
Yes	7 (38.9%)	8 (44.4%)		7 (22.6%)	4 (25.0%)	

PRA, panel reactive antibodies; Tx, transplantation

Microarray analysis

Transcriptional profiles of 40 renal allograft biopsy samples were analyzed using whole genome microarray slides. Expression profiles were investigated in the discovery cohort of 18 steroid resistant rejections and 18 steroid responsive rejections, and in 4 control biopsy samples. Six thousand eight hundred and thirty two genes with significant expression ($P<0.001$) above background were included for further analysis.

To test for associations between the gene expression profile in the biopsy samples and clinical parameters, we first clustered the patients based on similarities in the expression pattern of the corresponding kidney allograft biopsies (Figure 1A). This unsupervised hierarchical clustering analysis did not result in a clear distinction between steroid responsive and steroid resistant acute rejection biopsies. The control biopsies largely clustered together. Comparison of patients in different clusters did not reveal differences of the clinical parameters (Figure 1B).

Relation between molecular variables and steroid response

The expression of 249 genes differed significantly ($P<0.05$ with the unadjusted Mann-Whitney U-test) between steroid responsive and steroid resistant rejections: 158 transcripts were upregulated and 91 transcripts were downregulated in the steroid resistant rejections.

The 50 genes with the most significant differences between the two groups were included in two-way hierarchical clustering analysis (Figure 2). The dendrogram above the heatmap shows four clusters of patients: two clusters with mainly samples from steroid responsive patients (clusters 1 and 2), one cluster with mainly samples from steroid resistant patients (cluster 3), and one cluster containing only samples from steroid resistant patients (cluster 4). Comparison of the Banff scores from the steroid resistant patients in cluster 4 with the Banff scores of the steroid resistant patients in the other three clusters revealed no significant differences (data not shown). The dendrogram on the vertical axis revealed two distinct clusters of genes. A large number of genes from the metallothionein 1 (MT-1) family cluster together and show a similar expression pattern.

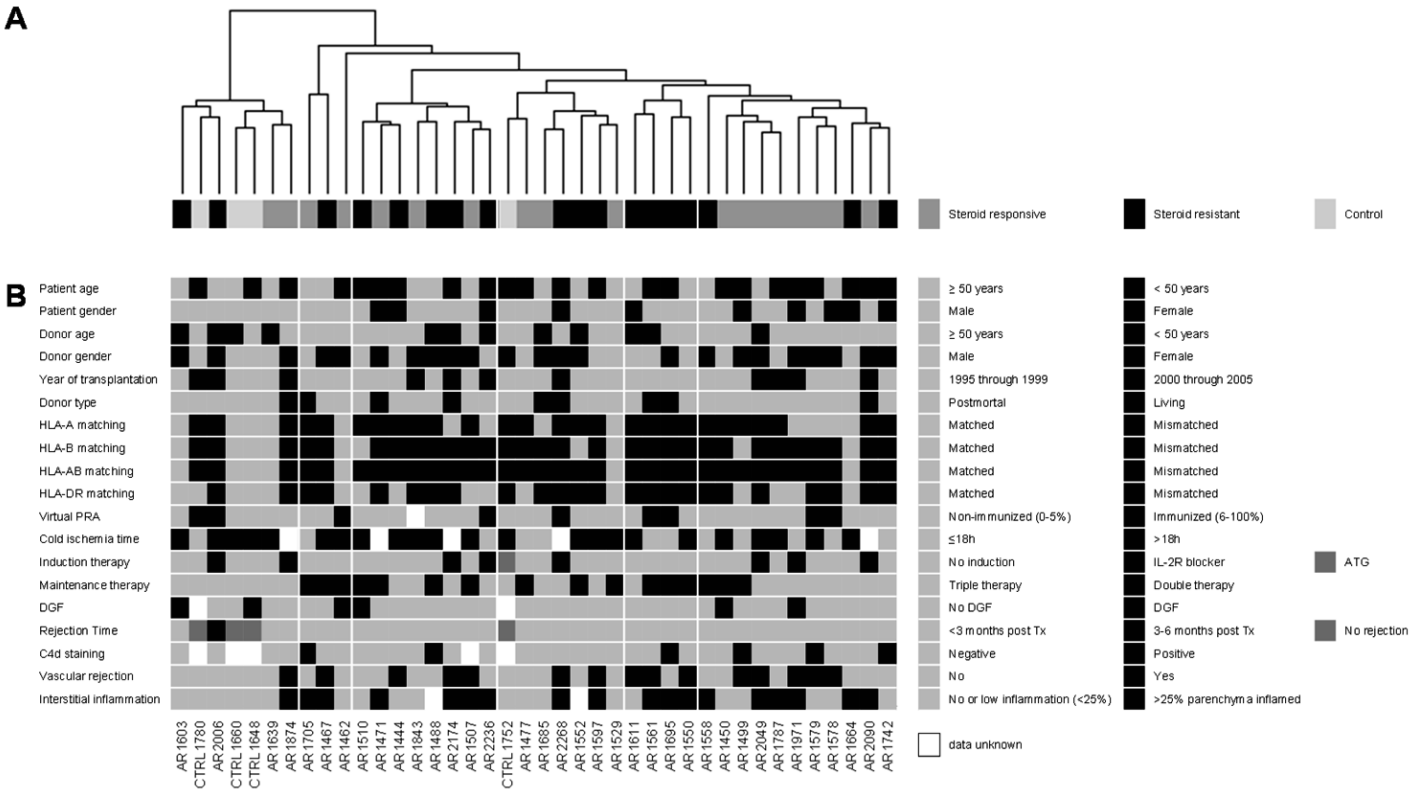


Figure 1. Analysis of associations between the gene expression profile in steroid resistant and steroid responsive kidney biopsy samples and clinical parameters. A) Unsupervised hierarchical clustering analysis of the kidney allograft biopsies in the discovery cohort and 4 control biopsy samples, based on similarities in the expression pattern of the 6,832 genes. B) Comparison of the clinical parameters of the patients in the identified clusters from the hierarchical clustering analysis.

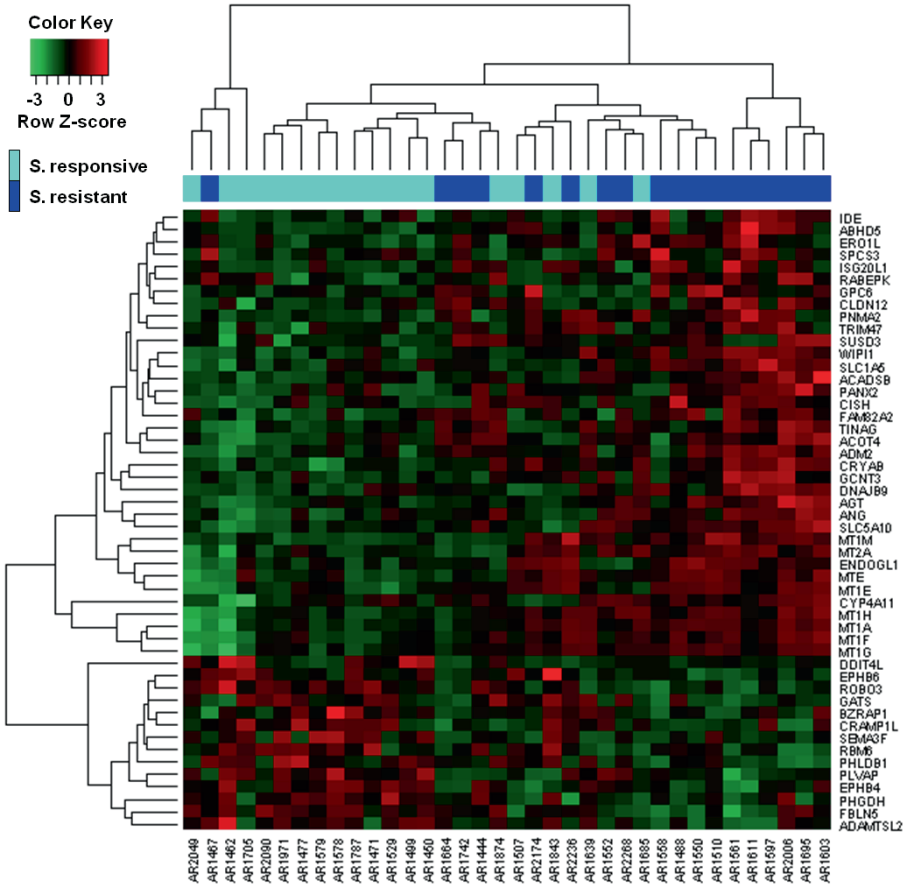


Figure 2. Hierarchical clustering and heatmap display of the 50 genes with the highest significant difference in expression between steroid responsive and steroid resistant acute rejection. Genes (rows) and samples (columns) were clustered in an unsupervised manner on the basis of the overall similarity in expression pattern between subjects in the discovery cohort. The dendrograms represent how close the relationship is between biopsy samples (x-axis) or different genes (y-axis). The color (scale indicated top left) in each cell reflects the level of expression of the corresponding gene in the corresponding sample. Color scale runs from bright green (relatively low mRNA expression) to bright red (relatively high mRNA expression). Patients with steroid responsive acute rejection are shown in light blue; patients with steroid resistant acute rejection are shown in dark blue.

Gene ontology

To gain insight into the function of differentially expressed genes, we included all 249 genes for pathway analysis by hypergeometric test in WebGestalt. One molecular function showed high enrichment in steroid resistant patients: cadmium ion binding (P=0.000000004). When adjusted for multiple testing, this finding remained highly

significant (ratio of enrichment of 20.1, P<0.000001; Table S2). Seven of the nine genes involved in this molecular function were significantly higher expressed in steroid resistant rejection, and they were all members of the MT-1 gene family (MT-1A, MT-1E, MT-1F, MT-1G, MT-1H, MT-1M, MT-1X). Other pathways concerned amongst others the extracellular region (P=0.0003), extracellular matrix part (P=0.0038), and copper ion binding (P=0.0082). The complete list of significant pathways is shown in Table S2.

Multivariate regression analysis

Multivariate analysis with lasso regression resulted in a model discriminating patient groups, containing as independent covariates MT-1G in combination with CYP4A11, TIMP1, FTHL7, and F2R. The predictive value of the multivariate model was significant (AUC=0.92, P=0.00002) when tested with receiver operating characteristics (ROC) curve analysis (Figure 3A). The predictive value of the multivariate model remained significant when validated with a leave-one-out cross-validated analysis (AUC=0.72, P=0.048; Figure 3B).

Quantitative PCR validation

Using real-time quantitative PCR (qPCR) we validated the microarray results for six mRNA transcripts, including the covariates from the multivariate lasso regression, in the acute rejection biopsy samples of the discovery cohort: MT-1, CYP4A11, TIMP1, F2R, AGT, and PHLDB1 (Figure 4). The qPCR results corresponded with the findings by microarray. The expression levels of MT-1 (P=0.018, Figure 4A) and TIMP1 (P=0.002, Figure 4C) were significantly increased in steroid resistant acute rejection, whereas those of PHLDB1 were significantly decreased (P=0.016, Figure 4F) compared with the steroid responsive group. The steroid resistant samples showed higher expression of AGT (P=0.058, Figure 4E) and of CYP4A11 (P=0.107, Figure 4B), and lower expression of F2R (P=0.195, Figure 4D).

The expression of the six transcripts was measured in pretransplant biopsies available from 23 of the patients studied during acute rejection (14 steroid resistant and nine steroid responsive). No significant differences were found in intragraft expression levels before transplantation, except for CYP4A11. The steroid resistant patients showed significantly higher CYP4A11 expression levels (P=0.0019, Figure 4B).

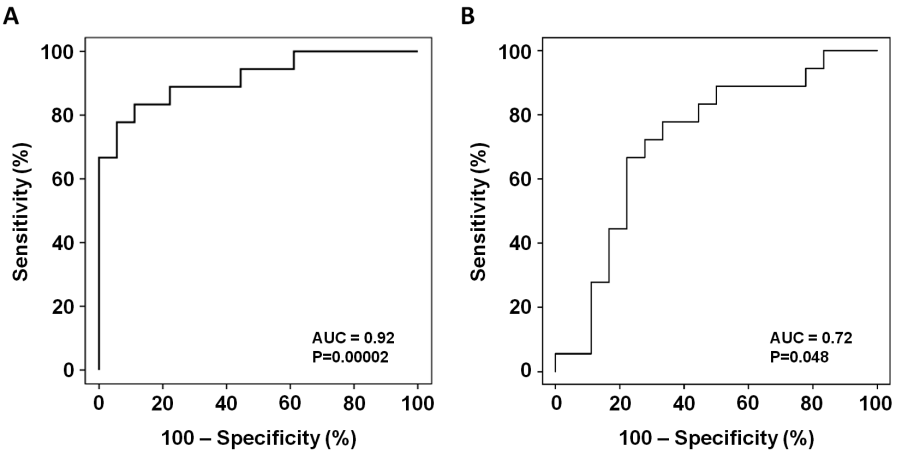


Figure 3. Predictive value of the lasso regression model for steroid resistant acute rejection. Multivariate regression analysis of the microarray data from the discovery set resulted in a predictive model with MT-1, CYP4A11, TIMP1, FTHL7, and F2R as independent covariates. A) The receiver operating characteristics (ROC) curve shows the true positive rate (sensitivity) and false positive rate (100 – specificity) for various cut-off levels of the multivariate model. The area under the curve (AUC) was 0.92 (P=0.00002). B) The predictive value of the multivariate model remained significant when validated with a leave-one-out cross-validated analysis (AU=0.72, P=0.048).

Validation cohort

To verify the findings that high MT-1 expression associates with resistance to steroid treatment, we used qPCR to analyze the expression of MT-1 in a validation cohort (n=47). The MT-1 expression level was significantly increased in steroid resistant acute rejection compared to steroid responsive acute rejection (P=0.029, Figure 5).

To validate the multivariate model obtained with microarray analysis, we performed multivariate logistic regression analyses on the qPCR measurements in the discovery and validation cohorts. The predictive value of the multivariate model, containing MT-1, CYP4A11, TIMP1, and F2R, was significant in both the discovery cohort (AUC=0.89, P=0.00008; Figure 6A) and the validation cohort (AUC=0.80, P=0.0009; Figure 6B).

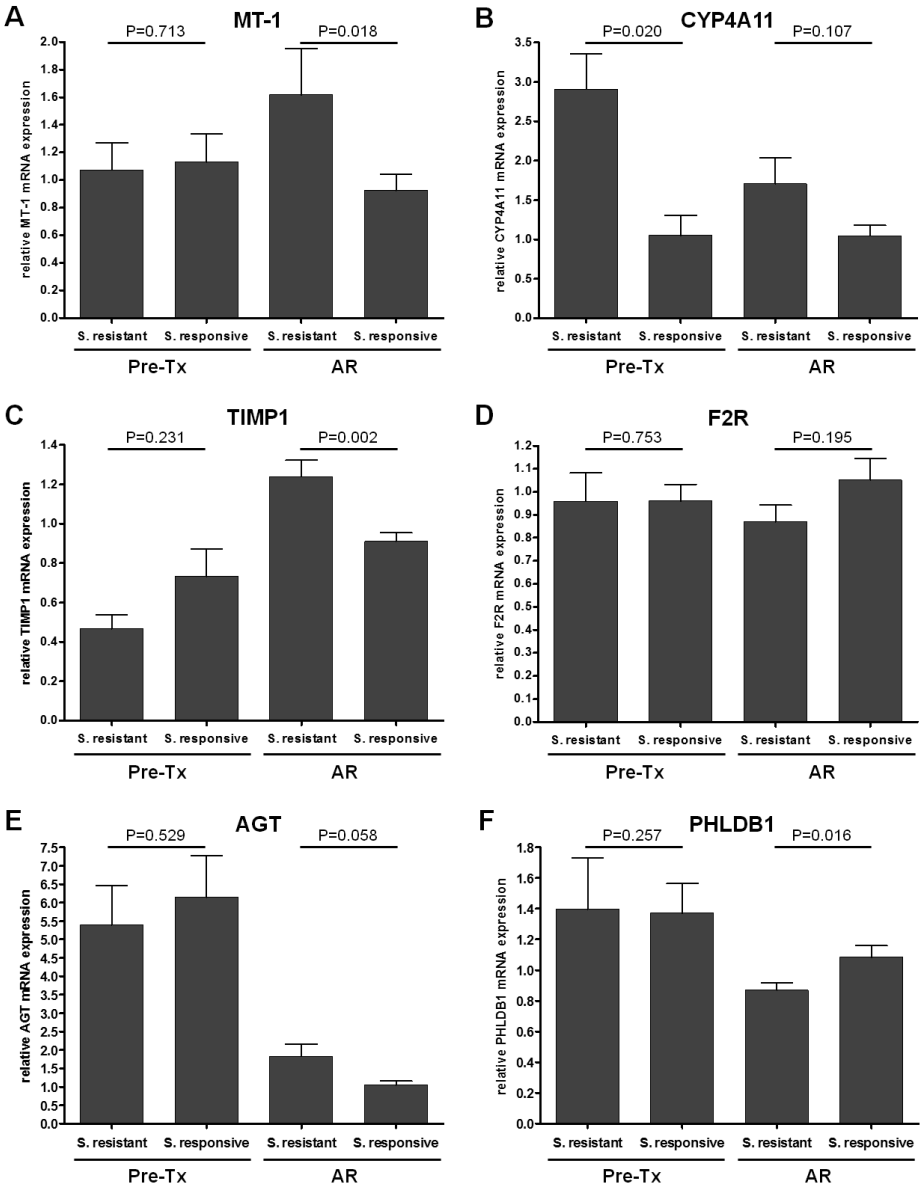


Figure 4. Quantitative real-time PCR validation of microarray results in the discovery patient cohort. RNA expression levels of MT-1 (A), CYP4A11 (B), TIMP1 (C), F2R (D), AGT (E), and PHLDB1 (F) measured in pretransplant biopsy samples (Pre-Tx) and acute rejection biopsy samples (AR) from the steroid resistant and steroid responsive patient groups in the discovery cohort. Data are expressed as mean ± SEM.

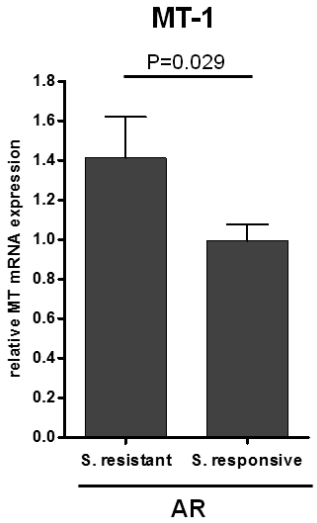


Figure 5. Metallothionein expression in the validation patient cohort. MT-1 mRNA levels were measured with qPCR in acute rejection (AR) biopsy samples from the steroid resistant and steroid responsive patients in the validation cohort. Data are expressed as mean ± SEM.

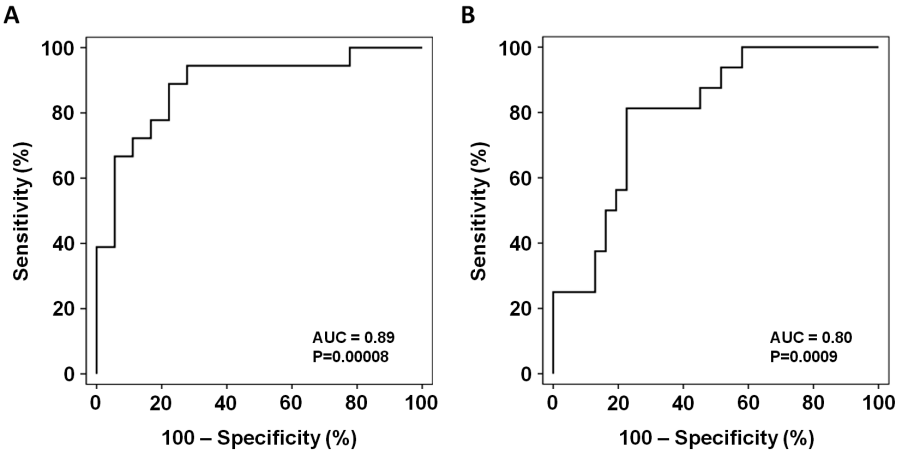


Figure 6. Predictive values of the multivariate regression model in the discovery set and validation set. Receiver operating characteristics (ROC) curve analysis of the multivariate regression model, containing MT-1, CYP4A11, TIMP1, and F2R, measured with qPCR. The area under the ROC curve was 0.89 (P=0.00008) in the discovery set (A) and 0.80 (P=0.0009) in the validation set (B). The ROC curves show the true positive rate (sensitivity) and false positive rate (100 – specificity) for various cut-off levels of the multivariate model.

Comparison of multivariate models

To investigate how the multivariate MT-1 model (MT-1, CYP4A11, TIMP1, and F2R) relates to the multivariate T-cell mediated model from our previous study (CD25:CD3e

ratio and LAG-3) [9], we combined the discovery and validation cohorts. The predictive value of the MT-1 model was slightly higher (AUC=0.81, P=0.000002; Figure 7) than the predictive value of the T-cell activation model (AUC of 0.79, P=0.000007; Figure 7). Combination of the two models resulted in a predictive model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates (AUC=0.88, P<0.0000001; Figure 7).

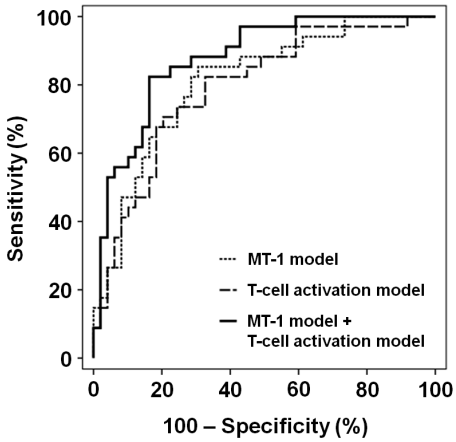


Figure 7. Comparison of the predictive values of the metallothionein and T-cell activation models for steroid resistant acute rejection. Receiver operating characteristics (ROC) curve analysis was used to compare the predictive value of the multivariate metallothionein-1 (MT-1) model (containing MT-1, CYP4A11, TIMP1, and F2R) and of the previously described multivariate T-cell activation model (containing CD25:CD3e ratio and LAG-3) [9]. The area under the ROC curve was 0.81 (P=0.000002, dotted line) for the MT-1 model and 0.79 (P=0.000007, striped line) for the T-cell activation model. Combination of the two models resulted in a multivariate model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates (AUC=0.88, P<0.0000001, solid line).

Localization of metallothionein expression in cell types and transplant biopsies

In order to determine which cell types express MT-1, we quantified MT-1 expression in a panel of various cell types (Figure 8). High MT-1 expression was seen in activated macrophages and proximal tubular epithelial cells (PTECs). The expression in PTECs was comparable to that in pretransplant biopsies (first bar). Endothelial cells from human aortas and umbilical veins showed intermediate MT-1 expression.

To investigate which cells express MT proteins within the grafts, we performed immunohistochemical stainings for MT on paraffin-embedded kidney allograft biopsy specimens. In accordance with the findings in the cell panel (Figure 8),

protein expression of MT was detected in tubular epithelial cells and inflammatory cells (Figure 9A-D). Co-localization of MT staining (Figure 9C and D) with expression of pan-macrophage marker CD68 (Figure 9E and F) and macrophage activation marker CD163 (Figure 9G and H) supported the observation that MT is expressed by activated macrophages. Stainings for the human Y-chromosome in a female-to-male transplantation revealed that MT is expressed by macrophages that are derived from the recipient (Figure 10).

No differences were found in the mRNA expression levels of macrophage marker CD68 in pretransplant (P=0.75) and acute rejection (P=0.95) biopsy samples, and in the CD68 protein expression (P=0.58) between the steroid response groups (data not shown).

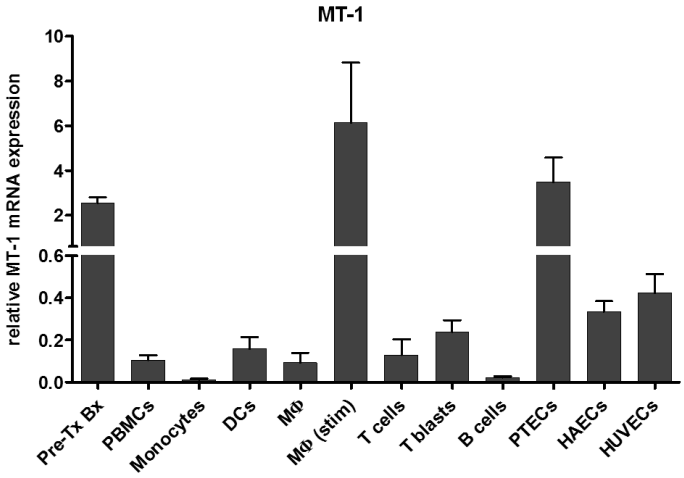


Figure 8. Localization of metallothionein (MT) expression in cell types. MT-1 expression was quantified by qPCR in mRNA from a panel of organs and cell types. High MT-1 expression was seen in activated macrophages (MΦ stim) and proximal tubular epithelial cells (PTECs). Data are expressed as mean ± SEM. Pre-Tx, pretransplant biopsy samples; PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; MΦ, differentiated macrophages; HAECs, human aorta endothelial cells; HUVECs, human umbilical vascular endothelial cells.

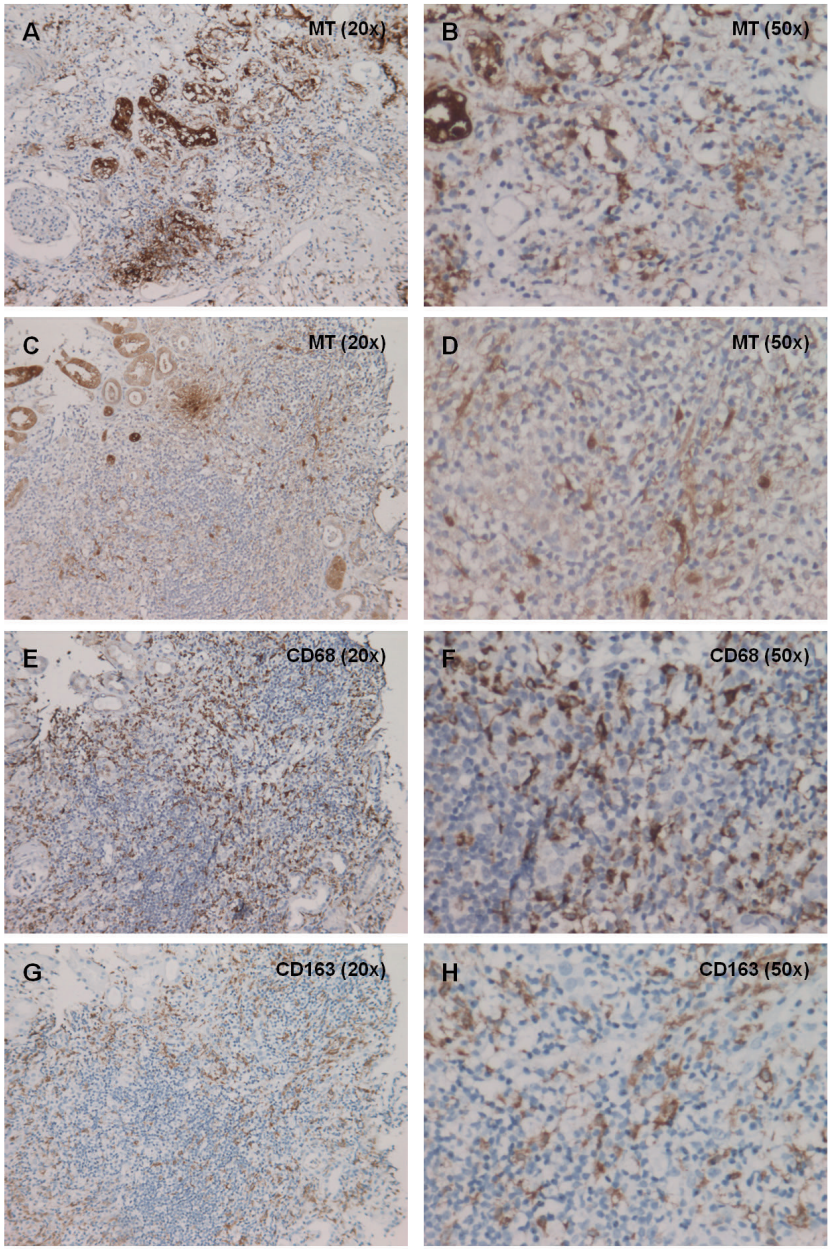


Figure 9. Immunohistochemical staining pattern of metallothionein (MT), CD68 and CD163 in sections from renal transplant biopsies with acute rejection. MT protein was detected in tubular epithelial cells and infiltrating lymphocytes (A-D). Sequential sections stained for MT protein (C and D), the pan-macrophage marker CD68 (E and F), and the macrophage activation marker CD163 (G and H) confirm that MT is expressed by activated macrophages. A, C, E, and G: overview recordings at 20x magnification; B, D, F, and H: enlarged recordings at 50x magnification.

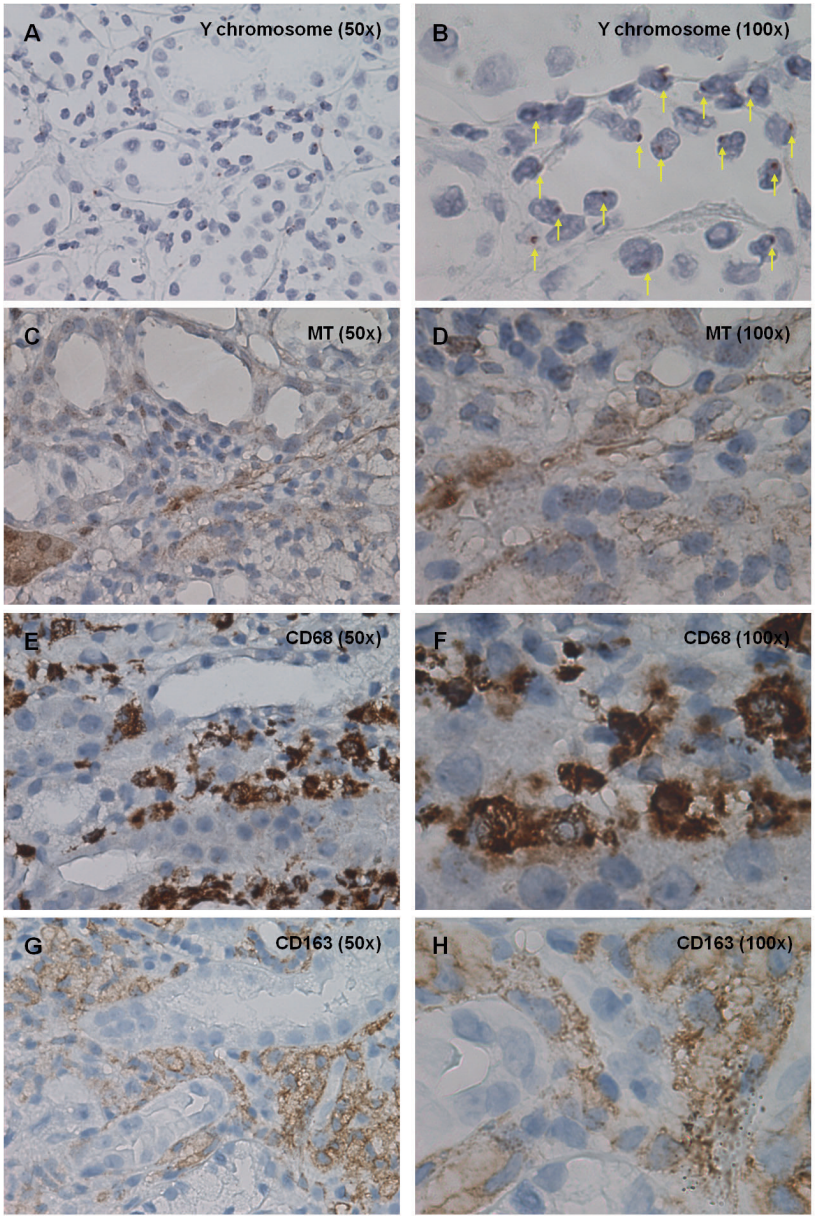


Figure 10. Analysis of the origin of MT positive macrophages. To detect the origin (donor or recipient) of the MT positive macrophages within renal transplant biopsies with acute rejection, we used in situ hybridization for the detection of the Y chromosome in transplant conditions where a male patient received a renal allograft from a female donor. Red-brown dots indicate the presence of Y chromosome-positive cells (yellow arrows) within the allograft (A-B). Sequential sections stained for MT protein (C-D), the pan-macrophage marker CD68 (E-F), and the macrophage activation marker CD163 (G-H) revealed that MT is expressed by macrophages that are derived from the patient. A, C, E, and G: overview recordings at 50x magnification; B, D, F, and H: enlarged recordings at 100x magnification.

***In vitro* methylprednisolone response experiments**

To further investigate the relationship between MT and response to steroid treatment of immune cells, peripheral blood mononuclear cells (PBMC) from healthy blood donors (n=11) were activated with PHA and cultured in the absence or presence of methylprednisolone. Using the *in vitro* cytokine response under the influence of methylprednisolone treatment, a distinction could be made between non-responders (n=3) and responders (n=8). Upon PHA stimulation, IFNγ and TNFα mRNA expression were increased compared to medium controls (Figure 11A and B). Responders to treatment (n=8) showed normalization of both IFNγ and TNFα expression levels when 10^{-4} M methylprednisolone was added during the PHA stimulation. Non-responders (n=3) still showed elevated IFNγ and TNFα mRNA expression even after methylprednisolone treatment (Figure 11A and B). Non-response to methylprednisolone treatment was associated with a significantly stronger increase in MT expression (48.8 fold) compared with what was seen in the responders (5.8 fold) (Figure 11C).

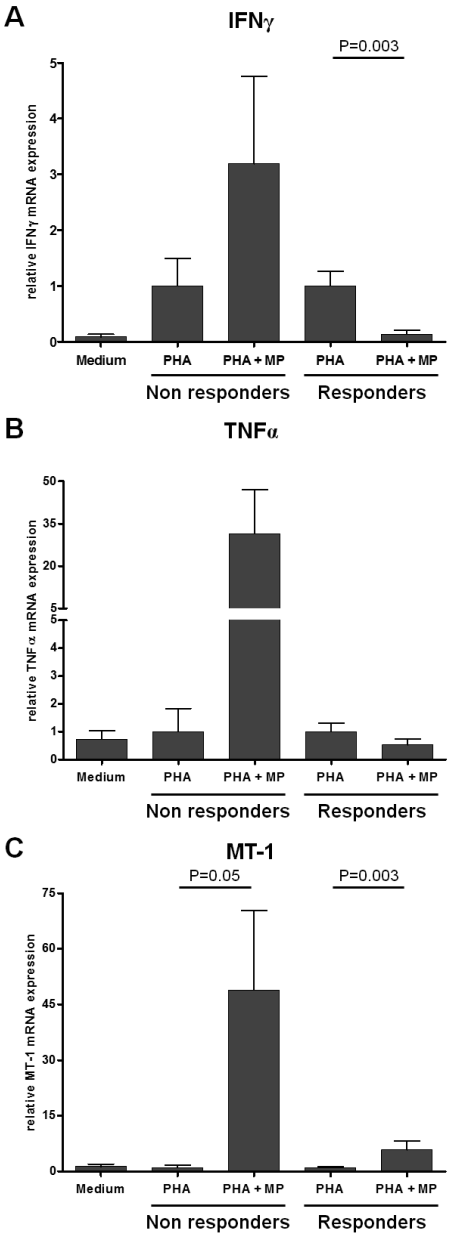


Figure 11. *In vitro* methylprednisolone (MP) response experiments. PBMC from healthy blood donors (n=11) were stimulated (50 h) with PHA or a combination of PHA and 10^{-4} methylprednisolone (PHA + MP). PBMC cultured in medium without a stimulus (50 h) were included as a control. After the culture period, RNA expression levels of IFNγ (A), TNFα (B), and MT-1 (C) were quantified by qPCR. Using the *in vitro* IFNγ and TNFα responses under the influence of methylprednisolone treatment, a distinction was made between non-responders (n=3) and responders (n=8). Data are expressed as mean ± SEM. Mean signals of PHA-stimulated PBMC were set to 1.

Discussion

We aimed to identify novel molecular markers associated with steroid-refractory acute rejection, and to gain insight into the mechanisms underlying steroid resistance. For this, we investigated intragraft gene expression profiles of renal allograft recipients with a first rejection episode. Our study reveals that the expression of metallothioneins (MT) within the renal allograft may distinguish patients with steroid resistant acute rejection from patients with steroid responsive acute rejection. The findings, which were generated in a discovery cohort of 36 patients, were validated in a validation cohort of 47 patients.

Until now, research in the field of steroid-refractory acute rejection in renal allografts has been focused on markers of inflammatory cells (reviewed in [8]). Recently, we found that the combination of T-cell activation markers CD25:CD3 ratio and LAG-3 offers a superior prognostic value for assessing steroid response compared with conventional parameters [9]. In the current study, we found that relatively high intragraft MT expression during acute rejection is associated with steroid resistance, and that MT-1 isoforms are expressed in activated macrophages and in tubular epithelial cells. These findings are in line with earlier findings in lung allograft recipients with steroid-refractory acute rejection. Yousem and colleagues found increased percentages of MT-positive macrophages in transbronchial biopsy samples of lung allograft recipients who experienced steroid-refractory acute rejection [25]. Interestingly, several studies in the oncological research field have also demonstrated that elevated expression of MT is related to treatment resistance [26-28].

In multivariate analysis, the combination of MT-1 with CYP4A11, TIMP1 and F2R represented the best predictive model. This multivariate MT-1 model has a slightly higher predictive value than the T-cell activation model found in our previous study (see Figure 7) [9]. Combination of the two models resulted in a predictive model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates, showing that our current findings strengthen the risk assessment of steroid resistance in patients having acute rejection. Patients with steroid-refractory acute rejection may benefit from immediate ATG treatment after the diagnosis of acute rejection.

Metallothioneins are a family of eleven proteins involved in the homeostasis of biologically essential metals [29-33]. Under physiological conditions MT are mainly expressed in the kidney and liver [30]. MT can bind zinc ions, and by functioning as a

zinc-donor or zinc-acceptor they can control cellular zinc distribution [29;30]. A variety of DNA-binding proteins rely on zinc finger motifs to bind to their target sequences [29;34]. Metallothioneins can influence the DNA-binding capacity of zinc-proteins by controlling the amount of zinc that is available for zinc finger domains [35;36].

The glucocorticoid receptor (GR) is also a zinc-dependent protein [37]. Glucocorticoid effects depend on GR-mediated transcriptional regulation of genes encoding for pro-inflammatory proteins. Binding of GR to glucocorticoid response elements (GRE) in the promoter region of target genes relies on two zinc finger motifs [29;34;37]. Increased expression of metallothioneins may lead to removal of zinc ions that are normally complexed in the zinc finger domains of GR, preventing its binding to GRE and inhibiting the immunomodulatory effects of the steroid-based anti-rejection treatment.

Another mechanism of action of the GR that may be affected by increased MT expression is its recruitment of histone deacetylase (HDAC)-2 [38]. Upon ligand binding the GR becomes a target for HDAC-2, which in turn allows the GR to associate with and suppress the pro-inflammatory transcription factor NF-κB [38;39]. In addition, the recruited HDAC-2 represses transcription from promoters through histone deacetylation, resulting in suppression of activated inflammatory genes within the nucleus [38;40]. As the recruitment of HDAC-2 to the GR relies on zinc, anti-inflammatory effects of this process may also be inhibited by MT.

In vitro tests with PBMC have been used to correlate gene expression profiles with clinical disorders, including steroid responsiveness [41-43]. Following this strategy, we compared the RNA level of pro-inflammatory cytokine responses (TNFα, IFNγ) between stimulated PBMC and stimulated PBMC treated with methylprednisolone. This allowed distinction between responders and non-responders to the steroid treatment. The concentration of 10^{-4} M methylprednisolone approximates the i.v. dosage of 1 g/day given in clinical practice. In all PBMC donors tested, treatment with methylprednisolone led to an increase in MT expression, which corresponds with earlier observations of MT as a steroid responsive gene [44;45]. However, non-responders displayed a significantly higher fold of MT upregulation upon methylprednisolone treatment than responders. These results are in line with the observation that patients who are steroid-resistant upon anti-rejection treatment have elevated MT expression. Further studies are needed to show that *in vitro* cultures of patient PBMC represent a useful indicator of the patient's response to steroid treatment *in vivo*.

Some comments need to be made with regard to the current findings. First, MT expression can be induced by the glucocorticoids [44]. To prevent an influence of steroid-based anti-rejection treatment on the intragraft mRNA and protein expression levels, the biopsy samples investigated in our study were collected before the patients received high-dose methylprednisolone. All patients did receive low-dose steroids as part of the maintenance therapy. Second, due to high homology in the gene sequence of the MT-1 subtypes, it has proven difficult to distinguish the MT-1 subtypes from each other. As our microarray assays rely on 50-bp long probes to measure specific mRNA transcripts, we were able to measure the mRNA expression of the individual MT-1 isotypes. However, no distinction could be made between the MT-1 isoforms with qPCR or immunohistochemistry. The finding by microarray, that most MT-1 isoforms were upregulated in steroid resistant acute rejection, shows that the difficulty to distinguish between MT-1 isoforms likely will not have affected our findings.

In conclusion, resistance to steroid treatment is associated with a relatively high intragraft expression of zinc-regulating MT during acute rejection. MT are mainly expressed by activated macrophages and tubular epithelial cells within the kidney. The relationship between MT and steroid resistance was confirmed *in vitro* by treatment of activated PBMC with corticosteroids. An increased expression of MT may lead to regulation of intracellular zinc concentrations, and to inactivation of the DNA binding capacity of the glucocorticoid receptor. The current findings point to MT as potentially novel therapeutic targets.

Acknowledgements

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Supplemental data

Table S1: Demographic and clinical data of the discovery set and validation set.

Variable	Discovery set (n=36), n (%)	Validation set (n=47), n (%)	P value
Patient age			1.00
≥ 50 years	16 (44.4%)	21 (44.7%)	
Patient gender			0.36
Female	10 (27.8%)	18 (38.3%)	
Donor age			0.36
≥ 50 years	11 (30.6%)	20 (42.6%)	
Donor gender			0.48
Female	22 (61.1%)	33 (70.2%)	
Year of transplantation			0.80
1995 through 1999	26 (72.2%)	36 (76.6%)	
2000 through 2005	10 (27.8%)	11 (23.4%)	
Donor type			0.79
Living	9 (25.0%)	10 (21.3%)	
Post mortal	27 (75.0%)	37 (78.7%)	
HLA-A matching			0.36
≥ 1 mismatch	25 (69.4%)	27 (57.4%)	
HLA-B matching			0.62
≥ 1 mismatch	28 (77.8%)	34 (72.3%)	
HLA-DR matching			1.00
≥ 1 mismatch	23 (63.9%)	30 (63.8%)	
Virtual PRA			1.00
Immunized (6-100%)	9 (25.7%)	13 (27.7%)	
Cold ischemia time			0.30
> 18 hours	20 (64.5%)	30 (76.9%)	
Induction therapy			1.00
Daclizumab	8 (22.2%)	11 (23.4%)	
None	28 (77.8%)	36 (76.6%)	
Maintenance therapy			0.66
Double therapy	16 (44.4%)	24 (51.1%)	
Triple therapy	20 (55.6%)	23 (48.9%)	
Delayed graft function			0.18
Yes	5 (13.9%)	13 (27.7%)	
Rejection time			1.00
<3 months post Tx	35 (97.2%)	46 (97.9%)	
3-6 months post Tx	1 (2.8%)	1 (2.1%)	
C4d staining			0.10
Positive	15 (41.7%)	11 (23.4%)	
Vascular rejection			1.00
Yes	7 (38.9%)	8 (44.4%)	

PRA, panel reactive antibodies; DGF, delayed graft function; Tx, transplantation

Table S2: Gene ontology molecular pathways overrepresented in the intra-graft expressed genes that significantly discriminate patient groups [a].

GO identification	Ontology	P value	Pathway/function	Genes
GO:0046870	MF	9.98E-07	Cadmium ion binding	*MT-1X, MT-1G, MT-1A, MT-1F, MT-1E, MT-1M, MT-1H*
GO:0005576	CC	0.0003	Extracellular region	*SVEP1, FRAS1, GPC6, LAMC3, LYG1, MMP11, ANG, TSKU, MFAP1, COL9A2, F12, REN, PROC, FXYD6, PLA2G7, IDE, TCN2, GC, CHRD, PLA2G15, AGT, ADAMTSL2, TINAG, PLG, FBLN5, TNFRSF1A, AZGP1, SEMA3F, CPN2, PRAP1, ADM2, SERPINA3, NUCB2, STC2, PLA1A, PHGDH, EPHB6, LAMA2, COL7A1, SPP1*
GO:0044421	CC	0.0011	Extracellular region part	*AGT, FRAS1, ADAMTSL2, TINAG, PLG, FBLN5, GPC6, LAMC3, SEMA3F, MMP11, ANG, MFAP1, COL9A2, F12, REN, PROC, NUCB2, FXYD6, PLA2G7, LAMA2, IDE, COL7A1, TCN2, GC, CHRD, SPP1*
GO:0044420	CC	0.0038	Extracellular matrix part	*MFAP1, COL9A2, FRAS1, TINAG, LAMC3, LAMA2, COL7A1, ANG*
GO:0005578	CC	0.0038	Proteinaceous extracellular matrix	*MFAP1, COL9A2, FRAS1, ADAMTSL2, TINAG, FBLN5, LAMC3, GPC6, LAMA2, COL7A1, MMP11, ANG*
GO:0005507	MF	0.0082	Copper ion binding	*MT-1X, MT-1G, MT-1A, MT-1F, MT-1E, MT-1M, MT-1H, ANG*
GO:0004091	MF	0.0082	Carboxylesterase activity	*PLA2G15, IPO4, NLGN1, PLA2G4F, ACOT4, PLA2G7, PLA1A, MGLL*
GO:0031012	CC	0.0122	extracellular matrix	*MFAP1, COL9A2, FRAS1, ADAMTSL2, TINAG, FBLN5, LAMC3, GPC6, LAMA2, COL7A1, MMP11, ANG*
GO:0005604	CC	0.0153	Basement membrane	*LAMC3, LAMA2, FRAS1, COL7A1, TINAG, ANG*
GO:0042598	CC	0.0175	Vesicular fraction	*CYP4A11, HSP90B1, CYP8B1, ERO1L, SEC11C, UGT1A9, UGT1A6, PLG, TALDO1, KCNJ8, SPCS3, RAB38*
GO:0032787	BP	0.0223	Monocarboxylic acid metabolic process	*PLA2G15, AGT, ACADSB, ACADL, AMACR, ALDH1L1, ACOT4, TNFRSF1A, ALDOB, CYP4A11, PCCB, RBP1, ABHD5, ECHS1, AGXT, AKR1C3, ACADS, MCCC2*
GO:0006544	BP	0.0223	Glycine metabolic process	*PHGDH, GCSH, SHMT2, AGXT*
GO:0006082	BP	0.0223	Organic acid metabolic process	*PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, DOCK6, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS*
GO:0019752	BP	0.0282	Carboxylic acid metabolic process	*PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS*
GO:0042180	BP	0.0282	Cellular ketone metabolic process	*PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS*

GO:0043436	BP	0.0282	Oxoacid metabolic process	*PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS*
GO:0009069	BP	0.0318	Serine family amino acid metabolic process	*PHGDH, GCSH, SHMT2, AGXT*
GO:0006629	BP	0.0318	Lipid metabolic process	*PLA2G15, AGT, ACADSB, IPO4, CYP27B1, ACADL, AMACR, ACOT4, TNFRSF1A, MGLL, AZGP1, ANG, CYP4A11, ABHD5, PCCB, RBP1, SERPINA3, ECHS1, PLA2G4F, PLA1A, PLA2G7, GDPD5, ETNK2, AKR1C3, GC, SPTLC3, HSD17B8, MCCC2, ACADS*
GO:0000267	CC	0.0325	Cell fraction	*PLA2G15, STK39, CRYAB, AGT, SLC1A5, ERO1L, GSTM3, SEC11C, CHST10, SPTAN1, PLG, FBLN5, KCNJ8, ACY3, SLC16A2, GCHFR, RAB38, CYP4A11, CYP8B1, HSP90B1, GCNT3, ZFP106, UGT1A9, TALDO1, UGT1A6, SPCS3, EFNB1, IDE, HSD17B8, CDH5*
GO:0005792	CC	0.0357	Microsome	*CYP4A11, HSP90B1, CYP8B1, ERO1L, SEC11C, UGT1A9, UGT1A6, PLG, TALDO1, KCNJ8, SPCS3*
GO:0006631	BP	0.0376	Fatty acid metabolic process	*CYP4A11, PLA2G15, ABHD5, PCCB, AGT, ACADSB, ECHS1, ACADL, ACOT4, TNFRSF1A, AKR1C3, MCCC2, ACADS*
GO:0007155	BP	0.0385	Cell adhesion	*SVEP1, SETBP1, AGT, FRAS1, AKAP8L, CHST10, CLDN8, CLDN12, TINAG, FBLN5, LAMC3, AZGP1, MUPCDH, BYSL, ACVRL1, CDH1, NLGN1, ADAM15, LAMA2, EFNB1, COL7A1, SPP1, CHRD, CDH5*
GO:0050327	MF	0.0463	Testosterone 17-beta-dehydrogenase activity	*AKR1C3, HSD17B8*
GO:0008453	MF	0.0463	Alanine-glyoxylate transaminase activity	*AGXT, AGXT2*
GO:0005243	MF	0.0463	Gap junction channel activity	*PANX2, GJB2*
GO:0005212	MF	0.0463	Structural constituent of eye lens	*CRYAA, CRYAB*
GO:0022829	MF	0.0463	Wide pore channel activity	*PANX2, GJB2*
GO:0005615	CC	0.0490	Extracellular space	*AGT, PLG, GPC6, SEMA3F, ANG, F12, REN, PROC, NUCB2, FXYD6, PLA2G7, IDE, GC, TCN2, CHRD, SPP1*

[a] All 249 genes significantly discriminating steroid responsive and steroid resistant patients were included for pathway analysis by hypergeometric test in WebGestalt. All P values are adjusted for multiple testing, using the Benjamini-Hochberg method. GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component.

Donor genotype and intragraft expression of CYP3A5 reflect the response to steroid treatment during acute renal allograft rejection

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Abstract

Steroid-refractory acute rejection is an important risk factor for inferior renal allograft outcome. We investigated the relationship between the genotype of glucocorticoid signaling and metabolism genes and the response to steroid treatment in renal allograft recipients with acute rejection. One hundred and fifty three kidney transplant recipients (1995-2005), who were treated with high dose i.v. methylprednisolone during a first acute rejection episode, were included in the retrospective study. Maintenance therapy consisted of a double regimen (prednisone, calcineurin inhibitor: Cyclosporine or Tacrolimus) or triple regimen (also with MMF). We analyzed single nucleotide polymorphism genotypes of genes involved in glucocorticoid signaling (Glucocorticoid Receptor, GLCCI1) and drug metabolism (CYP3A5, ABCB1, and PXR) in both recipients and donors. CYP3A5 genotype of the donor kidney was the only variable showing a significant association (P=0.039) with response to steroid treatment. In subanalyses, similar trends were observed between patients receiving Cyclosporine and patients receiving Tacrolimus. Patients who had received a renal allograft from a CYP3A5*1-allele-expressing donor had a 3.3 times lower risk of resistance to steroid treatment during acute rejection (odds ratio=0.3; 95% confidence interval, 0.10–1.0; P=0.047). CYP3A5*1 donor grafts (n=20) displayed significantly higher intragraft CYP3A5 mRNA expression levels than allografts from CYP3A5*3/*3 carriers (n=105; P<0.00001). Accordingly, significantly higher CYP3A5 mRNA levels were seen in steroid responsive patients compared to steroid resistant patients (P=0.006). By immunohistochemistry, distal and proximal tubules were found to abundantly express CYP3A5 protein. In a panel of different cell types, proximal tubular epithelial cells and activated macrophages showed the highest CYP3A5 mRNA expression. No differences were observed for glucocorticoid signaling or other drug metabolism-related genes. Our study shows that resistance to steroid treatment during acute rejection is associated with donor genotype and intragraft expression levels of CYP3A5. We speculate that recipients receiving kidneys with a high expressing CYP3A5*1 genotype are better protected against rejection injury and therefore are less resistant to steroid treatment.

Introduction

High-dose glucocorticoids (GC) is the preferred immunosuppressive treatment of acute rejection (AR) after kidney transplantation. Approximately 30% of the patients show no or an inadequate response to steroid AR therapy alone [1-3]. This occurrence of steroid-refractory AR is an important risk factor for inferior renal allograft outcome [4-7]. Current diagnostic procedures have demonstrated poor prediction for steroid resistance. The role of glucocorticoid signaling and drug metabolism in steroid-refractory AR has not been thoroughly examined. Differences in response to steroid treatment during AR may be explained by genetic polymorphisms in genes involved in glucocorticoid signaling and drug metabolism.

GC exert their anti-inflammatory and immunosuppressive action via the glucocorticoid receptor (GR) [8;9]. Single nucleotide polymorphisms (SNPs) in *NR3C1*, the gene encoding the GR, may affect the ability of this receptor to bind GC [10;11]. *NR3C1* polymorphisms have been associated with both enhanced sensitivity to GC (N363S, *Bcl*I) as well as GC resistance (ER22/23EK, GR-9β) (reviewed in [12]). A recently identified gene linked to steroid response is the glucocorticoid-induced transcript 1 gene (GLCCI1). The rs37972 SNP in the promoter region of GLCCI1 was associated with the response to glucocorticoid treatment in asthma [13], COPD [14], and bacterial meningitis [15].

Other potential genes involved in steroid responsiveness are drug metabolism-related genes. The widely expressed CYP3A enzymes metabolize 50% to 60% of all xenobiotics, including glucocorticoids [9;16]. CYP3A5 is the predominant drug metabolizing enzyme expressed within the kidney [17;18]. A SNP in intron 3 of the *CYP3A5* gene (CYP3A5*3) correlates with the level of renal CYP3A5 expression [19-21]. The efflux transporter P-glycoprotein, encoded by the *ABCB1* gene, is mainly expressed in the kidney and small intestine where it limits the absorption of xenobiotics, including glucocorticoids [16;22;23]. Three ABCB1 SNPs (C3435T, G2677T, and C1236T) have been associated with increased graft loss [24], increased susceptibility to chronic allograft damage [25] in renal transplant recipients, and with steroid resistance in patients with nephrotic syndrome [26]. The pregnane X receptor (PXR), encoded by the *NR1I2* gene, is a master transcriptional regulator of genes involved in metabolism and transport of xenobiotics [16;27;28]. Ligand activation of PXR leads to upregulation of a system of drug detoxification genes, including CYP3A5 and ABCB1 [29;30]. A SNP in *NR1I2* (A7635G) has

been associated with altered CYP3A4 regulation [31], and higher metabolic activity for prednisolone in renal transplant recipients [11;16].

In this study we investigated the impact of polymorphisms in genes involved in glucocorticoid signaling (GR and GLCCI1) and drug metabolism (CYP3A5, ABCB1, and PXR) upon the response to high-dose steroid treatment of AR and death-censored graft survival in renal allograft recipients.

Materials and methods

Patient information

All patients who underwent kidney transplantation in the Leiden University Medical Center (Leiden, the Netherlands) between 1995 and 2005, and who suffered from a histologically proven first rejection episode were considered for investigation. Only patients who received corticosteroids as anti-rejection treatment were included (n=153). Maintenance immunosuppression consisted of a calcineurin inhibitor (Cyclosporine A or Tacrolimus) and prednisone, with or without an antimetabolite (Mycophenolate Mofetil). Immunosuppression for the treatment of acute rejection consisted of pulse therapy for 3 days with a 1-g bolus of intravenous methylprednisolone daily.

Clinical endpoints

The primary clinical endpoint was response to anti-rejection treatment with methylprednisolone, as described previously [7]. Briefly, steroid resistant acute rejection was defined as lack of clinical response to steroid pulse therapy, and a requirement for anti-thymocyte globulin treatment within 14 days after the start of the steroid therapy. The response to steroid therapy was monitored by creatinine concentration measurements. A second clinical endpoint was graft survival, defined as time from transplant to graft failure, censoring for death with a functioning graft and grafts still functioning at time of analysis.

Patient materials

For SNP analysis, DNA was extracted from peripheral blood cells or splenocytes by the Chelex procedure or by classic salting out technique. DNA was available from 143

patients and 125 donors. From each patient, at least two renal biopsy cores were collected on clinical indication of acute rejection. One biopsy core was formalin fixed and embedded in paraffin. The second biopsy core was immediately snap frozen in liquid nitrogen and stored at -80°C. Rejection severity of all patients was blindly scored by two independent pathologists (I.B. and M.G.) on paraffin-embedded biopsy tissue according to Banff 2011 criteria [32].

All biopsy specimens obtained prior to the start of anti-rejection treatment were investigated further for mRNA and protein expression levels. Frozen biopsy material for gene expression analysis was available from 106 patients. Paraffin embedded biopsy material for protein stainings was available from 123 patients. In addition to the biopsies obtained during acute rejection (AR biopsy samples), snap frozen pretransplant (Pre-Tx) biopsy samples (stored at -80°C) were available from 77 patients.

Quantitative real-time PCR detection of SNPs

SNPs characterization was performed using quantitative real-time PCR (qPCR) assays. A total of 10 SNPs were investigated in DNA from both recipients and donors. Five SNPs were analyzed using SYBR Green-based qPCR assays, and 5 SNPs were analyzed using TaqMan-based qPCR assays. The primers and qPCR approach used are summarized in Table S1.

SYBR Green qPCR assay

Genotyping for the NR3C1 1184+646C>G (rs41423247) and G68A (rs6190) SNPs, and ABCB1 C3435T (rs1045642), G2677T (rs2032582), and C1236T (rs1128503) SNPs was performed using SYBR Green-based qPCR with in-house developed primers (see Table S1A). PCR was performed using a Bio-Rad MyiQ (Bio-Rad, Veenendaal, the Netherlands). Each PCR reaction contained 20 ng of DNA. The PCR program consisted of one cycle at 94°C for 10 min; 10 cycles of 94°C for 15 sec and 66°C for 45 sec; 30 cycles of 94°C for 15 sec, 63°C for 45 sec, and 72°C for 30 sec; and was finalized with a melting curve analysis.

TaqMan qPCR assay

Genotyping for the NR3C1 A1088G (rs56149945), NR3C1 A6167G (rs6198), GLCCI1 -1473C>T (rs37972), CYP3A5 A6986G (rs776746), and NR1I2 A7635G

(rs6785049) polymorphisms was performed using TaqMan SNP genotyping assays (C_26841917_40, C_8951023_10, C_970132_10, C_26201809_30, C_29280426_10; Applied Biosystems, Carlsbad, CA) and qPCR methodology on a StepOne Real-Time PCR System (Applied Biosystems). Each TaqMan assay was used according to the protocol recommended by the manufacturer. PCR reactions contained between 10 and 45 ng of DNA. The PCR program consisted of one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 50 cycles of 92°C for 15 sec and 60°C for 90 sec. Genotypes were determined based on the emission pattern of the fluorescent reporter dyes (VIC or FAM) attached to the primers (see Table S1B).

Gene expression analysis

Real-time qPCR was used to analyze the mRNA expression of CYP3A5, ABCB1, and glucocorticoid receptor α (GRα) in the pretransplant and AR biopsy samples. RNA isolation and cDNA synthesis procedures have previously been described [7]. Briefly, RNA was extracted from eight to ten 10-µm sections of renal cortex from each snap frozen biopsy core. On average, 0.89 ± 0.19 µg of isolated RNA was transcribed into cDNA. The cDNA samples were diluted 25 times. Primer design, reactions and PCR conditions have been described previously [7;33]. Primer sequences are provided in Table S2. PCR efficiencies were between 90% and 110%. Relative gene expression levels were determined on the basis of a standard curve (in duplicate) of five serial dilution points of reference cDNA (qPCR Human Reference Total RNA, Clontech, France). Differences in mRNA expression levels were normalized to the geometric mean signal of reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin.

CYP3A5 and ABCB1 expression in various tissues and cell types

Expression levels of CYP3A5 and ABCB1 were investigated in a panel of various tissues and cell types [34]. Included in this panel were liver, spleen, peripheral blood mononuclear cell fractions (n=9), cultured dendritic cells (GM-CSF and IL-4, without or with IFN-γ/IFN-β/LPS stimulation for 6 h, n=6), CD14+ monocytes (n=3), differentiated macrophages (5 to 8 days with GM-CSF or M-CSF, n=7), stimulated macrophages (10 ng IFN-γ or 100 ng LPS for 24 h, n=6), T cells (n=5), CD19+ B cells (n=6), proximal tubular epithelial cells (PTECs; n=2), human aorta endothelial cells (HAECs; n=5), and human umbilical vascular endothelial cells (HUVECs; n=5).

Measurements in pretransplant biopsy samples (n=77) and acute rejection biopsy samples (n=84) were included as references.

Protein expression of GR and metabolism-related genes

Immunohistochemical analyses were performed on paraffin-embedded biopsy samples from the patients. Protein stainings for CYP3A5 and ABCB1 were performed on sequential sections (4 µm). Polyclonal rabbit anti-human antibodies against CYP3A5 (Abcam, Cambridge, UK; ab22692) and monoclonal mouse anti-human antibodies against ABCB1 (Tebu-bio, Heerhugowaard, the Netherlands; clone JSB-1) were used for immunohistochemical stainings. Standardized protocols [35] were followed. Details concerning antigen retrieval and staining procedure are provided in Table S3. After endogenous peroxidase blocking with 0.1% H_2O_2, non-specific antibody binding was blocked with normal goat serum (NGS; Dako, Glostrup, Denmark). Visualization was performed with Rabbit-EnVision+ or Mouse-EnVision+ System-HRP (DAB) according to the manufacturers' (Dako) protocols. Protein stainings were scored by two independent researchers (N.R. and M.E.) using a semi-quantitative scoring system. Photos were taken with a Leica Leitz DMRD microscope with Leica DFC420 C camera and Leica Application Suite software (Leica Microsystems, Rijswijk, the Netherlands).

Statistics

Comparison of categorical data between steroid response groups was evaluated using the Pearson chi-square test. Comparison of SNP genotypes between steroid response groups was evaluated using the Pearson chi-square test or Fisher's Exact test, depending on the genotype distribution. Univariate logistic regression analysis was used to analyze the discriminative value of the SNPs. Kaplan-Meier curves with log-rank testing and Cox regression analyses were used to test for associations of SNP genotypes with death-censored graft loss. Genotypes were analyzed for deviations from Hardy-Weinberg equilibrium. Differences in mRNA expression between steroid response groups were analyzed with the independent samples t-test, and differences between mRNA levels measured in pretransplant and acute rejection biopsy samples were analyzed with the paired samples t-test. Differences in mRNA expression between SNP genotypes were analyzed with the Mann-Whitney U-test. The Spearman's rho and Kruskal-Wallis test were used to analyze the correlation between the mRNA expression levels and the protein stainings. Data from qPCR measurements

are presented as means ± standard error of the mean (SEM). All statistical tests were two-sided, and P values less than 0.05 were considered significant. Statistical analyses were performed using the SPSS statistical package (IBM SPSS Statistics., version 20.0.0, IBM, Amsterdam, the Netherlands).

Results

Demographics and clinical data

Demographic and clinical characteristics of the study cohort have been summarized in Table 1. No associations were found between the characteristics and the response to steroid treatment, except for vascular rejection. Steroid-resistant patients (n=60) experienced more vascular rejection compared with steroid-responsive patients (n=93) (P=0.08, Table 1). No significant differences were found in C4d positivity (P=0.29, Table 1).

Table 1. Demographic and clinical data of patients with steroid responsive and steroid resistant acute rejection.

Variable	Steroid responsive (N=93), n (%)	Steroid resistant (N=60), n (%)	P value
Patient age			0.54
≥ 50 years	45 (48.4%)	26 (43.3%)	
Patient gender			1.00
Female	31 (33.3%)	20 (33.3%)	
Donor age			0.34
≥ 50 years	38 (41.3%)	29 (49.2%)	
Donor gender			0.27
Female	61 (65.6%)	34 (56.7%)	
Year of transplantation			0.10
1995 through 1999	58 (62.4%)	45 (75.0%)	
2000 through 2005	35 (37.6%)	15 (25.0%)	
Donor type			0.50
Living	23 (24.7%)	12 (20.0%)	
Post mortal	70 (75.3%)	48 (80.0%)	
HLA-A matching			0.57
≥ 1 mismatch	66 (71.0%)	40 (66.7%)	
HLA-B matching			0.80
≥ 1 mismatch	68 (73.1%)	45 (75.0%)	
HLA-DR matching			0.45
≥ 1 mismatch	58 (62.4%)	41 (68.3%)	
Virtual PRA			0.31
Immunized (6-100%)	28 (30.4%)	23 (38.3%)	
Cold ischemia time			0.13
> 18 hours	48 (62.3%)	39 (75.0%)	
Induction therapy			0.40
Daclizumab	29 (31.2%)	14 (23.3%)	
None	64 (68.8%)	46 (76.7%)	
Maintenance therapy			0.15
Double therapy	31 (33.3%)	27 (45.0%)	
Triple therapy	62 (66.7%)	33 (55.0%)	
Delayed graft function			0.96
Yes	28 (30.4%)	18 (30.0%)	
Rejection time			0.96
<3 months post Tx	87 (93.5%)	56 (93.3%)	
3-6 months post Tx	6 (6.5%)	4 (6.7%)	
C4d staining			0.28
Positive	6 (9.7%)	8 (16.7%)	
Rejection severity [a]			0.08
Borderline rejection	39 (45.9%)	22 (40.0%)	
T cell mediated rejection	25 (29.4%)	10 (18.2%)	
Vascular rejection	21 (24.7%)	23 (41.8%)	

[a] Nine biopsies did not contain sufficient cortex to determine a Banff score. Four additional patients had a clinical rejection (with i- and t-scores of 0).
PRA, panel reactive antibodies; Tx, transplantation

Association of polymorphisms with graft outcome

Using quantitative real-time PCR (qPCR) we characterized the genotype of 10 SNPs in a cohort of renal allograft recipients (n=143) and the corresponding donors (n=125). Genotype distribution of the SNPs has been summarized in Table S4. Due to the low frequency of homozygosity for the mutant variant of the CYP3A5*1 SNPs (rs776746) and NR3C1 GR-9β (rs6198), we combined the homozygous mutant variants with the heterozygous groups of these two SNPs for all statistical analyses. The allele frequencies of the SNPs were in Hardy-Weinberg equilibrium, except for the recipient allele frequencies of NR3C1 *Bcl*1, NR3C1 GR-9β, and ABCB1 G2677T (see Table S5).

Analysis of the polymorphisms in drug metabolism and detoxification genes showed that the expression of the CYP3A5*1 allele variant in the donors is associated with steroid responsiveness (P=0.039, Table 2). Fifty five percent of the patients with a CYP3A5*3/*3 allograft were steroid responsive, while 80% of the patients who received a renal allograft from a CYP3A5*1 variant expressing donor were steroid responsive (Table 3). No association was observed between steroid response and the CYP3A5*1 variant in the recipients (Table 2). In univariate logistic regression analysis, the donor CYP3A5*1 variant predicted lower risk of steroid resistant AR (odds ratio = 0.3; 95% confidence interval, 0.10–1.0; P=0.047).

None of the ABCB1 or NR1I2 allele variants in the recipients or donors showed a significant association with response to steroid treatment (Table 2). We found no association between the response to steroid therapy and polymorphisms in genes involved in glucocorticoid signaling. None of the NR3C1 or GLCCI1 allele variants in the recipients or donors showed a significant association with steroid response (see Table 2).

Analysis of the relation between polymorphisms and death-censored graft survival at 6 years after transplantation revealed no associations between SNP genotypes in the recipients or donors and the risk of graft loss. In addition, graft survival showed no association with recipient- or donor-expression of the ABCB1 variant haplotype 1236T/2677T/3435T (data not shown).

Table 2. Associations between single nucleotide polymorphisms and response to steroid therapy.

Gene	SNP	rs number	Recipient	Donor
NR3C1	N363S	rs6195	1.00 [a]	0.44 [a]
NR3C1	ER22/23EK	rs6189/rs6190	0.37 [a]	0.74 [a]
NR3C1	*Bcl*1	rs41423247	0.68	0.41
NR3C1	GR-9β	rs6198	0.16 [b]	0.36 [b]
GLCCI1	-1473 C>T	rs37972	0.61	0.42
CYP3A5	CYP3A5*3	rs776746	0.49 [b]	0.039 [b]
ABCB1	C1236T	rs1128503	0.16	0.87
ABCB1	G2677T	rs2032582	0.45	0.99
ABCB1	C3435T	rs1045642	0.69	0.40
NR1I2	A7635G	rs6785049	0.87	0.87

[a] P values were obtained with the Pearson chi-square test, except for the N363S and ER22/23EK SNPs, which were obtained with the Fisher's Exact test. This was due to the low genotype frequency of the mutant variant (see Table S4).
[b] Due to too few patients homozygous for the mutant variant of the SNP, we combined this patient group with the heterozygous patient group for all statistical analyses.

Table 3. Associations between donor CYP3A5 genotype and response to steroid therapy.

Genotype	Steroid responsive (N=74), n (%)	Steroid resistant (N=51), n (%)	Total
Donor CYP3A5*1	16 (80.0%)	4 (20.0%)	20
Donor CYP3A5*3/*3	58 (55.2%)	47 (44.8%)	105
Total	74 (59.2%)	51 (40.8%)	125

Donor CYP3A5*1 genotype indicates that the donor expressed the CYP3A5*1 variant. This group includes both CYP3A5*1/*1 homozygous and CYP3A5*1/*3 heterozygous donors.

Genotype dependent CYP3A5 mRNA expression

We analyzed whether donor CYP3A5 genotype is associated with CYP3A5 mRNA expression levels in the graft. Comparison between genotype groups showed a significant effect of the donor CYP3A5*1 allele on CYP3A5 mRNA levels (Figure 1A). The expression levels of CYP3A5 in the AR biopsy samples were significantly higher in patients who had received a renal allograft from a CYP3A5*1/*1 (n=2; P=0.0042) or a CYP3A5*1/*3 (n=18; P=0.000001) donor compared with patient who had received a CYP3A5*3/*3 allograft (n=105). Similar results were observed in the pretransplant biopsy samples (P=0.0012, Figure 1A). No significant differences in characteristics were observed between donor CYP3A5*1 allele carriers and donor CYP3A5*3/*3 individuals (Table 4), except for the time of rejection (P=0.036).

Table 4. Characteristics according to the presence or absence of the donor CYP3A5*1 allele.

Variable	Donor CYP3A5 genotype [a]		P value
	CYP3A5*3/*3 (N=105), n (%)	CYP3A5*1 (N=20), n (%)	
Patient age			0.38
≥ 50 years	48 (45.7%)	7 (35.0%)	
Patient gender			0.86
Female	39 (37.1%)	7 (35.0%)	
Donor age			0.13
≥ 50 years	53 (51.5%)	14 (70.0%)	
Donor gender			0.92
Female	59 (56.2%)	11 (55.0%)	
Donor type			1.00
Living	11 (10.5%)	2 (10.0%)	
Post mortal	94 (89.5%)	18 (90.0%)	
HLA-AB matching			0.52
≥ 1 mismatch	86 (81.9%)	18 (90.0%)	
HLA-DR matching			0.44
≥ 1 mismatch	64 (61.0%)	14 (70.3%)	
Maintenance therapy			0.60
Double therapy	38 (36.2%)	6 (30.0%)	
Triple therapy	67 (63.8%)	14 (70.0%)	
Rejection time			0.036
<3 months post Tx	100 (95.2%)	16 (80.0%)	
3-6 months post Tx	5 (4.8%)	4 (20.0%)	
Rejection severity [b]			0.43
Borderline rejection	43 (44.8%)	6 (31.6%)	
T cell mediated rejection	19 (19.8%)	6 (31.6%)	
Vascular rejection	34 (35.4%)	7 (36.8%)	

[a] Donor CYP3A5*1 genotype indicates that the donor expressed the CYP3A5*1 variant. This group includes both CYP3A5*1/*1 homozygous and CYP3A5*1/*3 heterozygous donors.
[b] Nine biopsies did not contain sufficient cortex to determine a Banff score. Four additional patients had a clinical rejection (with i- and t-scores of 0).
Tx, transplantation

mRNA expression of metabolism-related genes and the glucocorticoid receptor

No significant differences were found in pretransplant intragraft mRNA expression levels of CYP3A5, ABCB1 and GRα between the steroid responsive and steroid resistant groups (Figures 1B and 2). CYP3A5 mRNA expression was significantly higher in the steroid responsive AR group compared with the steroid resistant AR group (P=0.006, Figure 1B). Steroid resistant patients showed significantly lower CYP3A5 mRNA levels in the AR biopsy samples compared with the pretransplant biopsy samples (P<0.001, Figure 1B). The expression of ABCB1 mRNA was significantly higher in the steroid

responsive AR group compared with the steroid resistant AR group (P=0.036, Figure 2A). No significant differences were observed in intragraft GRα mRNA expression between the steroid responsive and steroid resistant patient groups. The GRα expression level was significantly increased in the AR biopsy samples compared with the pretransplantation graft biopsies (P<0.00001, Figure 2B).

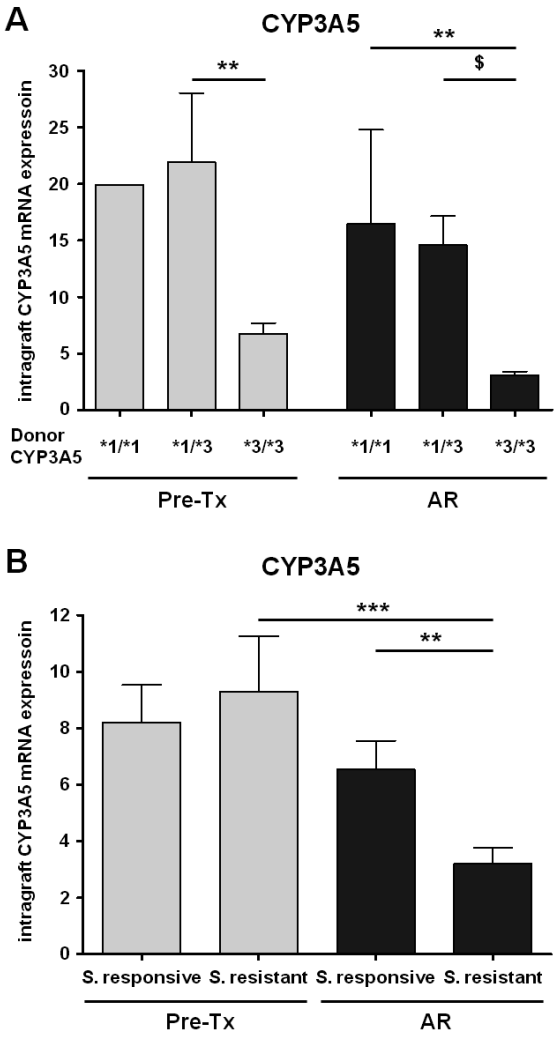


Figure 1. RNA expression of the metabolism-related gene CYP3A5 in renal allograft biopsy samples. Expression levels of CYP3A5 mRNA, measured with qPCR in pretransplant (Pre-Tx) and acute rejection (AR) biopsy samples, according to the respective donor CYP3A5 genotype group (A) and the steroid response groups (B). Data are expressed as means ± SEM. **, P<0.01; ***, P<0.001; $, P<0.00001.

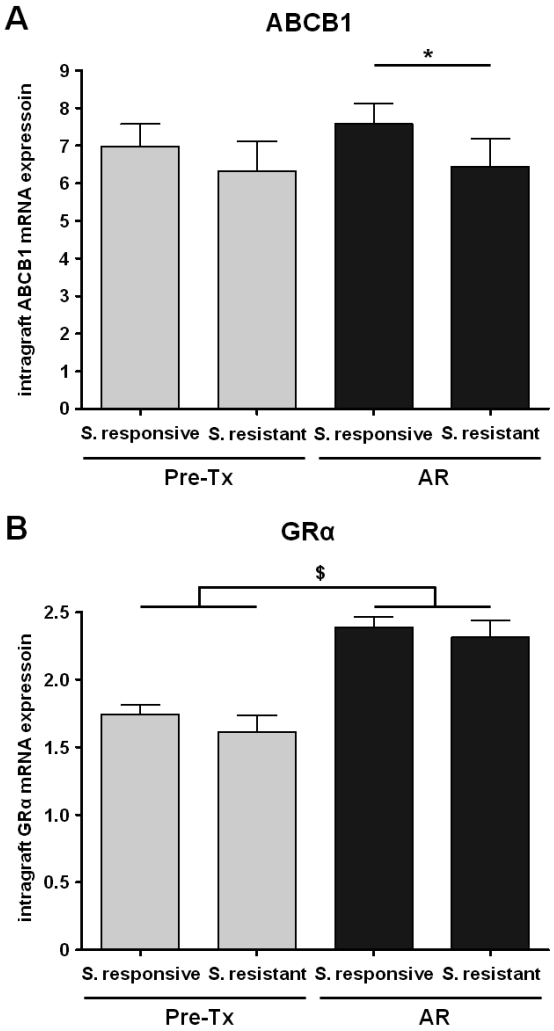


Figure 2. RNA expression of metabolism-related gene ABCB1 and the glucocorticoid receptor in renal allograft biopsy samples. RNA expression levels of ABCB1 (A) and GRα (B) measured in pretransplant (Pre-Tx) and acute rejection (AR) biopsy samples from steroid resistant and steroid responsive patients. Data are expressed as means ± SEM. *, P<0.05; $, P<0.00001.

Localization of CYP3A5 and ABCB1 expression

We quantified the expression of CYP3A5 and ABCB1 on a panel of mRNA samples from various tissues and cell types (Figure 3). We observed relatively high expression levels of both CYP3A5 and ABCB1 in pretransplant and AR rejection biopsies from renal allografts, which were similar to the expression levels seen in the liver. CYP3A5 expression was seen in macrophages and proximal tubular epithelial cells (PTECs)

(see Figure 3A), while ABCB1 was highly expressed by T cells and to a lesser extent by peripheral blood mononuclear cells, B cells and PTECs (see Figure 3B).

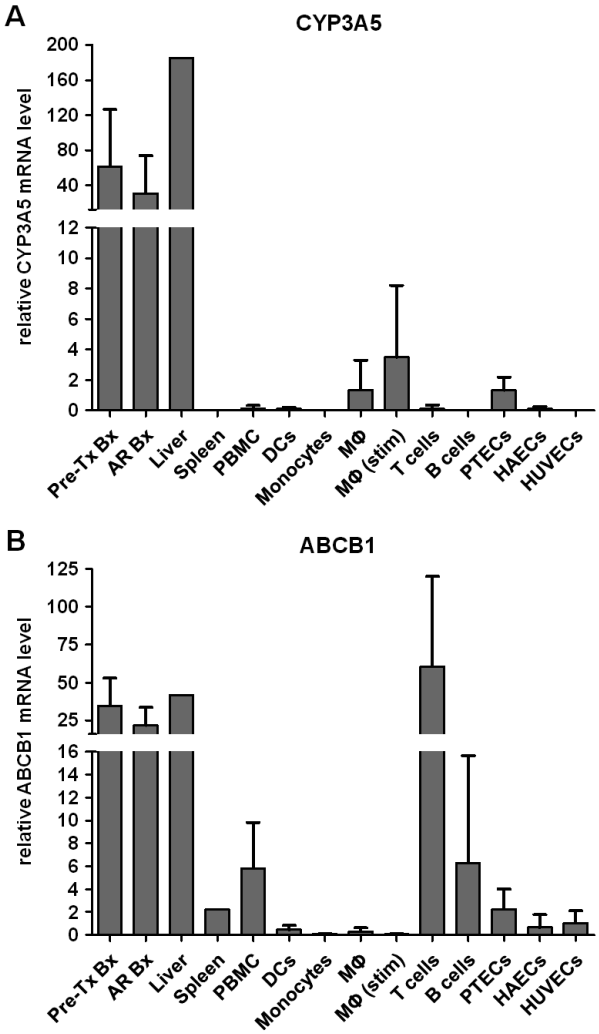


Figure 3. Localization of CYP3A5 and ABCB1 expression in various tissues and cell types. CYP3A5 (A) and ABCB1 (B) expression was quantified by qPCR in mRNA from a panel of organs and cell types. A) High CYP3A5 expression was seen in the liver, the kidney (both before and after transplantation), and in macrophages and proximal tubular epithelial cells (PTECs). B) ABCB1 was highly expressed in the liver, the kidney (both before and after transplantation), and in T cells. Intermediate ABCB1 expression was seen in peripheral blood mononuclear cells (PBMC), B cells and PTECs. Data are expressed as mean ± SEM.

Using immunohistochemical stainings we investigated the expression of CYP3A5 and ABCB1 proteins within the graft. In accordance with the mRNA expression levels in the cell panel, CYP3A5 protein expression was detected in inflammatory cells and tubular epithelial cells (Figure 4). The level of intragraft expression of CYP3A5 protein observed in the patient cohort varied between high (Figure 4A and B) and low (Figure 4C and D), but showed no significant difference between the steroid response groups (data not shown). Correlation analysis showed a weak correlation between the mRNA and protein expression of CYP3A5 ($P<0.05$, $\rho=0.21$). ABCB1 protein expression was detected in inflammatory cells and tubular epithelial cells (Figure 5). No correlation was found between ABCB1 staining and the response to steroid treatment (data not shown).

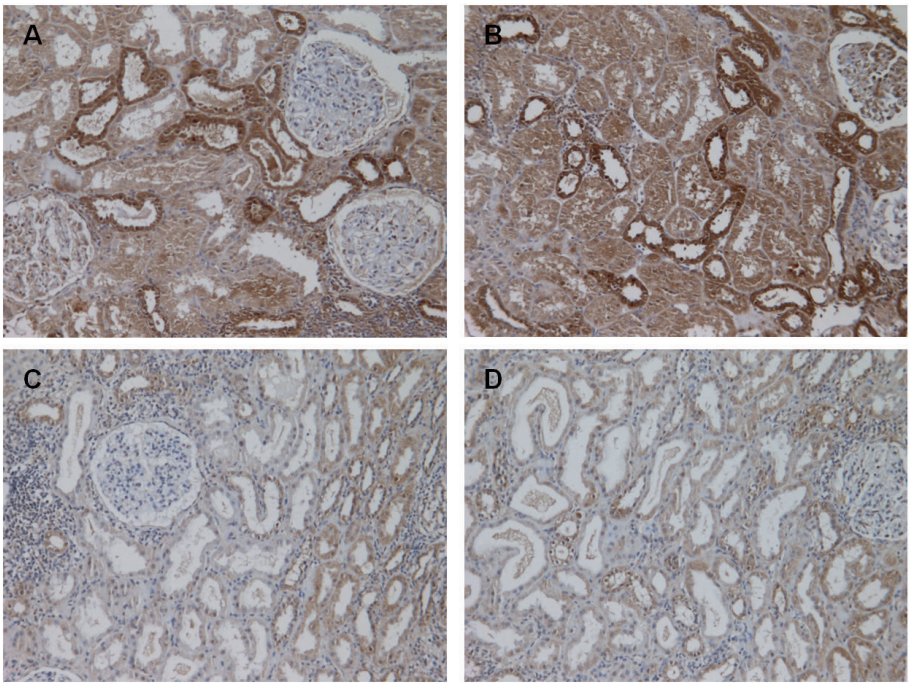


Figure 4. Immunohistochemical staining pattern of CYP3A5 in sections from renal transplant biopsies with acute rejection. CYP3A5 protein was detected in infiltrating lymphocytes and tubular epithelial cells. Intragraft expression of CYP3A5 protein varied from high (A-B) to low (C-D) in the patient groups. Recordings at 10x magnification.

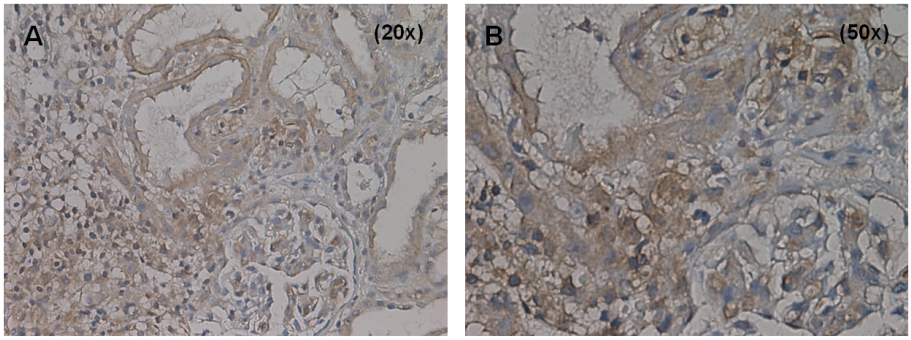


Figure 5. Immunohistochemical staining pattern of ABCB1 in sections from renal transplant biopsies with acute rejection. ABCB1 protein was detected in infiltrating lymphocytes and tubular epithelial cells. A: overview recording at 20x magnification; B: enlarged recording at 50x magnification.

Discussion

Research in the field of steroid-refractory AR in renal allografts has been mainly focused on markers of inflammatory cells [36]. The role of glucocorticoid signaling and drug metabolism as predisposing factors towards responsiveness for high-dose steroid therapy during AR has not been thoroughly examined. We investigated if polymorphisms in genes involved in glucocorticoid signaling (GR and GLCCI1) and drug metabolism (CYP3A5, ABCB1, and PXR) are associated with response to steroid treatment.

Our study shows for the first time that the donor CYP3A5*1 genotype and intragraft CYP3A5 expression levels are associated with responsiveness to steroid treatment. Earlier studies have shown that patients homozygous for CYP3A5*3 require lower doses of immunosuppression, such as Tacrolimus and Cyclosporine A, due to a lower capacity to metabolize the therapeutic compound [37;38]. We found that donor CYP3A5*3/*3 is associated with resistance to steroid treatment during AR. The CYP3A5*3 variant creates an alternative splice site resulting in truncated CYP3A5 protein. Carriers of the CYP3A5*1 variant were shown to produce high levels of CYP3A5 mRNA and protein, while individuals homozygous for CYP3A5*3 were low expressors of CYP3A5 [18;19;21]. In line with these findings, we observed a genotype-dependent level of CYP3A5 mRNA expression in the grafts from renal allograft recipients. Furthermore, intragraft expression of CYP3A5 protein was detected in inflammatory cells and tubular epithelial cells, which is in line with earlier findings in normal kidney tissue [21;39].

A potential mechanism for the correlation between CYP3A5 and the response to steroid treatment may be that CYP3A5*1 expression within the kidney results in improved metabolism of methylprednisolone into a more effective metabolite, increasing the immunosuppressive effect of the treatment. An alternate hypothesis is that the increased metabolic capacity of CYP3A5*1 carriers may prevent toxic side effects of the high concentration of steroids within the allograft, while leaving the immunosuppressive effects intact. Further research will be needed to gain insight into how CYP3A5 affects the response to steroid treatment.

Another highly expressed member of the CYP3A family of metabolizing enzymes is CYP3A4. Similar to CYP3A5, polymorphisms in the CYP3A4 gene may contribute to inter-individual variability in drug metabolism [18;40]. Due to the low expression levels of CYP3A4 within the kidney, we did not include the CYP3A4*1B SNP in our study [17;18;41]. Further research in a larger patient cohort may be needed to see if CYP3A4 associates with response to steroid treatment during AR.

Other important drug metabolism-related genes expressed in the kidney encode P-glycoprotein (ABCB1) and the pregnane X receptor (NR1I2) [16;23]. SNP genotyping revealed no associations between polymorphisms in ABCB1 or NR1I2 and steroid-refractory AR. We did find significantly higher ABCB1 mRNA expression in AR biopsy samples of steroid resistant patients. Earlier studies in children with nephrotic syndrome have linked the expression of ABCB1 polymorphisms and ABCB1 gene expression levels with resistance to steroid therapy [26;42]. Discrepancies with respect to the relation between ABCB1 polymorphisms and steroid responsiveness may be caused by differences in the age of the patients studied (children versus adults) and the type of steroids used as treatment. The nephrotic syndrome patients were treated with 60 mg / m^2 / d prednisone for four weeks, while acute renal allograft rejection is treated for 3 days with a 1-g bolus of methylprednisolone.

In addition to drug metabolism-related genes, we investigated genes involved in glucocorticoid signaling. We found no associations between steroid-refractory AR and the glucocorticoid receptor or GLCCI1. Both the genotype distribution of the NR3C1 and GLCCI1 polymorphisms and the GR mRNA expression in the renal allograft showed no correlation with the patient's response to methylprednisolone. Earlier studies showed that NR3C1 SNPs associated with increased (N363S [43] and *Bcl*I [44]) or reduced (23EK [45] and GR-9β [46]) sensitivity to glucocorticoids (reviewed in [12]), and that GLCCI1 SNPs were associated with reduced response to steroid treatment [13-15].

Some comments need to be made with regard to the current findings. In this retrospective study, we included 153 renal transplant patients with a first rejection episode who received corticosteroids as anti-rejection treatment. We handled strict inclusion criteria and clinical endpoint definitions for the patient selection. Although this is a relatively large cohort to study the response to steroid treatment, it is a relatively small patient cohort for the study of the effect of genotypes. In addition, recent studies have shown the importance of replicating the findings of molecular studies in kidney transplantation [47]. In order to increase the cohort size and test the validity of our findings, the study should be repeated in a second patient cohort. Furthermore, we observed only a weak correlation between the mRNA and protein expression of CYP3A5, and no correlation between ABCB1 mRNA and protein expression. This lack of correlation may have been caused by the biopsy material used to analyze the mRNA and protein expression. RNA was isolated from eight to ten 10-µm sections of renal cortex from snap frozen biopsy cores, while protein stainings were performed on 4-µm sections from formalin fixed and paraffin embedded biopsy cores.

In conclusion, we investigated the impact of genes involved in glucocorticoid signaling and drug metabolism as predisposing factors on the response to high-dose steroid treatment of AR in renal allograft recipients. Our study shows for the first time that resistance to steroid treatment during AR is associated with the donor CYP3A5*1 genotype and CYP3A5 expression levels within the renal allograft. The current findings show that gene variations in drug metabolism-related genes, but not in the GR, predispose to responsiveness to high-dose steroid therapy.

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Supplemental data

Table S1. Primers used for characterization of GR and metabolism SNPs.

A. SYBR Green PCR assays				
Gene	rs number	SNP	Primers (5' – 3')	
NR3C1	rs6189/rs6190	ER22/23EK	Forward 1	5'-GTGTGCTTGCTCAGGAGAG-3'
			Forward 2	5'-CAGTGTGCTTGCTCAGGAAAA-3'
			Reverse	5'-TTTGGACAGATCTGGCTGCT-3'
NR3C1	rs41423247	*Bcl*I	Forward	5'-TGACCTTTTTGGGTCAGACAA-3'
			Reverse 1	5'-AGACAAGTTATGTCTGCTGATC-3'
			Reverse 2	5'-AGACAAGTTATGTCTGCTGATG-3'
ABCB1	rs1128503	C1236T	Forward	5'-CCCATCTCGAAAAGAAGTTAAGG-3'
			Reverse 1	5'-TGCACCTTCAGGTTCAGG-3'
			Reverse 2	5'-TGCACCTTCAGGTTCAGA-3'
ABCB1	rs2032582	G2677T	Forward	5'-GGTTCCAGGCTTGCTGTAAT-3'
			Reverse 1	5'-TAGTTTGACTCACCTTCCTAGC-3'
			Reverse 2	5'-AGTTTGACTCACCTTCCCAGA-3'
ABCB1	rs1045642	C3435T	Forward	5'-AGGAGCCCATCCTGTTTGAC-3'
			Reverse 1	5'-CTCCTTTGCTGCCCTCACA-3'
			Reverse 2	5'-CTCCTTTGCTGCCCTCACG-3'

B. TaqMan PCR assays			
Gene	rs number	SNP	Primers (5' – 3')
NR3C1	rs6195	N363S	5'-TCCAGATCCTTGGCACCTATTCCAA[T/C] TTTCGGAACCAACGGGAATTGGTGG-3'
	(rs56149945)		C: VIC labeled T: FAM labeled
NR3C1	rs6198	GR-9β	5'-GTAATACCAGAACAGCAAATTTAAA[T/C] GAAAAAATAAAAGTTAAACATTTCC-3'
			C: VIC labeled T: FAM labeled
GLCCI1	rs37972	-1473 C>T	5'-CTCTTGAAAATTAATGAAAGTTATC[C/T] GATGAAGATCCTTACATTAATTGCT-3'
			A: VIC labeled G: FAM labeled
CYP3A5	rs776746	CYP3A5*3	5'-ATGTGGTCCAAACAGGGAAGAGATA[T/C] TGAAAGACAAAAGAGCTCTTTAAAG-3'
		(A6986G)	C: VIC labeled T: FAM labeled
NR1I2	rs6785049	A7635G	5'-AGGAGCCATCCTCCCTCTTCCTCTC[A/G] CCCCCAACTTCTGGATTATGGGATG-3'
			A: VIC labeled G: FAM labeled

ABCB1, ATP-binding cassette B1; CYP3A5, cytochrome P450 3A5; GLCCI1, glucocorticoid-induced transcript 1; NR3C1, nuclear receptor 3C1 (glucocorticoid receptor); NR1I2, nuclear receptor 1I2 (pregnane X receptor)

Table S2. Primers used for gene expression analysis.

Gene	Forward primer	Reverse primer
ABCB1	5’-aggccaacatacatgccttc-3’	5’-ccaccagagagctgagttcc-3’
CYP3A5	5’-cagcctggtcctctatc-3’	5’-cagcacagggagttgacctt-3’
GRα	5’-taccctgcatgtacgaccaa-3’	5’-tccttccctcttgacaatgg-3’
GAPDH	5’-acccactcctccacctttgac-3’	5’-tccaccaccctgttgctgtag-3’
β-actin	5’-accacaccttctacaatgag-3’	5’-tagcacagcctggatagc-3’

ABCB1, ATP-binding cassette B1; CYP3A5, cytochrome P450 3A5; GRα, glucocorticoid receptor α (NR3C1); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table S3. Characteristics of antibodies.

Protein	Antigen retrieval	Primary antibody	Dilution	Blocking	Incubation	Secondary antibody	Detection
CYP3A5	Citrate (pH 6.0)	Rabbit polyclonal	1:4000	0.1% H2O2, 20'; 5% NGS, 25'	o/n at RT	Rabbit-EnVision+	DAB
ABCB1	Tris/EDTA (pH 9.0)	Mouse monoclonal	1:100	0.1% H2O2, 20'; 10% NGS, 20'	o/n at RT	Mouse-EnVision+	DAB

DAB, diaminobenzidine; NGS, normal goat serum; RT, room temperature.

Table S4. Single nucleotide polymorphisms studied and genotype distribution.

Gene	Location	rs number	Nucleotide change	Recipients (%)			Donors (%)		
				+/+	+/-	-/-	+/+	+/-	-/-
NR3C1	exon 2	rs6195	A>G	95.2	4.8	0.0	94.4	5.6	0.0
NR3C1	exon 2	rs6189/rs6190	G>A	96.6	3.4	0.0	92.8	7.2	0.0
NR3C1	intron 2	rs41423247	C>G	11.2	35.1	53.7	16.5	44.6	38.8
NR3C1	exon 9β	rs6198	A>G	75.4	17.2	7.5	67.5	27.5	5.0
GLCCI1	1473 bp upstream of ATG site	rs37972	C>T	26.8	47.2	26.0	32.2	47.5	20.3
CYP3A5	intron 3	rs776746	G>A	86.6	13.4	0.0	84.0	14.4	1.6
ABCB1	exon 12	rs1128503	C>T	35.1	47.8	17.2	33.3	46.7	20.0
ABCB1	exon 21	rs2032582	G>T	40.9	39.4	19.7	32.0	45.1	23.0
ABCB1	exon 26	rs1045642	C>T	27.5	49.3	23.2	23.0	46.7	30.3
NR1I2	intron 5	rs6785049	A>G	27.1	50.7	22.2	38.4	43.2	18.4

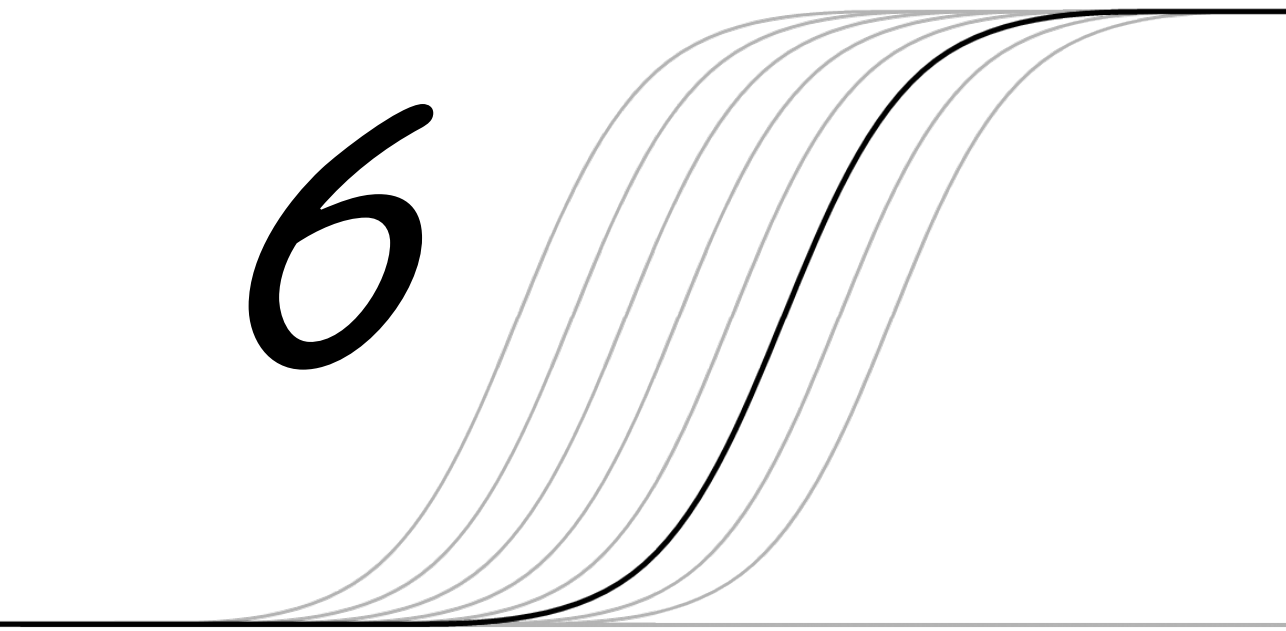
Distribution of wild type (+/+), heterozygous (+/-), and mutant (-/-) genotype variants in donors and recipients is depicted in percentages. ABCB1, ATP-binding cassette B1; CYP3A5, cytochrome P450 3A5; GLCCI1, glucocorticoid-induced transcript 1; NR3C1, nuclear receptor 3C1 (glucocorticoid receptor); NR1I2, nuclear receptor 1I2 (pregnane X receptor)

Table S5. SNP genotypes analyzed for deviations from Hardy-Weinberg equilibrium.

Gene	SNP	rs number	Recipient	Donor
NR3C1	N363S	rs6195	0.95	0.92
NR3C1	ER22/23EK	rs6189/rs6190	0.99	0.90
NR3C1	*Bcl*1	rs41423247	0.029	0.57
NR3C1	GR-9β	rs6198	0.014	0.59
GLCCI1	-1473 C>T	rs37972	0.54	0.69
CYP3A5	CYP3A5*3	rs776746	0.76	0.68
ABCB1	C1236T	rs1128503	0.67	0.61
ABCB1	G2677T	rs2032582	0.021	0.43
ABCB1	C3435T	rs1045642	0.64	0.66
NR1I2	A7635G	rs6785049	0.98	0.58

ABCB1, ATP-binding cassette B1; CYP3A5, cytochrome P450 3A5; GLCCI1, glucocorticoid-induced transcript 1; NR3C1, nuclear receptor 3C1 (glucocorticoid receptor); NR1I2, nuclear receptor 1I2 (pregnane X receptor)

Beneficial effects of myeloid related S100 proteins in kidney transplantation



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Abstract

Occurrence of acute rejection is a risk factor for adverse kidney graft outcome. We found in a recent patient cohort that relatively high S100A8 and S100A9 mRNA expression during acute rejection (>2 times the median; n=36) led to a 12-year graft survival of 91.5%, whereas low expression (n=61) led to 69.2% graft survival ($P<0.05$). S100A9 mRNA expression levels corresponded with the extent of S100A9 protein in the grafts. High S100A8 and A9 expression was related to elevated expression of anti-inflammatory IL-10 ($P<0.01$) and regulatory T cell marker FoxP3/CD3 ($P<0.0001$). Triple immunostaining showed that 85% of the total myeloid cell population (CD68+) consisted either of S100-positive cells or CD163+ activated macrophages. In cultured monocytes S100 expression was especially elevated when the cells were stimulated with IL-10. Overexpression of the S100A8 and S100A9 genes in the monocytic cell line THP-1 led to *de novo* production of IL-10 for at least four days after transfection. These findings indicate that renal allograft survival is related to the presence of distinct myeloid cell subsets within the graft during acute rejection, and that high expression of S100 positive cells leads to increased local regulation of the immune response and a reduction in renal allograft damage.

Introduction

Kidney transplantation is the preferred treatment for patients suffering from end-stage renal disease. At present, more than 36,500 kidney transplantations are performed annually in Europe and the United States [1;2]. Fifteen percent of renal allograft recipients experience an acute rejection (AR) episode [3;4], and approximately 40% of the renal grafts fail within ten years after kidney transplantation [2]. During AR immune cells of the host infiltrate the donor kidney and cause injury to the allograft. The occurrence of AR is an important risk factor for adverse graft outcome and graft loss [5-9]. Until now, it has proven difficult to differentiate between patients having a high or low risk profile for adverse graft outcome. Identification of biomarkers for assessing the risk of graft failure may lead to improved monitoring and intervention strategies and may contribute to improved long-term renal allograft outcome.

Molecular markers in transplant biopsies with acute rejection may be helpful to predict the risk of graft loss (reviewed in [10]). Previously, we found that patients experiencing AR with relatively high intragraft expression levels of S100A8 and S100A9 show favorable long-term graft outcome [11]. S100A8 and S100A9 belong to a large family of calcium-binding proteins and are expressed by neutrophils, monocytes, and early differentiated macrophages, but absent in resident macrophages and lymphocytes [12-16]. Both S100A8 and A9 have been found to be biomarkers for a variety of inflammatory disorders, including complications in organ transplantation [12;13;17]. Increased serum levels of these S100 molecules predict the occurrence of AR [18;19]. The elevated levels prior to an AR episode could indicate either counter-regulatory responses or pro-inflammatory effects by these molecules.

In the current study on a large cohort of patients suffering from AR, we verified that the presence of relatively high levels of S100A8 and S100A9 in inflammatory infiltrates during AR is associated with favorable graft outcome. We found that S100A8 and S100A9 proteins are expressed by a specific subset of myeloid cells. In further experiments we provide evidence that the S100 proteins are directly related to immune regulatory mechanisms, which may account for the favorable long-term outcome observed.

Materials and methods

Patients

The study cohort consisted of 142 patients who received a renal allograft in our center between 1995 and 2005. Biopsy samples were taken within 6 months after transplantation because of acute decrease in graft function: 137 patients suffered from a histologically proven acute rejection, and five patients showed no histomorphologic indication of rejection [20]. All rejection biopsy samples were obtained prior to the start of anti-rejection treatment. As a control group for graft survival analyses, 100 renal allograft recipients from our center who did not experience an acute rejection episode, and matched for transplantation date and donor type (living, deceased), were selected.

The second cohort consisted of 65 patients from the CANNES trial, who were enrolled in an open-label randomized control trial on calcineurin inhibitor based maintenance immunosuppressive therapies [21;22]. The 65 patients received a renal allograft in our center between October 2000 and October 2002, and had undergone biopsies before transplantation (pretransplant biopsy), and at 6 and 12 months after transplantation. None of these patients had experienced an acute rejection episode. All patients gave informed consent to take part in the study.

Medication

Maintenance immunosuppressive medication consisted of double- (25.1%) or triple-drug (74.9%) therapy: glucocorticoids (prednisone) and a calcineurin inhibitor (Cyclosporine A or Tacrolimus), with or without an antimetabolite (Mycophenolate Mofetil). One hundred and five (50.7%) patients received induction therapy with an IL-2 receptor blocker (Daclizumab or Basiliximab). High-dose intravenous methylprednisolone was used as initial treatment of acute rejection (1-g bolus/day x 3 days). Steroid resistant acute rejection was treated with anti-thymocyte globulin. Patients from the CANNES trial with subclinical acute rejection did not receive additional medication.

Clinical endpoints

The primary clinical endpoint was graft survival, defined as time from transplant to graft failure. Death with a functioning graft and grafts still functioning at time of analysis were censored.

Biopsy samples

Biopsy cores were obtained by percutaneous, ultrasound-guided biopsy with an 18-gauge needle. At least two cores were collected from each patient. One core was formalin fixed, embedded in paraffin, and used for histological analysis (Banff score) and immunohistochemical stainings. The second biopsy core was immediately snap frozen in liquid nitrogen and stored at -80°C.

Banff score

In the patient cohort suffering from acute rejection, rejection severity was assessed on paraffin-embedded biopsy tissue according to Banff 2011 criteria [23], as described previously [20].

Protocol biopsy samples fulfilling the criteria of acute rejection in the absence of clinical symptoms were classified as subclinical acute rejection (SAR). SAR was defined by a tubulitis score of ≥ 2 and interstitial infiltrate score ≥ 0. Eleven cases of SAR were observed in the protocol biopsy specimens: four at 6 months and seven at 12 months post transplantation.

RNA extraction and cDNA synthesis

RNA extraction was performed on snap frozen biopsy samples from 99 patients having acute rejection, five patients with no indication of rejection, and 65 patients from the CANNES trial [20;21]. Eight to ten 10-µm thick sections of renal cortex were cut with a cryomicrotome from each frozen biopsy core. Total RNA from the renal tissue was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. RNA concentration was measured by NanoDrop spectrophotometer (Nanodrop Products, Wilmington, DE) and RNA quality was determined on Nano LabChips with the Aligent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The A260/A280 ratio was between 1.8 and 2.1. A maximum of 1 µg of isolated RNA from each frozen biopsy core was transcribed into cDNA, according to a previously described protocol [24]. The cDNA samples were 25-times diluted.

Quantitative real-time PCR analysis

Primer sequences for real-time qPCR assays are provided in Table S1. To prevent amplification of genomic DNA, forward and reverse primers for each transcript targeted separate exons, spanning at least one intron with a size of 800 bp or more.

All primer sets were tested before use on control cDNA and genomic DNA to ensure optimal performance and no amplification of genomic DNA. Reactions and PCR conditions have been described previously [24]. All PCR efficiencies were above 90%. Relative gene expression levels were determined on the basis of a standard curve (in duplicate) of five serial dilution points of reference cDNA (qPCR Human Reference Total RNA, Clontech, France). Differences in mRNA expression levels were normalized to the geometric mean signal of the reference genes GAPDH, 18S rRNA and β-actin (inter-correlation ranged between 0.88 and 0.95).

Microarray Analysis

Gene expression profiles were analyzed in 36 acute rejection biopsy samples with Illumina HumanRef-8 v3.0 BeadChips (Illumina, San Diego, CA), as previously described [25]. All samples had a RNA quality index > 8. Illumina GenomeStudio software was used to verify the adequacy of signal to background ratio and hybridization controls. All data have been deposited in the Gene Expression Omnibus public database, accession number: GSE47097.

Immunohistochemistry

Immunohistochemical analyses were performed on 123 paraffin-embedded biopsy samples from the patients having acute rejection. Sequential serial sections (4 μm) of formalin-fixed paraffin-embedded biopsy samples were used to detect S100A9 monomers, S100A8/A9 heterodimers (Ab 27E10), CD68, and CD163. Primary monoclonal rabbit anti-human antibodies against S100A9 and primary monoclonal mouse anti-human antibodies against 27E10 have been described previously [26]. Primary monoclonal mouse anti-human antibodies against CD68 were obtained from Dako (Glostrup, Denmark; clone KP1), and against CD163 were obtained from Abcam (Cambridge, UK; clone 10D6, prediluted). Antibodies and staining protocols have been described previously [26;27]. Details concerning antigen retrieval and staining procedure are provided in Table S2. For S100A9 and 27E10, peroxidase-conjugated second-stage antibodies were purchased from Dianova (Hamburg, Germany), and peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich, St. Louis, MO). For CD68 and CD163, Mouse-Envision+ System (DAB) was used according to the manufacturers (Dako) protocol. Photos were taken with a Pannoramic MIDI slide scanner (3DHISTECH, Budapest, Hungary) with AxioCam MRm

camera (Carl Zeiss Microscopy GmbH, Germany) and Pannoramic Viewer software (3DHISTECH). Quantification of immunohistochemical stainings was performed using Image-J software (National Institutes of Health, Bethesda, MD).

Immunofluorescence staining

To investigate the presence of macrophage subtypes, a triple immunofluorescence staining was performed for S100A9, CD163 and CD68. Paraffin-embedded sections (4 µm) were deparaffinized in xylene and hydrated via graded ethanols to purified H_2O. After blocking of endogenous peroxidase with 0.1% H_2O_2, heat-induced antigen-retrieval was performed with a citrate buffer (pH 6.0) for 30 minutes, followed by washing in PBS. Sections were incubated overnight with a mix of primary antibodies: monoclonal rabbit anti-human IgG against S100A9 (dilution 1:4000; Abcam; clone EPR3555), monoclonal mouse anti-human IgG1 against CD163 (dilution 1:20; Abcam; clone 10D6, prediluted), and monoclonal mouse anti-human IgG2a against CD68 (dilution 1:100; Abcam; clone 514H12). Thereafter, the sections were washed with PBS three times for 5 minutes each, and incubated for 30 minutes with a mix of secondary antibodies: Alexa Fluor 488 goat-anti-mouse IgG1 (dilution 1:200; Life Technologies, Bleiswijk, the Netherlands), Alexa Fluor 546 goat-anti-rabbit IgG (dilution 1:200; Life Technologies), and Alexa Fluor 647 goat-anti-mouse IgG2a (dilution 1:200; Life Technologies). After rinsing of the sections three times with PBS, slides were covered with Vectashield (Vector Laboratories ltd., Burlingame, CA). Photos were taken with Leica TCS SP8 confocal microscope (Leica Microsystems, Rijnsburg, the Netherlands). S100A9 and CD163 staining patterns were graded by N.V.R. and M.E. together on a – tot ++ scale. On average, 994 ± 279 cells were scored positive in 14 ± 3 locations.

S100A8 and S100A9 expression in cultured human monocytes

Procedures for isolation of monocytes and generation of different subsets of polarized human macrophages have been described previously [28]. Monocytes were isolated from peripheral blood of six healthy volunteers and cultured in 6-well culture plates (0.5×10^6 / ml) in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Breda, the Netherlands) containing 10% fetal bovine serum (FBS; PAA Laboratories, Cölbe, Germany). Monocytes were cultured for 4 days in medium only or were polarized with GM-CSF (50 ng/ml; R&D Systems, Abingson, UK), M-CSF (50 ng/ml; R&D Systems), IFN- γ (50 ng/ml; R&D Systems), IL-4 (40 ng/ml; Miltenyi Biotec, Bergisch Gladbach,

Germany), or IL-10 (50 ng/ml; R&D Systems). LPS (100 ng/ml; E. coli 0111:B4, Sigma Aldrich, Zwijndrecht, the Netherlands) was added in cultures with IFN- γ, IL-4, and IL-10 as an additional stimulant. After the culture, the cells were harvested and RNA was extracted. Expression of S100A8 and S100A9 was measured by qPCR.

Transient overexpression of S100A8 and S100A9 in monocytes

THP-1 (human leukemic monocyte cell line) cells were cultured in IMDM (Lonza, Verviers, Belgium) containing 10% heat-inactivated FBS (Life Technologies), 1 U/ml penicillin (Lonza), 1 U/ml streptavidin (Lonza) and 3 mM L-glutamine (Life Technologies). For the construction of pVITRO-S100A8/A9, both hS100A8 and hS100A9 PCR fragments were cloned into pVITRO2-hygro-mcs (InvivoGen, San Diego, CA) according to previously described protocols [29]. Empty pVITRO2-hygro-mcs vector was used to control for potential effects of the transfection procedure. Lipofectamine LTX transfection reagent was obtained from Invitrogen (Karlsruhe, Germany). The transfection was performed as described previously [29], with some modifications. THP-1 cells were seeded in 24-well plates (100 x 10^6 cells/well) 24 hours before transfection. Six µl Lipofectamine and 1.5 µg of total plasmid DNA were diluted to a final volume of 800 µl with pure IMDM and added to the cells. The medium was changed 5 hours after the transfection. The cells were cultured for 24 hours, 48 hours, 4 days, or 6 days. Expression of S100A8, S100A9, TNFα, and IL-10 was measured by qPCR.

Statistical analysis

Differences in mean mRNA expression between patient groups were tested with the Mann-Whitney U-test. Comparison of categorical data between patient groups was evaluated using the Pearson chi-square test. Association with death-censored graft loss was tested using Kaplan-Meier curves and log-rank testing. All statistical tests were two-sided, and P values less than 0.05 were considered as significant. Statistical analyses were performed using the SPSS statistical package (IBM SPSS Statistics., version 20.0.0, IBM, Amsterdam, the Netherlands).

Results

S100A8 and S100A9 expression in renal allograft biopsy samples

We quantified S100A8 and S100A9 mRNA expression levels in six groups of biopsy specimens obtained at different time points before and after transplantation. Biopsy samples with histologically confirmed AR had significantly higher mRNA expression levels of S100A8 and S100A9 compared with biopsies taken before transplantation (Pre-Tx) (P<0.00001), biopsies with no morphologic signs of rejection (P<0.001), and protocol biopsies taken at 6 months (P<0.00001) and 12 months (P<0.00001) (Figure 1 and Table S3). AR biopsy samples did not differ from biopsies with subclinical acute rejection (SAR) with respect to S100A8. We conclude from these results that S100A8 and S100A9 expression levels in the graft are specifically elevated during conditions of increased inflammation.

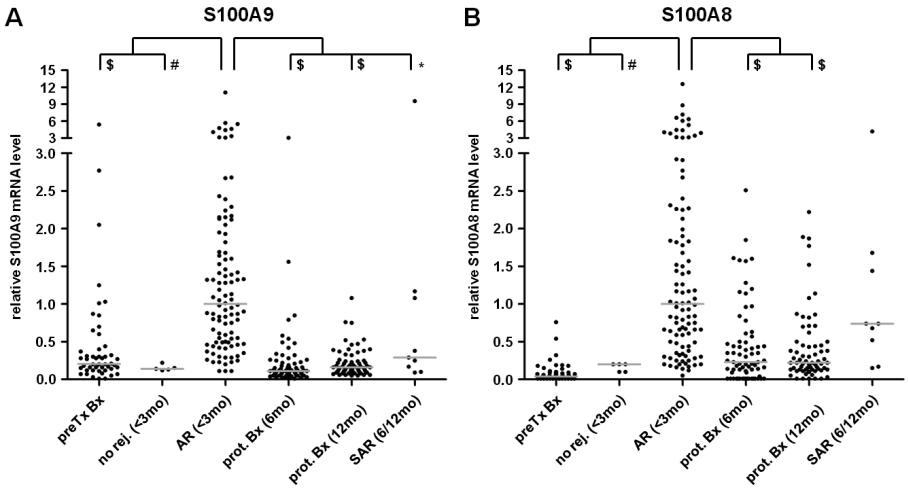


Figure 1. Expression levels of S100A9 and S100A8 in renal allograft biopsy specimens. The mRNA expression S100A9 (A) and S100A8 (B) measured with qPCR in six groups of biopsy specimens obtained at different time points before and after transplantation: pretransplant biopsy samples (Pre-Tx Bx), biopsies taken on clinical indication within 3 months post transplantation with no signs of rejection (No rej. (<3 mo)), or with histologically confirmed acute rejection (AR (<3 mo)), protocol biopsies taken at 6 months (Prot. Bx (6 mo)) or 12 months (Prot. Bx (12 mo)) with no signs of rejection, or with histological indication of subclinical acute rejection SAR (6/12 mo). The line shows the median. *, P<0.05; #, P<0.001; $, P<0.00001.

Relationship of S100A8 and S100A9 mRNA expression during acute rejection with graft outcome

In a small case-control study of patients transplanted between 1986 and 1992 [11], relatively high intragraft expression levels of S100A8 and S100A9 during AR were found to be related to improved graft outcome at a later time point. We could confirm and extend these data in a much larger and more recent cohort of patients, transplanted between 1995 and 2005: S100A9 mRNA expression levels were 3.3 fold higher in patients retaining their graft for at least 6 years (non-progression [NP] group) compared to patients showing progression to graft loss (PR group) (P<0.05; Figure 2).

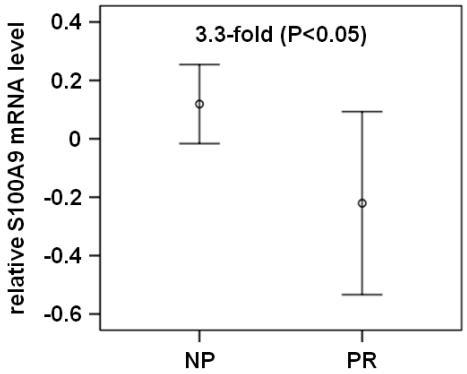


Figure 2. Association between S100A9 expression and graft outcome in patients with acute renal allograft rejection. Comparison of the intragraft S100A9 mRNA expression level during AR between patients retaining their graft for at least 6 years (non-progression [NP] group) and patients showing progression to graft loss (PR group).

The patients suffering from AR were divided into a $S100A9^{high}$ (n=36; at least two times the median S100A9 expression of the whole group) and a $S100A9^{low}$ (n=61; expression level lower than 2 times the median) expresser group. Six years after transplantation, graft survival was 97.2% in the $S100A9^{high}$ group and 79.9% in the $S100A9^{low}$ group (P=0.032, Figure 3A). At the end of follow-up, i.e. 12.5 years after transplantation) this difference remained significant, with 91.5% graft survival in the $S100A9^{high}$ group and 69.2% graft survival in the $S100A9^{low}$ group (P=0.046, Figure 3A). The graft survival in the $S100A9^{high}$ group was similar to the graft survival observed in a group of 100 matched patients, who were transplanted within the same time period but who did not experienced an acute rejection episode (P=0.65,

Figure 3A). The S100A9^{low} group showed significantly more graft loss compared to the patients without acute rejection (P=0.010, Figure 3A). Demographic and clinical data were not significantly different between the patients in the S100A9^{low} and S100A9^{high} groups (Table 1), even though that virtual PRA did show an association with graft survival (see Table S4 and Figure S1). In the whole group, S100A9 expression is highly correlated to S100A8 expression (r=0.94, P<0.000001 and Figure 3B). Thus, S100A9 and S100A8 levels in renal allografts with acute rejection predict prognosis. Acute rejection episodes with relatively high levels of these molecules have a similar impact on late graft outcome as situations where no acute rejection episode was experienced by the patient.

We next investigated whether expression of markers related to immune regulation and immunity are associated with the S100 expression. Expression of the anti-inflammatory cytokine IL-10 (P<0.01, Figure 3C) and regulatory T cell marker FoxP3:CD3e ratio (P<0.0001, Figure 3D) was significantly higher in the S100A9^{high} group compared to the S100A9^{low} group. In contrast, kidney injury molecule (KIM)-1 (P<0.05, Figure 3E), pan-T cell marker CD3e (P<0.005, data not shown), and T helper cell marker CD4 (P<0.05, data not shown) showed lower expression in the S100A9^{high} group compared to the S100A9^{low} group.

S100A9 protein expression in renal allograft biopsy samples

Intragraft S100A9 mRNA expression was related to the extent of S100A9 protein in the AR biopsy specimens: significantly higher protein expression was seen in the S100A9^{high} group compared to the S100A9^{low} group (P<0.005, Figure 4A). Infiltrating lymphocytes in the tubulointerstitial compartment stained positive for S100A9 (Figure 4). Similar findings were observed for staining of 27E10, which is the epitope specifically recognized on S100A8-A9 heterodimer protein (data not shown). Protocol biopsy samples obtained at 6 or 12 months after transplantation expressed low levels of S100A9 protein (Figure 4B). The AR biopsy samples showed a wide range in the extent of staining for S100A9 (10.32% ± 10.34% of the tissue surface positive; Figure 4C and D).

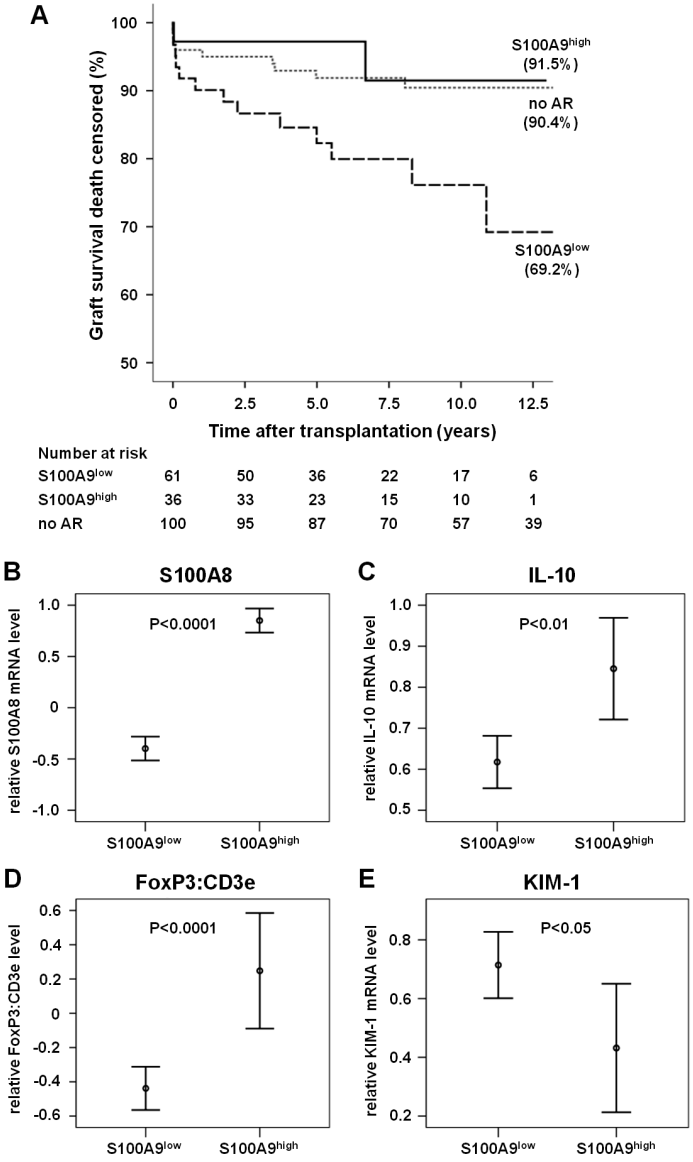


Figure 3. S100A9 expression and renal allograft survival. (A) Graft survival (censored for death with functioning graft) analyzed in renal allograft recipients with low (striped line) or high (straight line) mRNA expression levels of S100A9 during acute rejection, and in patients having no acute rejection (dotted line). The graft survival of the S100A9^{high} expresser group was significantly better compared to the S100A9^{low} expresser group. Patients with high S100A9 expression showed significantly better graft survival compared to S100A9^{low} expressers (log-rank = 3.978; P=0.046). The graft survival in the S100A9^{high} group was similar to the graft survival observed in patients without acute rejection (log-rank = 0.206; P=0.65). The S100A9^{low} group showed significantly more graft loss compared to the patients without acute rejection (log-rank = 6.651; P=0.010). (B-E) Relative mRNA expression levels of S100A8 (B), IL-10 (C), FoxP3:CD3e ratio (D), and KIM-1 (E) measured with qPCR in the S100A9^{low} and S100A9^{high} expresser groups.

Table 1: Demographic and clinical data of AR patients with high or low S100A9 mRNA expression

Variable	$S100A9^{low}$ (n=63), n (%)	$S100A9^{high}$ (n=36), n (%)	P value
Patient age			0.91
≥ 50 years	27 (42.9%)	15 (41.7%)	
Patient gender			0.38
Female	19 (30.2%)	14 (38.9%)	
Donor age			0.61
≥ 50 years	26 (41.3%)	13 (36.1%)	
Donor gender			0.29
Female	37 (58.7%)	25 (69.4%)	
Year of transplantation			0.18
1995 through 1999	39 (61.9%)	27 (75.0%)	
2000 through 2005	24 (38.1%)	9 (25.0%)	
Donor type			0.32
Living	12 (19.0%)	10 (27.8%)	
Post mortal	51 (81.0%)	26 (72.2%)	
HLA-AB matching			0.64
≥ 1 mismatch	52 (82.5%)	31 (86.1%)	
HLA-DR matching			0.75
≥ 1 mismatch	40 (63.5%)	24 (66.7%)	
Virtual PRA			0.66
Immunized (6-100%)	18 (29.0%)	12 (33.3%)	
Cold ischemia time			0.63
> 18 hours	38 (71.7%)	20 (66.7%)	
Induction therapy			0.10
Daclizumab	22 (34.9%)	7 (19.4%)	
None	41 (65.1%)	29 (80.6%)	
Maintenance therapy			0.32
Double therapy	25 (39.7%)	18 (50.0%)	
Triple therapy	38 (60.3%)	18 (50.0%)	
Delayed graft function			0.06
Yes	19 (30.6%)	5 (13.9%)	
Rejection time			0.18
<3 months post Tx	60 (95.2%)	36 (100.0%)	
3-6 months post Tx	3 (4.8%)	0 (0%)	
C4d staining			0.19
Positive	6 (10.3%)	7 (20.0%)	
Interstitial inflammation			0.09
Low inflammation (<25%)	33 (55.0%)	12 (36.4%)	
>25% parenchyma inflamed	27 (45.0%)	21 (63.6%)	
Rejection severity [a]			0.11
Borderline rejection	28 (49.1%)	9 (27.3%)	
T cell mediated rejection	12 (21.1%)	8 (24.2%)	
Vascular rejection	17 (29.8%)	16 (48.5%)	

[a] Six biopsies did not contain sufficient cortex to determine a Banff score. Three additional patients had a clinical rejection (with i- and t-scores of 0).
PRA, panel reactive antibodies; Tx, transplantation

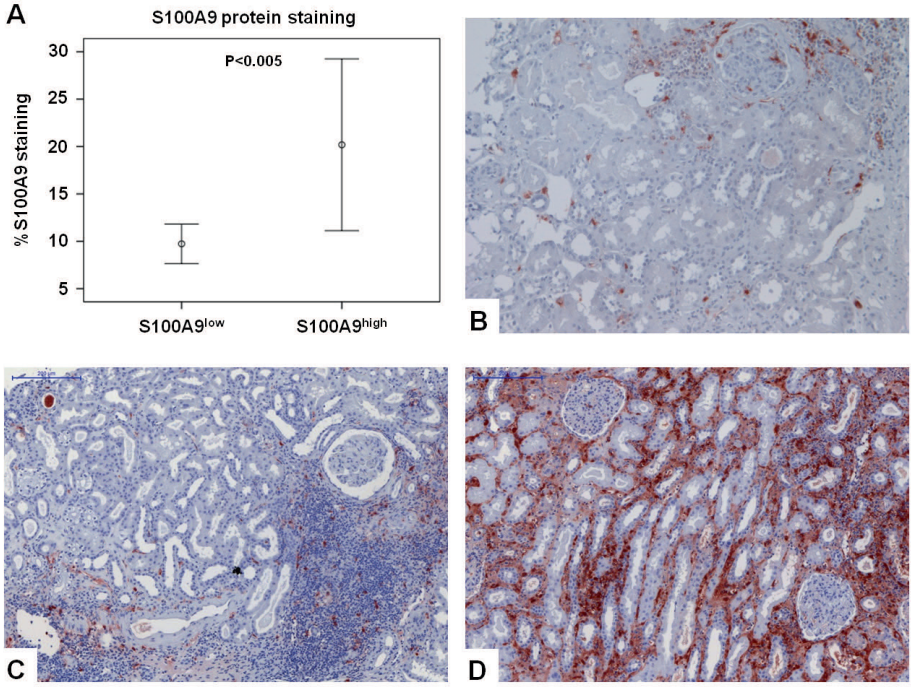


Figure 4. S100A9 protein expression in renal allograft biopsy samples. (A) Comparison of S100A9 protein expression between AR biopsy samples with low and high S100A9 mRNA expression. (B-D) Immunohistochemical staining pattern of S100A9 in patient groups. S100A9 protein was mainly detected in infiltrating lymphocytes in the interstitial compartment. (B) Protocol biopsy samples obtained at 6 (pictured) or 12 months (not shown) after transplantation expressed low levels of S100A9 protein. (C-D) Infiltrating lymphocytes in the tubulointerstitial compartment stained positive for S100A9. The AR biopsy samples showed a wide range in the extent of staining for S100A9. Recordings at 10x magnification.

Macrophage markers CD163 and CD68

As S100A8 and S100A9 are mainly expressed by myeloid cells [13-16], we investigated the expression of activated macrophage marker CD163 and pan-macrophage marker CD68 in the AR biopsy specimens. Patients with acute rejection having relatively high CD163 mRNA expression (n=27; at least two times the median of the whole group) had a significantly higher risk of losing their graft compared to patients having relatively low CD163 mRNA expression (n=74; lower than two times the median). Six years after transplantation the graft survival was 75.0% in the $CD163^{high}$ group and 89.4% in the $CD163^{low}$ group (P=0.054, Figure 5A). Immunohistochemical analysis in the AR biopsy specimens showed a significant correlation between CD163 mRNA expression and the extent of CD163 protein (P<0.001, Figure 5B). CD163 staining

was seen in infiltrating lymphocytes in the tubulointerstitial compartment (Figure 5C and D). We found no significant differences in CD68 protein staining between the S100A9^{high} and S100A9^{low} groups, or between the CD163^{high} and CD163^{low} groups (data not shown).

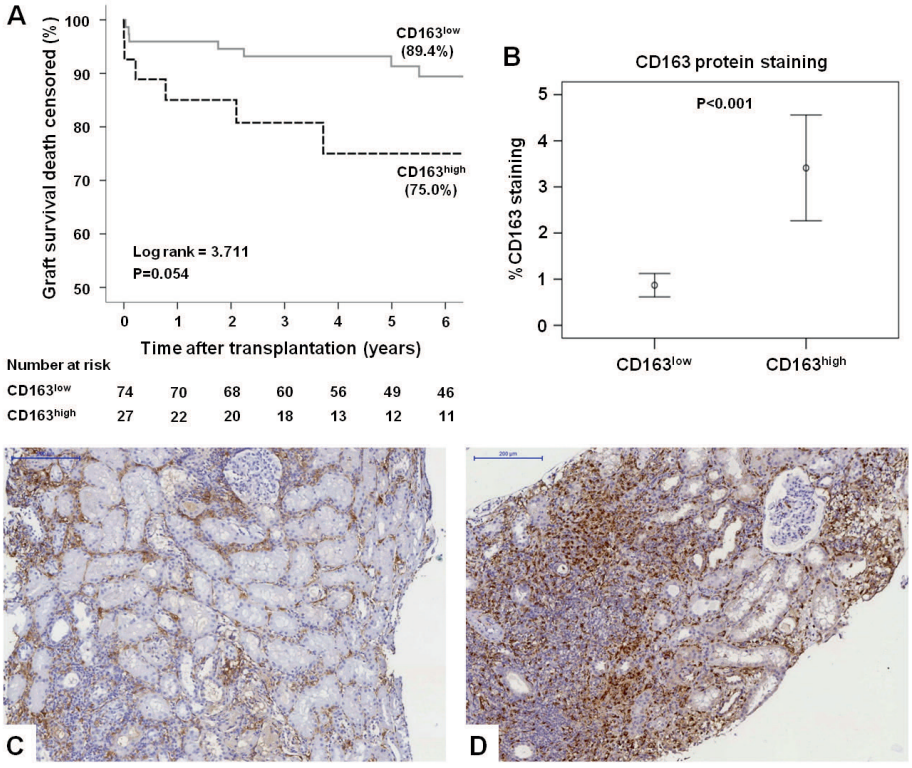


Figure 5. CD163 expression in biopsy samples with acute rejection. (A) Graft survival (censored for death with functioning graft) analyzed in renal allograft recipients with low (straight line) or high (striped line) mRNA expression levels of CD163 during acute rejection. (B) Comparison of CD163 protein expression between AR biopsy samples with low and high CD163 mRNA expression. (C-D) Immunohistochemical staining of CD163 protein was detected in infiltrating lymphocytes. AR biopsy samples showed a wide range in the extent of staining for CD163. Recordings at 10x magnification.

Macrophage subtypes in AR biopsy samples

To gain further insight into the relationship between S100A9 and CD163 expression, double immunofluorescence staining was performed on a subset of the AR biopsy samples (n=6) (Figures 6A-C and 7A-C). Counting of almost 1,000 positively-stained cells on average per biopsy showed that most cells either expressed only S100A9

(32.2% ± 9.2%, Figure 6D, red bar) or only CD163 (50.3% ± 12.4%, Figure 6D, green bar). A limited number of cells showed expression of both S100A9 and CD163 (Figure 6D, grey bars). At the same time, we saw by triple immunofluorescence staining including CD68, that each cell positive for S100A9, CD163 or both also stained positive for CD68 (see Figure 7D).

The results suggest that S100A9 and CD163 represent different myeloid cell subsets within the total CD68+ cell population infiltrating the graft during acute rejection.

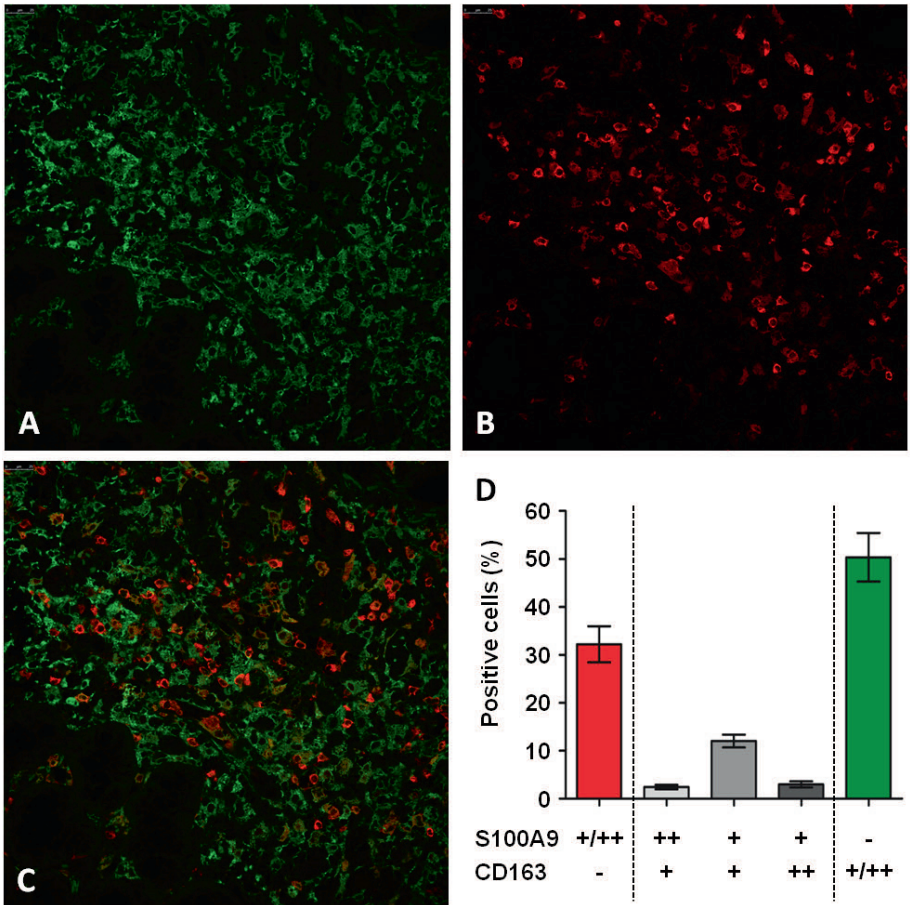


Figure 6. Relationship between S100A9 and CD163 expression. Immunofluorescence staining on sections from renal transplant biopsies with acute rejection for S100A9 (red) and CD163 (green). Shown are the single stainings for CD163 (A) and S100A9 (B), the combined double staining containing S100A9 and CD163 (C), and the quantification of the percentage single and double positive cells for S100A9 and CD163 expression (D). Bars represent the mean ± SEM of 6 biopsy samples. Recordings at 40x magnification. ++, strong positive; +, positive; -, negative.

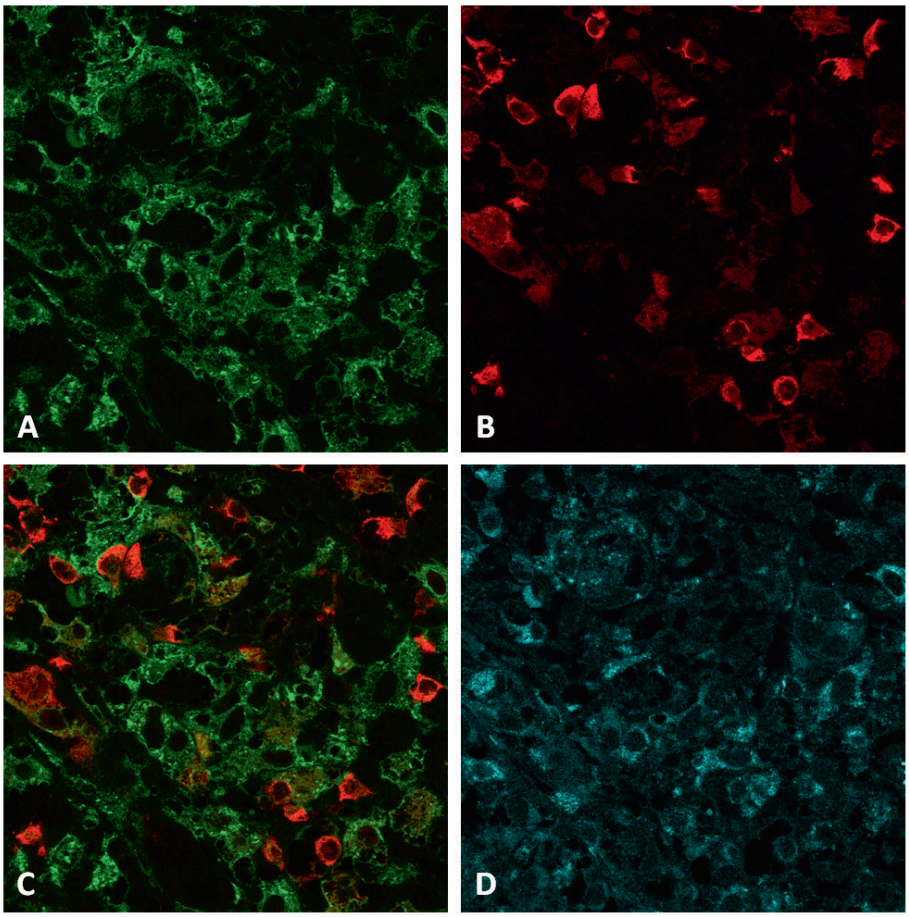


Figure 7. Colocalization of S100A9, CD163, and CD68 protein expression. Triple immunofluorescence staining on sections from renal transplant biopsies with acute rejection for S100A9 (red), CD163 (green), and CD68 (blue). Shown are the single stainings for CD163 (A), S100A9 (B), and CD68 (D), and the combined double staining containing S100A9 and CD163 (C). Recordings at 100x magnification.

S100 expression is related to genes involved in immune regulation

We wanted to acquire further insight in the biologic effect of the S100 proteins in vivo in the grafts. Therefore, we analyzed multiple acute rejection biopsies (n=36) by microarray analysis, and characterized genes of which the expression correlated best with that of S100A9. Thirty-nine genes showed an r≥0.65. As expected, the highest correlation was found with S100A8 (r=0.93). The theme of the list reflects genes expressed by the granulocyte-monocyte lineage (CD11b, CEBPB, FPR1, HCK, TNFR1, CCL23, MNDA, ANXA1, and PIM1) (see Table S5). NCF4/p40-PHOX (r=0.67)

is a component of the NADPH oxidase complex, which generates reactive oxygen species (ROS) by myeloid cells. Several of the genes on the list are involved in immune regulation (Table S5). For instance, the FPR1-ANXA1 axis (r=0.78 and r=0.66, respectively) regulates the biosynthesis of inflammation mediators, exerting anti-inflammatory effects through a similar pathway as glucocorticoids do [30]. CD44 mediates matrix adhesion and activation of leukocytes, and plays a critical role in resolving inflammation, as found in the lung [31]. MAP3K8/TPL2 (r=0.68) is a negative regulator of Th1 adaptive immunity [32].

Effect of cytokines and growth factors on S100 expression in human monocytes

We wanted to know whether cytokines and growth factors affect the S100A8 and S100A9 expression in monocytes. When compared with untreated monocytes in culture, the largest extent of increase in expression of the S100A8 and S100A9 was seen when monocytes were stimulated with IL-10, either in the presence or absence of LPS (Figure 8). Stimulation with IL-4 resulted in a decreased expression of the S100 molecules.

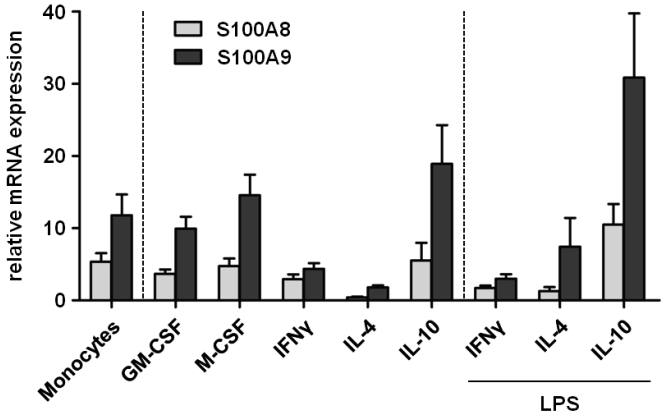


Figure 8. Effect of cytokines and growth factors on S100A8 and S100A9 expression in *in vitro* cultured monocytes. Healthy peripheral blood monocytes were cultured in medium alone (monocytes) or polarized with GM-CSF, M-CSF, IFNγ, IL-4, or IL-10 for 4 days, with or without additional LPS stimulation. IL-10 stimulation resulted in the largest extent of increase in S100A8 (light grey bars) and S100A9 (dark grey bars) mRNA expression, IL-4 stimulation resulted in a decreased expression of S100 mRNA. Data are expressed as mean ± SEM.

Effect of overexpression of S100A8 and S100A9 in human macrophages

Transfection of the pVITRO-S100A8/A9 expression vector into THP-1 monocytic cells resulted in a sustained overexpression of S100A8 and S100A9 (Figure 9A). This led to *de novo* IL-10 mRNA expression by the cells for at least four days after the transfection (Figure 9B). We also observed an increase of TNFα mRNA after transfection, but the extent of upregulation was less pronounced compared to IL-10, and had normalized by day four (data not shown).

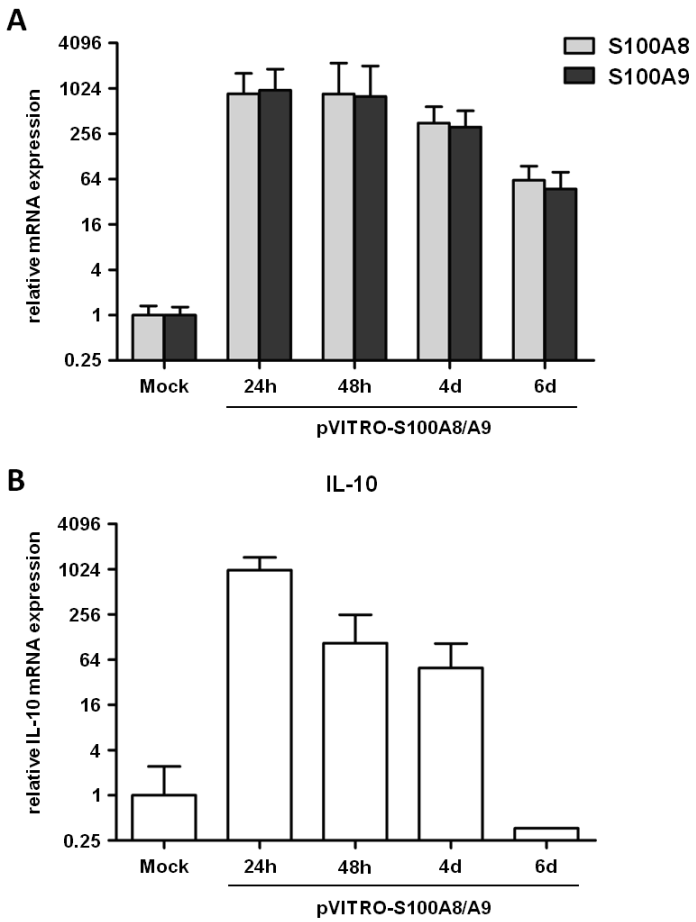


Figure 9. Effect of S100A8 and S100A9 overexpression upon IL-10 mRNA expression. RNA expression levels of S100A8 and S100A9 (A), and IL-10 (B) were measured with qPCR in THP-1 monocytic cells after transfection with the pVITRO-S100A8/A9 expression vector. Cells were transfected with empty vector (mock) or pVITRO-S100A8/A9 expression vector, and incubated for 24 hours, 48 hours, 4 days, or 6 days. Mean signal of the mock transfected THP-1 cells with 24h incubation were set to 1. Data are expressed as mean ± SEM.

Discussion

We aimed to study the relevance of intragraft S100A9 and S100A8 expression levels as predictive markers for graft outcome and to investigate the mechanism by which these molecules exert their biologic effects. In this study we show that determination of S100A8 and S100A9 expression levels in renal allograft tissue is clinically relevant and may be used for assessing the risk of adverse graft outcome.

It has been shown that increased serum levels [18;19] of S100A9 and S100A8 are associated with the occurrence of AR. In line with this, we found significantly higher S100A9 and S100A8 expression in biopsy samples from patients with histologically proven AR compared with patients having taken biopsy specimens that showed no signs of AR (Figure 1). As S100A9 and S100A8 are mainly expressed during the early stages of monocyte/macrophage differentiation [13;15], the observed increase in expression is likely a reflection of the infiltration of recipient-derived myeloid cells into the allograft during AR. This was indeed confirmed by immunohistochemistry for the S100 proteins.

We showed that during acute rejection intragraft expression of S100A9 and S100A8 was significantly higher in patients who retained their graft long-term than in patients who lost their graft over time (Figures 2 and 3). Graft survival of the patients in the S100A9^{high} group was similar to that of patients who had received a kidney transplant within the same time period and did not experience an AR episode. The high correlation between S100A9 and S100A8 expression levels points toward co-expression of the two genes in the graft. These observations confirm and extend the data from a previous patient cohort [11]. Overall, the results suggest that intragraft S100A9 and S100A8 expression during AR within the first months after transplantation provides prognostic information for the patient with respect to graft survival.

We also investigated the expression of FoxP3, IL-10, and KIM-1. FoxP3 is expressed by regulatory T cells, which play an important role in suppressing immune responses and immunologic tolerance after organ transplantation [33;34]. Multiple studies have shown that FoxP3 expression was significantly higher among renal allograft recipients with AR who had stable graft function than in patients who eventually showed graft loss [35;36]. Similarly, expression of the anti-inflammatory cytokine IL-10 was found to be increased in renal allograft recipients with stable graft function compared with patients with allograft dysfunction [37]. In line with these findings, the patients with

relatively high S100A9 expression and favorable graft outcome showed significantly higher expression of the FoxP3:CD3e ratio and IL-10 (Figure 3), and at the same time lower expression of markers reflecting total T cell burden (CD3, CD4) in the graft. S100A9^{high} expressers also expressed significantly lower levels of KIM-1, which is a transmembrane receptor on proximal tubules that is upregulated during acute and chronic kidney injury [38]. Urinary KIM-1 expression is associated with renal function decline and graft failure [39]. Our findings suggest that patients with relatively high S100A9 and S100A8 expression during acute rejection have improved graft survival due to increased local regulation of the immune response and less renal allograft damage. Compared to biopsies with no or minimal inflammatory infiltrate, S100A8 and S100A9 expression was elevated in biopsies with subclinical rejection (Figure 1). We propose that inflammatory infiltrate during acute rejection episodes, which contains relatively low numbers of S100-positive myeloid cells, are resolved less adequately and/or persist in the graft for prolonged time. Studies with sequential protocol biopsies during the first year after transplantation have shown that (untreated) subclinical inflammation and subclinical acute rejection resulted into increased interstitial fibrosis and tubular atrophy [40-42], which account for chronic allograft dysfunction and graft loss.

Immunohistochemical triple labeling experiments showed that S100A9 protein is expressed by CD68+ cells in the tubulointerstitial compartment (Figure 7), which was also previously suggested to be the case based on stainings for these markers on sequential tissue sections [26;43]. Myeloid cells comprise a heterogeneous cell population with both pro- and anti-inflammatory subtypes [44-49]. S100A9-positive cells in the grafts to a large extent seem to represent a myeloid cell subset with distinct effects from those of CD163-positive cells. CD163 has previously been proposed as a marker of anti-inflammatory macrophages [50;51]. However, our observation that patients with high CD163 expression in situ have a higher risk of losing their graft compared to patients with low expression (Figure 5) rather point toward CD163 as a marker of activated macrophages [52;53]. Further data from cultured macrophages supported our *in vivo* finding that high S100A8/A9 expression associates with immunoregulation. S100A9 and S100A8 were mainly expressed by IL-10 polarized macrophages (Figure 8), which are known for their immunoregulatory properties [28]. Vice versa, we showed that overexpression of the S100A8 and S100A9 genes in the monocytic cell line THP-1 led to *de novo* production of IL-10 for at least four days after transfection (Figure 9).

Overall, this shows that there may be a positive feedback loop between IL-10 and maintenance of high S100 levels. These findings provide support that renal allograft survival is dependent on the presence of different macrophage subtypes within the graft.

The biologic effects of S100A9 and S100A8 in disease are dual and concern both pro- and anti-inflammatory activities [54;55]. The S100 proteins can act as ligands for Toll-like receptor 4, driving progression of septic shock [56] and autoimmune disease [57]. On the other hand, the S100 proteins can mediate wound repair [58] and regulate accumulation of myeloid-derived suppressor cells that are capable of inhibiting T cell responses [59;60]. S100A8 and S100A9 proteins may potentiate activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [29;61;62]. The NADPH oxidase is a multi-unit enzyme complex expressed by phagocytic cells like monocytes and macrophages, which generates reactive oxygen species (ROS) upon activation. ROS acts physiologically as a signaling molecule, which at low amounts can result in immune regulation [63;64]. Kraaij and colleagues have shown that macrophage-derived ROS can suppress T cell responses via the induction of regulatory T cells [64]. An essential factor in the formation and activation of NADPH oxidase is arachidonic acid [65]. S100A9 and S100A8 bind and transfer arachidonic acid to the membrane-bound NADPH oxidase [61]. Overexpression of S100A8/A9 in epithelial cells and B lymphocytes results in increased activation of NAPDH oxidase and ROS generation [29;62]. Based on previous studies and current findings, we hypothesize that increased S100A9 and S100A8 expression during AR leads to increased ROS production by myeloid cells and induction of regulatory immune mechanisms.

Our study shows that determination of S100A8 and A9 positive macrophages in renal allograft tissue is clinically relevant and may be useful for assessing the risk of adverse graft outcome. However, its usefulness is slightly limited by the invasive nature of the biopsy procedure. A potential alternative to intragraft assessment may be to measure the expression of S100A8 and S100A9 in serum or urine from the patients [17]. Similar to our findings in the graft, the level of S100 expression in the serum may help predict if a patient has high or low risk of adverse graft outcome.

In conclusion, we show that patients with relative high S100A9 and S100A8 expression during acute rejection have improved graft survival. We found that S100A9 and S100A8 are expressed by a subset of myeloid cells. The clinical effect may be explained by local effects of S100A8 and S100A9 on regulation of the immune response, which limits renal allograft damage and prevents graft loss.

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Supplemental data

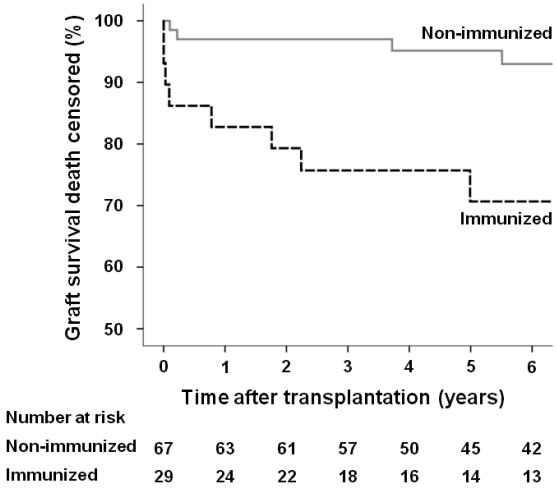


Figure S1: Virtual panel reactive antibodies (PRA) and renal allograft survival. Graft survival (censored for death with functioning graft) analyzed in non-immunized renal allograft recipients (virtual PRA of 0-5%; straight line) and immunized renal allograft recipients (virtual PRA of 6-100%; striped line). Non-immunized patients showed significantly better graft survival compared to immunized patients (log-rank = 9.410; P=0.002).

Table S1: Primer sequences

Gene	Forward primer	Reverse primer
S100A9	5′-cagctggaacgcaacataga-3′	5′-tcagctgcttgtctgcattt-3′
S100A8	5′-gggaatttccatgccgtct-3′	5′-ccttttttcctgatatactgaggacact-3′
CD3e	5′-ccgccatcttagtaaagtaacag-3′	5′-aataccacccatttcttcattacc-3′
CD68	5′-ttcccctatggacacctcag-3′	5′-ttgtactccaccgccatgta-3′
CD163	5′-tggagtgacctgctcagatg-3′	5′-caccgtccttggaatttgat-3′
FoxP3	5′-acagcacattcccagagttc-3′	5′-caggtggcaggatggtttc-3′
IL-10	5′-gcgctgtcatcgatttcttcc-3′	5′-gtagatgcctttctcttggagctta-3′
TGFβ	5′-cccagcatctgcaaagctc-3′	5′-gtcaatgtacagctgccgca-3′
GAPDH	5′-acccactcctccacctttgac-3′	5′-tccaccaccctgttgctgtag-3′
18S rRNA	5′-agtccctgccctttgtacaca-3′	5′-gatccgagggcctcactaaac-3′
β-actin	5′-accacaccttctacaatgag-3′	5′-tagcacagcctggatagc-3′

Table S2: Characteristics of antibodies.

Protein	Antigen retrieval	Type	Dilution	Blocking	Incubation	Secondary antibody	Detection
S100A9	Proteinase K (pH 7.5)	Rabbit monoclonal	1:500	0.1% H2O2, 30 min [a]	o/n at 4°C	Rabbit / peroxidase	AEC
27E10	Proteinase K (pH 7.5)	Mouse monoclonal	1:10	0.1% H2O2, 30 min [a]	o/n at 4°C	Mouse / peroxidase	AEC
CD68	Tris/EDTA (pH9.0)	Mouse monoclonal	1:1000	Mouse-EnVision+, 5 min	1h at RT	Mouse-EnVision+	DAB
CD163	Citrate (pH 6.0)	Mouse monoclonal	1:10	Mouse-EnVision+, 5 min	1h at RT	Mouse-EnVision+	DAB

[a] 0.1% H2O2 was diluted in methanol. AEC, 3-amino-9-ethylcarbazole; DAB, 3,3'-diaminobenzidine

Table S3: Intragraft mRNA expression of S100A8 and S100A9 in biopsy samples obtained at various time points before and after kidney transplantation.

Group	S100A9 mRNA expression					S100A8 mRNA expression				
				Percentile					Percentile	
	Median	Min	Max	25th	75th	Median	Min	Max	25th	75th
Pre-Tx Bx	0.206	0.006	5.410	0.136	0.413	0.043	0.013	0.762	0.014	0.097
No rej. (<3 mo)	0.136	0.118	0.220	0.123	0.184	0.201	0.101	0.204	0.102	0.204
AR (<3 mo)	0.987	0.109	11.063	0.489	1.681	0.974	0.047	12.557	0.437	2.162
Prot. Bx (6 mo)	0.113	0.006	3.053	0.062	0.225	0.232	0.013	2.507	0.037	0.515
Prot. Bx (12 mo)	0.155	0.049	1.083	0.106	0.251	0.225	0.013	2.218	0.132	0.501
SAR (6/12 mo)	0.378	0.090	9.550	0.173	1.084	0.743	0.146	8.588	0.522	1.684

The mRNA expression S100A9 and S100A8 was measured with qPCR in renal allograft biopsy samples obtained at various time points before and after kidney transplantation: pretransplant biopsy samples (Pre-Tx Bx), biopsies taken on clinical indication within 3 months post transplantation without indications of rejection (No rej. (<3 mo)) or with histologically confirmed acute rejection (AR (<3 mo)), protocol biopsy samples obtained at 6 months (Prot. Bx (6 mo)) or 12 months (Prot. Bx (12 mo)) post transplantation without indications of rejection, or with histological indication of subclinical acute rejection SAR (6/12 mo).

Table S4: Association between graft outcome and demographic and clinical data of patients with acute renal allograft rejection

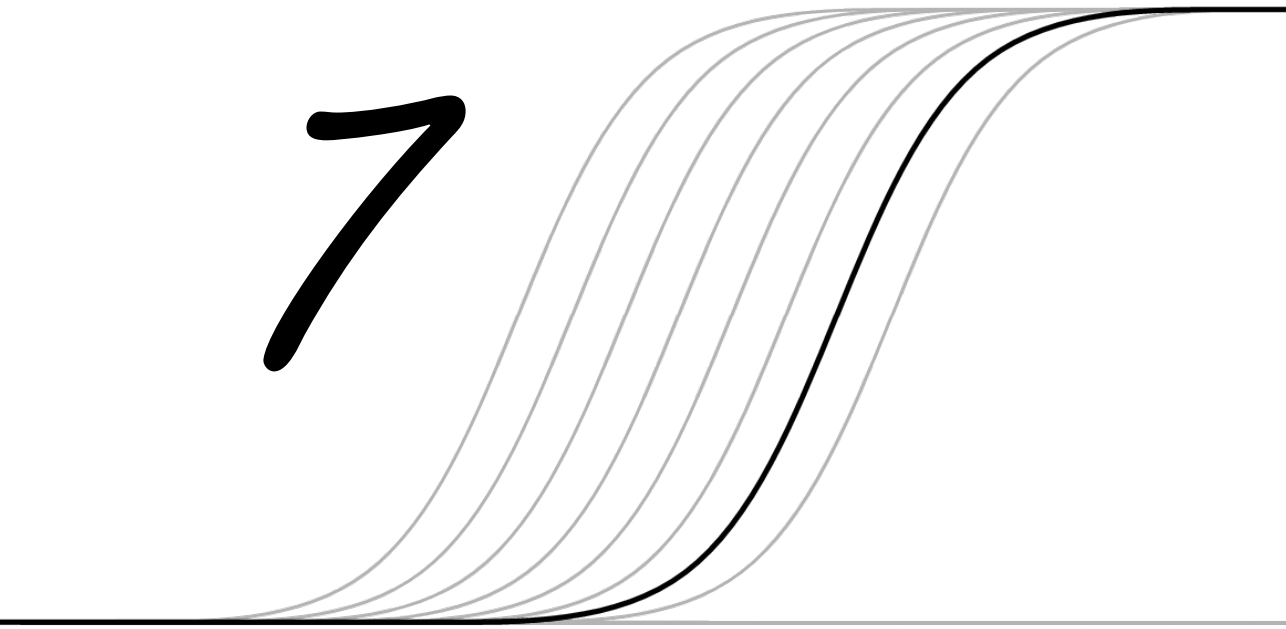
Variable	6 year outcome P value	12 years outcome P value
Patient age	0.17	0.53
Donor age	0.31	0.10
Year of transplantation	0.69	0.65
Donor type	0.21	0.33
HLA-AB matching	0.44	0.75
HLA-DR matching	0.37	0.55
Virtual PRA	0.002	0.0003
Cold ischemia time	0.40	0.52
Induction therapy	0.96	0.92
Maintenance therapy	0.13	0.06
Delayed graft function	0.08	0.23
C4d staining	0.69	0.99
Interstitial inflammation	0.46	0.42
Tubulitis	0.18	0.32
Intimal arteritis	0.93	0.99
Interstitial fibrosis	0.14	0.17
Tubular atrophy	0.09	0.15

PRA, panel reactive antibodies

Table S5: Correlation between mRNA expression of S100A9 and genes with known immune functions.

Gene	Function	Correlation
S100A8		0.93
ITGAM / CD11b	Matrix adhesion; lymph node homing; promotes adhesion of granulocytes	0.84
CEBPB / IL6DBP	Effector of IFNB-signaling in primary macrophages	0.82
CD44 / Pgp1	Leukocyte adhesion; lymph node homing critical role in resolving lung inflammation	0.80
TIMP1	Inhibition of matrix degradation	0.78
SERPINA3	Plasma protease inhibitor	0.78
FPR1	Interacts with bacterial N-formyl peptides and activates phagocytic cells in vitro	0.78
BATF	Enforced expression in CD8+ T cells impairs proliferation and cytokine signaling	0.73
TGM2	Role in apoptosis	0.73
TGFBI / BIGH3	Transforming growth factor beta-induced gene	0.72
GLIPR2	Inducer of autophagy	0.70
HCK	Expressed in mature granulocytes and monocytes; functional role in phagocytosis	0.69
TNFRSF1A / TNFR1	Tumor necrosis factor receptor	0.69
CCL23 / MPIF1	Chemotactic activity on resting leukocytes; inhibits hematopoietic progenitor cells that give rise to granulocyte and monocyte lineages	0.68
MAP3K8 / TPL2	Negative regulator of Th1-type immunity (by inhibiting IL-12 from accessory cells)	0.68
NCF4 / p40-phox	Stimulates NADPH oxidase activity	0.67
MNDA	Significantly upregulated in human monocytes that were exposed to interferon alpha	0.66
ANXA1 / LPC1	Acts through the FPR on human neutrophils to terminate neutrophil adhesion; mediates anti-inflammatory activities of glucocorticoids	0.66
NDST2	Processing of heparin polymers; production of histamine and protease in mast cells	0.66
SLAMF1 / CD150	Involved in T cell stimulation	0.65
RGS19	Affects survival and cell cycle of macrophages	0.65
PIM1	Role in hematopoietic development	0.65

Summary and general discussion



Summary and general discussion

Kidney transplantation is the preferred renal replacement therapy for patients suffering from end-stage renal disease. Unlike other renal replacement therapies, such as hemodialysis and peritoneal dialysis, renal transplantation results in the complete replacement of the native renal functions [1]. Kidney transplantation provides superior long-term patient survival compared to dialysis and improves the patient's quality of life [2;3]. Despite the success of renal transplantation, the number of transplants performed each year is hampered by shortage of donors [1;4]. This limitation stresses the need to improve long-term graft survival and prevent adverse graft outcome.

The occurrence of acute rejection is one of the most important risk factors for adverse graft outcome, as it is associated with reduced graft survival [5-9] and the development of chronic transplant dysfunction [10-13]. Once the diagnosis acute rejection has been made, it remains difficult to predict the risk of graft loss and the response to anti-rejection treatment using clinical parameters and histopathologic assessment. Availability of biomarkers could provide complementary parameters for assessing the risk of adverse graft outcome. Although various markers for graft outcome have been proposed [14-23], the heterogeneity in transcriptional regulation observed among acute rejection biopsies makes interpretation of the findings difficult. The aim of the work described in this thesis was to identify biomarkers during acute rejection, which may predict the response to high-dose steroid therapy and renal allograft survival.

Optimization of molecular techniques used for measurement of biomarkers

Molecular techniques are vital for the identification and validation of biomarkers for graft outcome. Molecular methods, such as quantitative real-time polymerase chain reaction (qPCR) [24-28] and microarray analysis [28-30], offer a rapid, sensitive, and specific tool to detect differences between patient groups. The reliability of molecular techniques can be affected by the quality and quantity of RNA that is isolated from the patient samples [31-33]. After kidney transplantation, the most valuable source of patient material is biopsy samples. Histopathological evaluation of biopsy samples can provide information on the location, severity, and characteristics of inflammatory infiltrates present in the renal allograft, which is important for diagnosis of the cause of graft dysfunction. In addition, renal biopsies form an important source of patient material for RNA extraction and subsequent gene expression analyses. Biopsy cores of kidney tissue are usually less than 1 millimeter in diameter and up to 1 centimeter

long. The limited availability of biopsy material and the high sensitivity of molecular techniques stress the importance to optimize the yields and quality of RNA obtained from the patient samples, and to minimize the extent of RNA degradation. In **chapter 2**, we investigated the impact of sample storage on RNA degradation, and compared the efficiency of various procedures for obtaining RNA and cDNA.

In order to conserve the renal biopsy samples for the actual biomarker discovery, peripheral blood cells were used as a model system for the optimization of the molecular techniques. Peripheral blood mononuclear cells (PBMC) and peripheral blood lymphocyte (PBL) blasts obtained from healthy individuals form a readily available source of RNA for research, and contain a full repertoire of immune cells known to infiltrate the graft during an acute rejection episode [14;34;35]. The use of PBMC and PBL blasts for the optimization experiments also minimized the fluctuations in cells composition between test conditions. We and others have observed a considerable heterogeneity in the extent and composition of the interstitial cellular infiltrate among acute rejection biopsies (see **Chapters 3 and 4**) [35-37]. These differences in cell composition could hamper the reliability of the optimization. In addition to the potential implications for biomarker discovery after transplantation, our finding may also be of interest for other research fields. Peripheral blood cells are frequently used for immunological research.

Impact of sample storage on RNA integrity

An essential requisite for the identification of biomarkers is the storage of patient material. Even though the storage of patient samples is often required for obtaining a meaningful sample cohort, it may also have an impact on RNA integrity. Therefore, we determined the impact of sample storage on RNA degradation. We found that RNA integrity is maintained during conventional procedures of storing peripheral blood cells at -180°C and thawing them thereafter, when compared to RNA extracted from freshly isolated cells. Incorporation of benzonase, a recombinant nuclease used for prevention of cell clumping [38], in the thawing procedure has no adverse effect on the preservation of high-quality RNA. In addition, thawed cells can be stored again in RNA-preserving reagents for RNA extraction on a later day. Our findings confirm that freezing of patient samples is a good approach to conserve RNA integrity. A limitation of our study is that we did not investigate the storage duration. Most frozen samples are stored for an extended period, which might influence the RNA integrity.

RNA extraction and cDNA synthesis

Other important aspects affecting the reliability of gene expression profiling are the molecular techniques used for RNA extraction and subsequent cDNA synthesis. Comparison of five RNA extraction protocols revealed that the yield and quality of the isolated RNA was similar for all procedures. When the qPCR signals per nanogram of total RNA and convenience are taken into account, the use of NucleoSpin and *mir*Vana extraction kits is preferable. However, these RNA extraction kits were not yet available at the start of the retrospective cohort studies described in this thesis. RNA extraction from the renal allograft biopsies was performed with the RNeasy spin columns, which resulted in high yields and high-quality RNA. Comparison of protocols for synthesizing cDNA from RNA revealed that the SuperScript III protocol provides the highest yields of cDNA. Robust cDNA synthesis increases the sensitivity of gene expression measurements.

Impact of RNA degradation on gene expression analysis

Assessment of RNA quality is a critical step for reliable quantification of gene expression levels. The integrity of RNA samples is an important element for the overall success of RNA-based analyses. We have shown that the RNA quality index (RQI), also known as RNA integrity number (RIN), provides a robust and reliable indicator of RNA integrity, which corresponds with earlier observations [33;39]. To establish the effects of RNA degradation, we investigated the impact of RNA integrity on gene expression analysis. Our study revealed a negative impact of RNA degradation on mRNA expression levels [31-33]. When normalized to endogenous reference genes, the expression of target mRNA transcripts can reliable be measured in moderately degraded RNA samples (RIN of 4 or higher). This finding corresponds with an earlier study, in which a RIN of 5 or higher was regarded as suitable for downstream qPCR application [31]. The use of partly degraded RNA samples may result in suboptimal qPCR conditions and reduced sensitivity, which may hamper the measurement of mRNA levels from genes that are expressed to low extent, including various cytokines and chemokines. Therefore, we recommend the use of high-quality RNA for molecular experiments.

Furthermore, we investigated the stability of microRNA transcripts. The studies revealed that microRNA expression levels remain relatively stable in degraded RNA. MicroRNA transcripts could be reliably quantified in severely degraded RNA (having

a RIN as low as 2). These observations hold promise for reliable quantification of microRNA expression in patient material that is prone to RNA degradation, such as urine samples. MicroRNA expression analyses could provide new insights into molecular mechanisms, and may lead to novel molecular markers of graft outcome after kidney transplantation.

Response to high-dose corticosteroid therapy for treatment of acute rejection

One of the main parameters determining graft outcome after renal transplantation is the sensitivity of the patient to high-dose corticosteroid therapy upon an acute rejection episode [40-42]. Approximately 25 to 30% of first rejection episodes cannot be reversed with corticosteroid therapy alone [17;42-45]. In such cases of steroid resistance, the patient requires more rigorous therapy with anti-lymphocyte antibodies to reverse the acute rejection episode. 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 [40-42;45;46].

The therapeutic effects of synthetic glucocorticoids (GC) for the treatment of acute renal allograft rejection, such as prednisone and methylprednisolone, are mainly attributed to their anti-inflammatory and immunosuppressive effects. They protect the allograft by inhibiting T-cell proliferation and cytokine gene transcription. The broad spectrum of immunomodulatory effects of GC are exerted through a complex system of molecular mechanisms.

Complexity of corticosteroid signaling

The actions of GC are mediated by the intracellular glucocorticoid receptor (GR), a ligand-dependent transcription factor of the nuclear receptor superfamily, which is ubiquitously expressed in most human cells [47;48]. The genomic structure of the GR consists of 9 exons (Figure 1) [49-51]. Alternative splicing in exon 9 generates two C-terminal receptor isoforms, termed GRα and GRβ [49]. The predominantly expressed GRα is activated by GC binding and mediates most of the known immunomodulatory effects, whereas the GRβ isoform expresses a different C-terminal region which inhibits GC binding [52-54]. The expression of GRβ is induced by cytokines [55]. GRβ can exert a dominant negative effect upon GRα-induced transcription. However, the functional importance of the GRβ isoform has not yet been determined. The GR protein structure consists of three domains (Figure 1): an N-terminal domain (NTD),

which directs target gene activation and the interaction with other transcription factors; a central DNA-binding domain (DBD), responsible for binding with glucocorticoid response elements (GRE) in the promoter region of target genes; and a ligand-binding domain (LBD), which contains specific GC and heat shock protein (Hsp) binding sites [56-59].

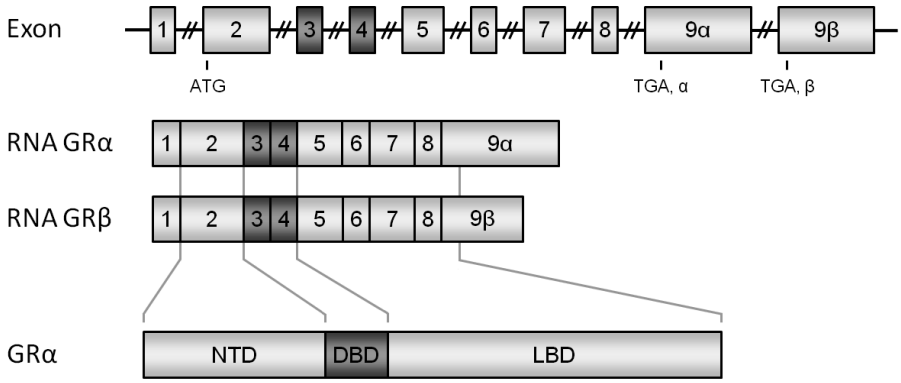


Figure 1. Genomic and protein structure of the human glucocorticoid receptor. The GR gene consists of 9 exons and is located in chromosome 5. Exon 1 is untranslated region, exon 2 codes for the N-terminal domain (NTD), exons 3 and 4 code for the DNA-binding domain (DBD), and exons 5 to 9 code for the ligand-binding domain (LBD). The GR gene contains two terminal exons 9 (exons 9α and 9β) alternatively spliced to produce the classic GRα and non-ligand binding GRβ.

After administration, GC diffuse across the cell membrane and bind to the cytoplasmic GR. In its ligand-free state, the cytoplasmic GRα is associated with an inhibitory complex, in which two Hsp90 molecules and one molecule each of Hsp70 and FKBP52 are included (Figure 2) [47;51;60]. This association stabilizes the hormone-responsive form of the receptor and inhibits nuclear localization. Upon ligand-induced activation, the GR dissociates from the molecular chaperone proteins and undergoes conformational changes [60-62]. This results in a rapid translocation of the GC-GR complex to the nucleus, where it regulates the transcription of target genes through direct or indirect signaling pathways (see Figure 2).

GR dimers bind via two zinc finger motifs in the DBD to GRE in the promoter region of target genes [63-65]. To initiate transcription, the GR uses transcriptional activation domains, located in the NTD and LBD. The GR interacts with the promoter region and recruits transcriptional co-activators and basal transcription machinery to the transcription start site [51;60]. This group of co-activators includes steroid receptor co-

activator-1 (SRC-1), CREB-binding protein (CBP), and GR-interacting protein 1 (GRP-1), which induce histone acetylation and subsequent transcription of anti-inflammatory genes (trans-activation) [62;66]. Less commonly, the GC-GR complex interacts with negative GRE (nGRE), which results in the repression of pro-inflammatory genes containing GR-binding sites (cis-repression) [60;61].

The major action of corticosteroids is the indirect suppression (trans-repression) of pro-inflammatory genes that are activated during acute rejection [48;50;62;67]. The GC-GR complex interferes with activating transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and cyclic AMP-responsive element-binding (CREB), and their transcriptional co-activator molecules. In addition, GR increase the transcription of IκB and MAP kinase phosphatase (MKP)-1, which inhibit NF-κB and mitogen-activated protein (MAP) kinase, respectively [47;48]. Furthermore, activated GR recruit histone deacetylase (HDAC)-2 to the activated inflammatory gene complex, resulting in deacetylation of nuclear histones and inhibition of gene transcription [48;50;62]. The signaling pathways of the GC-GR complex inhibit the transcription of pro-inflammatory molecules, such as cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors. Alterations in the molecular mechanisms of GR signaling may lead to steroid resistance.

Molecular biomarkers of steroid resistance

Prediction of steroid resistance at the time of biopsy could reduce unnecessary exposure to high-dose corticosteroids, and prevents irreversible nephron loss during the period that acute rejection is undertreated with steroids alone, which may affect long-term graft outcome.

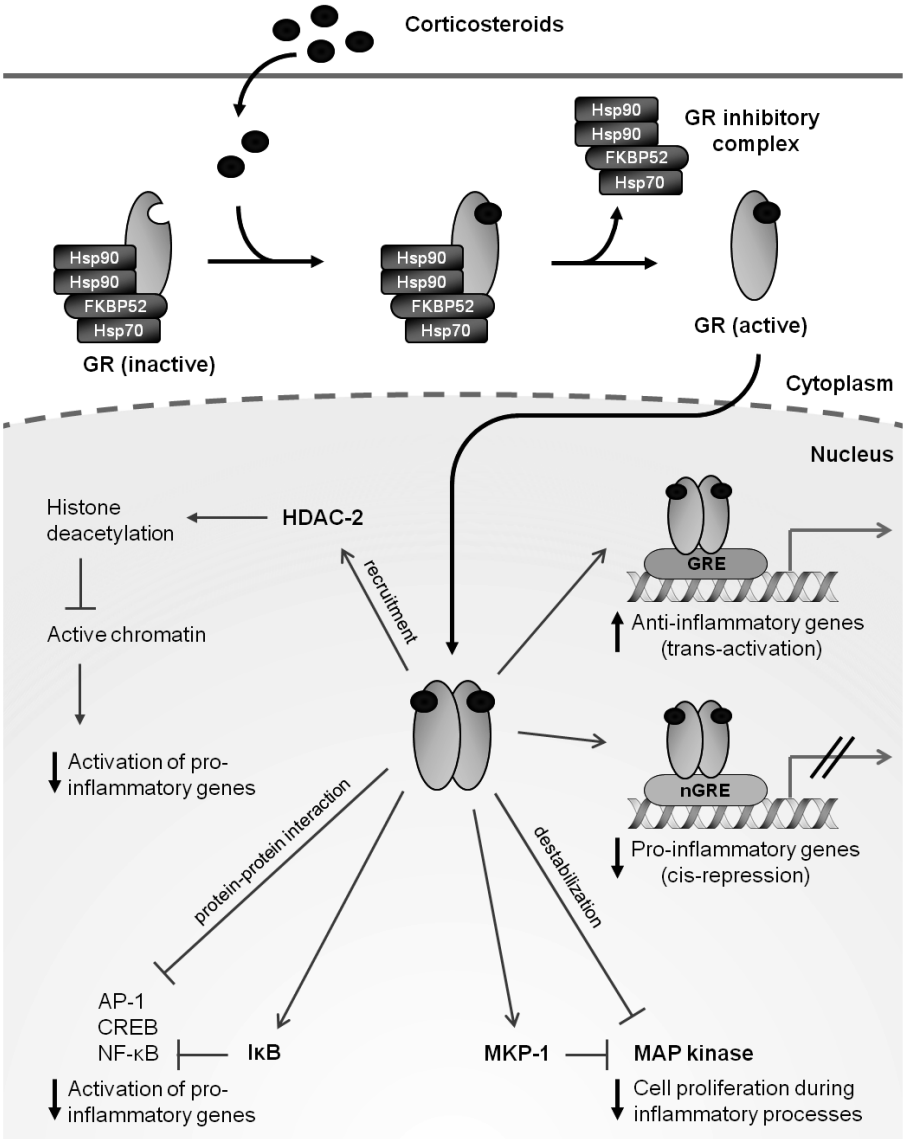


Figure 2. Complexity of corticosteroid signaling pathways. AP-1, activator protein-1; CREB, cyclic AMP-responsive element-binding; FKBP, FK506-binding protein; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; HDAC-2, histone deacetylase-2; Hsp, heat shock protein; IκB, inhibitor of κB; MAP kinase, mitogen-activated protein kinase; MKP-1, MAP kinase phosphatase 1; NF-κB, nuclear factor-κB; nGRE, negative glucocorticoid response elements.

To identify cellular and molecular markers associated with steroid resistance during 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 allografts. The selected panel of immune-related genes, which included markers previously reported in association with steroid response [14-23;68-70], reflected the full immune repertoire that may be present in the grafts. The panel contained cytokines, chemokines, and surface and activation markers of various cell types including T cells, macrophages, and B cells. We showed that differences in intragraft expression profiles reflect variability in the response to anti-rejection treatment with high-dose steroids. We found that the combination of T cell activation markers CD25:CD3e ratio and LAG-3 offers an improved prognostic value for assessing steroid response, compared to conventional clinical parameters and histopathologic assessment. These two signal transduction molecules are involved in the regulation of T cells: CD25, the α-subunit of the IL-2 receptor (IL-2R), is an important regulator of T cell survival and proliferation [71;72], while the activation-induced lymphocyte activation gene-3 (LAG-3) is involved in the negative regulation of homeostasis and T cell function [73;74]. High expression of cytotoxic T cells has been associated with resistance to steroid treatment of acute renal allograft rejection [16]. In addition, T cell characteristics, through disparities in IL-2 responses, have been correlated with steroid resistance [68-70].

These findings indicate that steroid resistance may reside in specific activated T cell populations. However, the prognostic value of the T cell activation markers was hampered by the molecular heterogeneity among the biopsy samples with acute rejection. This observation may be a reflection of the complexity of the mechanisms involved in response to steroid therapy. In contrast, it might also be that the differences in T cell characteristics observed between patients with steroid-refractory and steroid responsive acute rejection are the result of other upstream alterations in steroid signaling.

To gain further insight into the mechanisms underlying steroid resistance, and to identify novel molecular markers associated with steroid-refractory acute rejection, we performed genome wide expression profiling. Data presented in **chapter 4** show that relatively high intragraft expression of metallothioneins (MT) during acute rejection is associated with steroid resistance. Seven members of the MT-1 gene

family were expressed to a significantly higher extent in steroid resistant rejection. MT expression was mainly detected in activated macrophages and tubular epithelial cells within the kidney. These findings are in line with earlier findings in lung allograft recipients [75]. Increased percentages of MT-positive macrophages were found in transbronchial biopsy samples of lung allograft recipients with steroid-refractory acute rejection. MT are cysteine-rich proteins involved in the homeostasis of biologically essential metals, of which the regulation of zinc ions is the most important [63;76-79]. By functioning as a zinc-donor or zinc-acceptor, MT can control cellular zinc distribution [63;76]. Increased intragraft MT expression may lead to removal of zinc ions that are normally used in GC signaling (see Figure 3). The binding of activated GR to GREs relies on two zinc finger motifs located in the DBD [63-65]. Increased MT levels may lead to removal of zinc ions complexed in the zinc finger motifs [80;81], preventing GR binding to GREs and inhibiting the immunomodulatory effects of trans-activation and cis-repression (Figure 3). Another GC signaling pathway that may be affected by MT is the zinc-dependent recruitment of HDAC-2 by the GC-GR complex [48;50;62]. Increased expression of MT may lead to inhibition of the anti-inflammatory effects of this process (Figure 3).

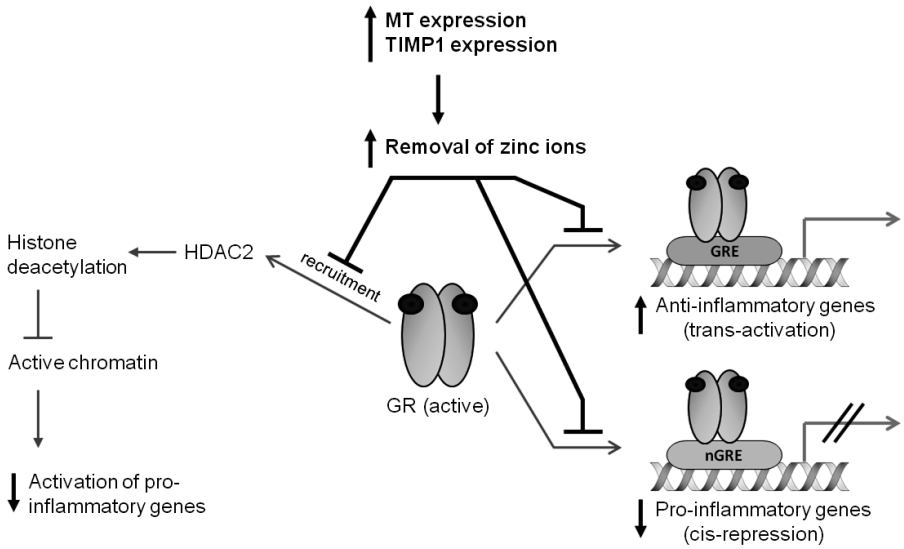


Figure 3. Effects of zinc-regulating molecules upon corticosteroid signaling. GR, glucocorticoid receptor; GRE, glucocorticoid response elements; HDAC-2, histone deacetylase-2; nGRE, negative glucocorticoid response elements.

Multivariate analysis of the genome wide expression profiles revealed the combination of MT-1 with CYP4A11, TIMP1 and F2R as the best predictive model for steroid-refractory acute rejection. This multivariate MT model offers a significantly improved prognostic value for assessing responsiveness to steroid therapy compared to conventional parameters, and has a slightly higher predictive value than the multivariate T cell activation model found in chapter 3. Combination of the two models resulted in a superior predictive model with MT-1, TIMP1, F2R, CD25:CD3e ratio, and LAG-3 as independent covariates, showing that the models reinforce each other's prognostic value. This finding indicates that there is no association between the observed differences in T cell characteristics and the intragraft regulation of zinc ions.

Besides MT and T cell activation markers, the combined multivariate model contains two other novel markers for steroid resistance: tissue inhibitor of metalloproteinase-1 (TIMP1) and coagulation factor II receptor (F2R). TIMP1 has been identified as an endogenous inhibitor of matrix metalloproteinases (MMP) [82]. TIMP1 inhibits MMP activity through coordination of the zinc ions of the MMP active site by the conserved cysteine residues in its N- and C-terminal domains [83;84]. Similar to MT, TIMP1 may diminish the zinc-requiring anti-inflammatory effects of the GR through regulation of the intracellular zinc concentrations (Figure 3). In addition, more recent studies have implicated TIMP-1 in the regulation of cell growth and apoptosis [84], which may influence the effects of GC signaling. F2R is a regulator of numerous intracellular signaling pathways, which include NF-κB and MAP kinase pathways [85-87]. Differences in F2R may influence the pro- and anti-inflammatory effects of GC. Further research will be needed to unravel the mechanism through which MT-1, TIMP1 and F2R affect the response to steroid therapy.

Finally, we investigated the role of glucocorticoid signaling and drug metabolism as predisposing factors towards responsiveness for high-dose steroid therapy during acute renal allograft rejection. Genetic polymorphisms in genes involved in these processes may explain the differences in response to steroid treatment. Data presented in **chapter 5** show for the first time that resistance to steroid treatment during acute renal allograft rejection may be associated with differences in the drug metabolizing enzyme CYP3A5. An adequate response to high-dose steroid therapy is associated with expression of the CYP3A5*1 gene variant by the donor tissue,

and with higher intragraft expression levels of CYP3A5. Carriers of the CYP3A5*1 variant have been shown to produce high mRNA and protein levels of CYP3A5, while individuals homozygous for CYP3A5*3 are low expressors of CYP3A5 [88-90]. In line with earlier findings, we observed genotype-dependent CYP3A5 expression levels in biopsies samples obtained during acute renal allograft rejection. This expression was mainly detected in inflammatory cells and tubular epithelial cells. A potential explanation for this observed correlation may be that CYP3A5*1 expression within the graft results in improved metabolism of methylprednisolone into a more effective metabolite. This in turn may increase the immunosuppressive effect of the steroid therapy (see Figure 4). Alternatively, it may be that toxic side effects of high steroid concentration within the allograft are prevented by the higher metabolic capacity of the CYP3A5*1 variant, while the anti-inflammatory effects of therapy are preserved. Further research will be needed to gain insight into the mechanism for the correlation between CYP3A5 and the response to steroid treatment.

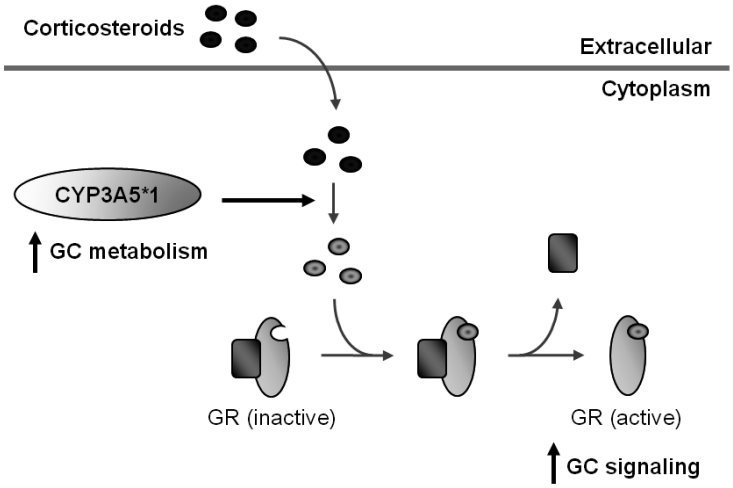


Figure 4. Effect of donor CYP3A5*1 gene variant upon corticosteroid signaling. GC, glucocorticoids; GR, glucocorticoid receptor.

Polymorphisms in other important drug metabolism-related genes, namely the efflux transporter P-glycoprotein (ABCB1) and the drug metabolism regulating pregnane X receptor (NR1I2), showed no correlation with response to steroid therapy, although polymorphisms in these genes have been associated with differences in GC metabolism [91-94], renal allograft outcome [95;96], and steroid resistance in children

with nephrotic syndrome [97]. Another important finding is that steroid-refractory acute rejection is not correlated with polymorphisms in GC signaling genes. Earlier studies have shown that genetic polymorphisms in NR3C1, the gene encoding the GR, may alter a patient's sensitivity to GC [98-102]. However, data presented in this thesis demonstrate that neither the genotype distribution of NR3C1 nor the intragraft expression levels of GR are correlated with the response to methylprednisolone during acute rejection. Combined, these findings provide data that gene variations in drug metabolism-related genes, but not in the glucocorticoid receptor, predispose to responsiveness to steroid therapy.

Clinical implications

The aim of the work described in this thesis was to identify cellular and molecular mediators that determine the response to high-dose steroid treatment during acute renal allograft rejection. The data from these studies demonstrate that resistance to steroid therapy is a complex and multifactorial condition, in which both immunological and non-immunological factors can be involved.

Investigations of immune-related biomarkers revealed that both T cells and macrophages play an important role in the response to steroid therapy. Renal allograft recipients with a steroid-refractory acute rejection episode express higher levels of T cells with characteristic activation markers compared to patients who are responsive to the treatment of acute rejection. Furthermore, macrophages derived from the transplant recipient were found to be an important aspect of the immune-infiltrate during acute rejection. The level of MT-expressing macrophages was correlated with steroid response. These findings support earlier observations correlating steroid resistance with the expression level and characteristics of T cells [16;68-70], and with the infiltration of macrophages into the renal allograft [20-23]. Earlier studies have also described that B cells may play a role in steroid response [14;15]. We investigated the predictive value of B cell specific transcripts, but found no correlation between the expression of B cell related genes and the response to steroid therapy. This is in line with earlier attempts to verify the impact of B cells infiltration on treatment response during acute renal allograft rejection [103-106]. Combined, these findings indicate that steroid resistance resides in specific cell populations and is not a feature of all lymphocytes. This may guide the therapeutic approaches for treatment of steroid-refractory acute rejection episodes.

A novel finding of our studies was that zinc regulation may play an important role in the response to steroid therapy during acute renal allograft rejection. Expression profiling revealed a correlation between steroid responsiveness and the expression levels of seven MT-1 family members and TIMP1. Both MT and TIMP1 are cysteine rich molecules involved in the regulation of intracellular zinc concentrations [76-79;83;84]. Increased expression of these molecules may lower the availability of zinc ions required during steroid signaling. This may cause inactivation of the DNA binding capacity of the GC-GR complex and inhibition of HDAC-2 recruitment, which in turn reduces the immunomodulatory effects of the high-dose corticosteroid therapy. Renal allograft recipients who express high intragraft levels of MT and TIMP1 during acute rejection might benefit from extra zinc intake for optimal GC signaling. A second, non-immunological factor that may affect GC signaling is the induction of metabolic changes in the administered methylprednisolone. Gene variations in the drug metabolism gene CYP3A5 expressed in the renal allograft may alter the metabolic capacity of CYP3A5, and improve the responsiveness to steroid therapy during acute rejection.

Thus, in summary, expression analyses revealed multiple markers which are associated with steroid resistant acute rejection. No single biomarker on itself predicts the response to steroid treatment with both high sensitivity and high specificity. The presence of multiple mechanisms underlying steroid resistance probably accounts for the restricted predictive power of single markers. Molecular heterogeneity among biopsy samples may also explain the difficulties in validating the prognostic value of previously proposed biomarkers for steroid resistance. In addition, it demonstrates the importance of using internal and external validation techniques to verify the robustness of potential biomarkers.

We also demonstrate that a multivariate prediction model, containing biomarkers related to different aspects of GC signaling, offers a superior prognostic value for assessing steroid response compared to conventional clinical parameters and histopathologic assessment, and to single biomarkers. Combined analysis of the intragraft MT-1, TIMP1, F2R, CD25:CD3e ratio, and LAG-3 expression levels can predict the responsiveness to high-dose corticosteroid therapy with high sensitivity and high specificity. This multivariate approach can identify patients who will not respond to steroid treatment and may benefit from immediate anti-thymocyte globulin (ATG) treatment. Introduction of our multivariate biomarker model in the clinic may

reduce unnecessary exposure of renal allograft recipients to high-dose corticosteroid therapy. More importantly, it may help avoid the development and progression of irreversible nephron loss during the period that steroid resistant acute rejection is undertreated with steroids alone. In addition, our multivariate model provides insight into the causative mechanisms in steroid-refractory acute rejection episodes, which may guide the development of novel therapeutic approaches. However, our multivariate model did not reach one hundred percent specificity and sensitivity for the prediction of steroid-refractory acute rejection. This suggests that additional, yet unidentified factors influence the response to high-dose steroid treatment of acute renal allograft rejection.

Prediction of renal allograft survival: expression of S100 molecules

Using the current diagnostic procedures, it has proven difficult to differentiate between renal allograft recipients with a high or low risk for adverse graft outcome. Incorporation of molecular markers, determined in transplant biopsy specimens, could help to assess the risk of developing AR, and whether such AR episode subsequently leads to graft loss over time. Two potential biomarkers for graft outcome are members of the S100 family of calcium-binding proteins. S100A8 and S100A9 are predominantly expressed in myeloid cells, such as neutrophils and early stages of monocyte/macrophage differentiation, but are absent in resident macrophages and lymphocytes [107-110]. S100A8 and S100A9 have been implicated as biomarkers for a variety of acute inflammatory disorders, including complications in organ transplantation [107;111-113].

In **chapter 6**, we evaluated the usefulness of intragraft S100A8 and S100A9 expression levels as prognostic markers for graft outcome. It has been shown that increased serum levels of S100A8 and S100A9 may predict the occurrence of acute rejection [113;114]. In line with these observations, we found increased expression levels of S100A8 and S100A9 in biopsy samples with histologically proven acute rejection compared with biopsy specimens without signs of acute rejection. As these molecules are mainly expressed in myeloid cells, the observed increase in expression likely reflects the infiltration of myeloid cells into the graft. This was indeed confirmed by analysis of S100A8 and S100A9 protein expression in renal biopsies. Our findings indicate that tissue expressed S100A8 and S100A9 are biomarkers of the development of acute renal allograft rejection.

Incorporation of biomarkers could help in assessing if an acute rejection episode subsequently leads to graft loss over time. We showed that patients with relative high S100A8 and S100A9 expression during acute rejection have improved graft survival. These observations confirm the data from a previous patient cohort [103]. The graft survival of patients with high S100 expression was similar to the graft survival of transplant recipients who had not experienced an acute rejection episode. Various studies have shown that the occurrence of acute rejection within the first few months is associated with an increased risk for graft loss. Our findings suggest that intragraft expression levels of S100A8 and S100A9 during acute renal allograft rejection may provide prognostic information for the patient with respect to graft survival.

The biologic effects of S100A8 and S100A9 in inflammatory disorders are dual and involve both pro- and anti-inflammatory actions [115-121]. To gain insight into the mechanism by which these molecules exert their biologic effects during acute renal allograft rejection, we investigated the association of S100 expression with the expression of pro- and anti-inflammatory factors. Patients with relatively high S100 expression and favorable graft outcome showed increased expression of immune regulatory markers, and at the same time low expression of markers reflecting kidney injury and total T cell burden in the graft, which is in line with earlier observations [19;122-124]. Furthermore, characterization of the S100 positive cell population revealed that S100A8 and S100A9 are expressed by a subset of CD68 positive myeloid cells in the tubulointerstitial compartment of the kidney. Although CD68 is widely regarded as a selective monocyte/macrophage marker [125-128], it can also be expressed by other myeloid cell types, such as granulocytes and dendritic cells [129;130]. The S100 positive cells seem to represent a myeloid cell subset with distinct effects from those of CD163 positive myeloid cells. CD163 has previously been proposed as a marker of anti-inflammatory macrophages [131;132]. However, our observations point toward CD163 as a marker of activated macrophages [133;134]. We propose that renal allograft survival is related to the presence of different myeloid cell subsets within the graft during acute rejection, and that high expression of S100 positive cells leads to increased local regulation of the immune response and a reduction in renal allograft damage.

Clinical implications

Our study shows that determination of S100A8 and S100A9 expression in renal allograft tissue is clinically relevant and may be used for assessing the risk of adverse

graft outcome. Screening of intragraft expression levels of S100 molecules may provide a complementary approach for diagnosing acute renal allograft rejection, and to determine whether such acute rejection episode subsequently leads to graft loss over time.

The extent of S100 expression during acute rejection correlates with the patient's risk of renal allograft loss over time. This is of interest in terms of the immunological mechanisms involved in the acute rejection episode. We demonstrate that high intragraft expression of S100 positive myeloid cells leads to increased local immune regulation, accompanied by a decrease in total T cell burden in the graft. This shift towards regulation reduces the intensity of the acute inflammatory response directed against the renal allograft, which in turn is reflected by a reduction in allograft damage and favorable long-term graft outcome. Based on these findings, we hypothesize that inflammatory infiltrate during acute rejection episodes, which contain relatively low numbers of S100 positive myeloid cells, are resolved less adequately and/or persist in the graft for prolonged time. Further studies will be needed to unravel the mechanism through which S100A8 and S100A9 positive myeloid cells induce the observed immunomodulatory effects.

Thus, in summary, the identification of S100A8 and S100A9 as predictive markers for adverse graft outcome could lead to improved monitoring and intervention strategies after renal transplantation, and may contribute to improved long-term renal allograft outcome.

Strengths and considerations of study design

Although various cellular and molecular markers have been proposed as prognostic biomarkers for renal allograft outcome [14-23], heterogeneity in transcriptional regulation observed among acute rejection biopsies makes interpretation of the findings difficult. The prognostic value of earlier proposed biomarkers could not be verified in later studies [103-106]. Important aspects that may influence the reproducibility of biomarker identification are differences in patient cohort characteristics (such as type of immune suppression, and the time between transplantation and the rejection episode), clinical endpoint definition, sample processing, and the molecular techniques used for expression analysis. This stresses the importance of the study design for the reliable and sensitive identification of biomarkers for renal allograft outcome. In the retrospective cohort studies described in this thesis, we handled

strict inclusion criteria, clinical endpoint definitions, and quality controls for sample processing and expression assays.

Due to that the main focus of our research was on the response to corticosteroid therapy, we only included patients who received pulse therapy with methylprednisolone as anti-rejection treatment. Furthermore, to prevent effects of anti-rejection treatment upon the composition of the renal allograft, only patients from whom renal biopsies had been obtained prior to the start of anti-rejection treatment were included. In addition, we excluded patients who received ATG as induction therapy because of the long-lasting alterations in lymphocyte subset composition induced by ATG therapy [135].

Another factor that affects the interpretation of biomarker discovery findings is the definition of clinical endpoints. The definition of steroid resistance varies widely between studies, and frequently cases of steroid-refractory rejection are poorly defined [14-23]. To improve the reproducibility of our findings, we used a stringent clinical endpoint definition. Steroid resistant acute rejection was defined as lack of clinical response to steroid pulse therapy with methylprednisolone, and a requirement for ATG treatment within 14 days after the start of the steroid therapy. Response to steroid therapy was monitored by serum creatinine concentration measurements and was defined as decrease in creatinine level to within 120% of the pre-rejection baseline value. This definition of steroid resistance is similar to the clinical endpoint definition used in earlier studies [21;106;136;137]. To ensure a reliable distinction between steroid-resistant and steroid-responsive cases, we set a minimal time period of five days before assessing the response to steroids [43].

As previously indicated, the reliability of biomarker discovery can be affected by the quality of the molecular techniques used for sample processing and expression analysis. In our retrospective cohort studies, we performed stringent controls to ensure the quality of our measurements. Biopsy samples with very low RNA yield or severe RNA degradation were excluded from the study. To ensure high sensitivity of qPCR measurements, all primer sets were tested for optimal performance before use, and melting curve analysis was used for assessment of the characteristics of each PCR amplicon. Suboptimal qPCR results were excluded from the study. For reliable gene expression measurements, we only included samples with an RQI of 8 or higher.

Future perspectives

Possible immune mechanisms of S100A8 and S100A9

The identification of S100A8 and S100A9 as prognostic biomarkers for adverse graft outcome could lead to improved long-term renal allograft survival. However, the immune mechanisms through which the S100 molecules induce this effect have not yet been uncovered. S100A8 and S100A9 may potentiate activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytic cells like monocytes and macrophages [138-140]. Activation of NADPH oxidase leads to generation of reactive oxygen species (ROS), which act as signaling molecules and may induce immune suppression via the induction of regulatory T cells [141;142]. An essential factor in the formation and activation of NADPH oxidase is arachidonic acid [143]. S100A8 and S100A9 bind and transfer arachidonic acid to the membrane-bound NADPH oxidase [138]. Increased expression of S100A8 and S100A9 during acute renal allograft rejection could lead to increased ROS production by myeloid cells and induction of regulatory immune mechanisms [139;140]. This local effect of S100 molecules on regulation of immune responses may limit renal allograft damage and prevent graft loss. Further studies will be needed to elucidate the mechanisms through which S100A8 and S100A9 modulate the immune response during acute renal allograft rejection.

Non-invasive methods for monitoring of graft outcome

Although evaluation of renal biopsies remains the gold standard for the diagnosis of graft outcome, its usefulness is slightly limited by the invasive nature of the biopsy procedure. Due to the associated risk of procedural complications, biopsy material for diagnosis of acute rejection and performance of molecular analysis is generally only taken on clinical indication [144;145]. A potential alternative to intragraft assessment may be the use of more non-invasive sources of material such as peripheral blood and urine. Identification of molecular markers in blood and urine may provide means to monitor graft function in time, which could lead to earlier detection of graft dysfunction and timely intervention of the immune process. However, it is not completely clear whether expression levels of molecular markers in blood or urine are as reliable as measurements of molecular markers in renal biopsies.

In addition to monitoring of the expression levels of molecular biomarkers, peripheral blood samples may also provide means to assess if a renal allograft

recipient is responsive to steroid therapy. *In vitro* tests with PBMC exposed to GC have been used to correlate gene expression profiles with clinical disorders, including steroid responsiveness [67;146-148]. However, the experimental design of these in vitro tests varied between studies, and the findings could not always be reproduced. Following this strategy, we used *in vitro* cultures of PBMC to investigate differences in response to methylprednisolone exposure. Comparison of molecular transcript levels allowed the distinction between responders and non-responders to steroid therapy. Further studies will be needed to confirm if *in vitro* cultures of patient PBMC represent a useful indicator of the patient's response to steroid treatment *in vivo*.

Validation of the prognostic value of our biomarkers

An essential requirement in biomarker discovery is validation and verification of the predictive value of proposed biomarkers. The diagnostic and/or prognostic value of biomarkers is generally better in the original patient cohort used for biomarker discovery compared to other patient cohorts. Validation techniques are used to ensure accurate and appropriate data collection and verify the usefulness of proposed biomarkers. We incorporated a variety of validation techniques in our retrospective studies to validate the prognostic value of potential biomarkers, including cross-validation and the use of a discovery and validation cohort. Although these techniques confirm the prognostic value of our univariate and multivariate prediction models to a large extend, additional validation studies in unrelated patient cohorts will be needed to verify our findings. In addition, the prognostic value of the biomarkers should be confirmed in a prospective study before they can be introduced into the clinic.

Analysis of microRNA expression

Another target for the identification of potentially informative biomarkers is the expression of microRNA transcripts. MicroRNAs are a class of small, non-coding RNA molecules that negatively regulate mRNA expression by degradation or translational repression [149;150]. Since the initial discovery in the early 1990s, over 1,000 microRNA transcripts have been identified. It is estimated that expression of more than one third of all genes is regulated by at least one microRNA [151;152]. In recent years, microRNAs have gained interest for their involvement in hematopoiesis and immune cell function [153-156], and for their role in allograft rejection [157-159]. Because of their relatively stable

expression, microRNAs are emerging as potential biomarkers. Analysis of microRNA expression profiles in renal biopsies may lead to the identification of novel prognostic biomarkers for the outcome of acute renal allograft rejection.

Conclusion

The results presented in this thesis demonstrate the potential of molecular and cellular biomarkers for the diagnosis and prediction of the outcome of acute renal allograft rejection. We demonstrated that resistance to steroid therapy is a complex and multifactorial condition, in which both immunological and non-immunological factors can be involved. The response to high-dose corticosteroid therapy for the treatment of acute renal allograft rejection correlates with the expression level and characteristics of T cells and macrophages infiltrating into the renal allograft. These findings indicate that steroid resistance resides in specific cell populations and is not a feature of all lymphocytes. Zinc regulation and drug metabolism may play a role in the response to steroid therapy during acute renal allograft rejection. Increased expression of zinc-regulating molecules may diminish the zinc-requiring anti-inflammatory effects of corticosteroid therapy. Therefore, kidney transplant recipients may benefit from additional zinc intake to optimize GC signaling. Genetic variations in drug metabolism genes may predispose to the responsiveness to steroid therapy during acute rejection. Furthermore, we demonstrated that no single biomarker on itself predicts the response to steroid treatment with both high sensitivity and high specificity. A multivariate prediction model, containing biomarkers related to different aspects of GC signaling, offers the best prognostic value for assessing steroid response. Combined analysis of the intragraft MT-1, TIMP1, F2R, CD25:CD3e ratio, and LAG-3 expression levels predicts the responsiveness to high-dose corticosteroid therapy with high specificity and high sensitivity. The prognostic value of this multivariate model is superior compared to that of conventional clinical parameters and histopathologic assessment, and to single biomarkers. Use of our multivariate biomarker model could reduce unnecessary exposure of renal allograft recipients to high-dose corticosteroid therapy, and prevent irreversible nephron loss during the period that steroid resistant acute rejection is undertreated with steroids alone. Finally, we investigated cellular and molecular mediators that may be used to predict graft survival. We demonstrated that determination of S100A8 and S100A9 expression in renal allograft tissue is clinically relevant and can be used for assessing

the risk of adverse graft outcome. Intragraft S100 expression levels can be used as an indicator for the development of acute rejection and for determining risk of renal allograft loss over time. High S100A8 and S100A9 expression during acute renal transplant rejection correlates with better long-term graft survival.

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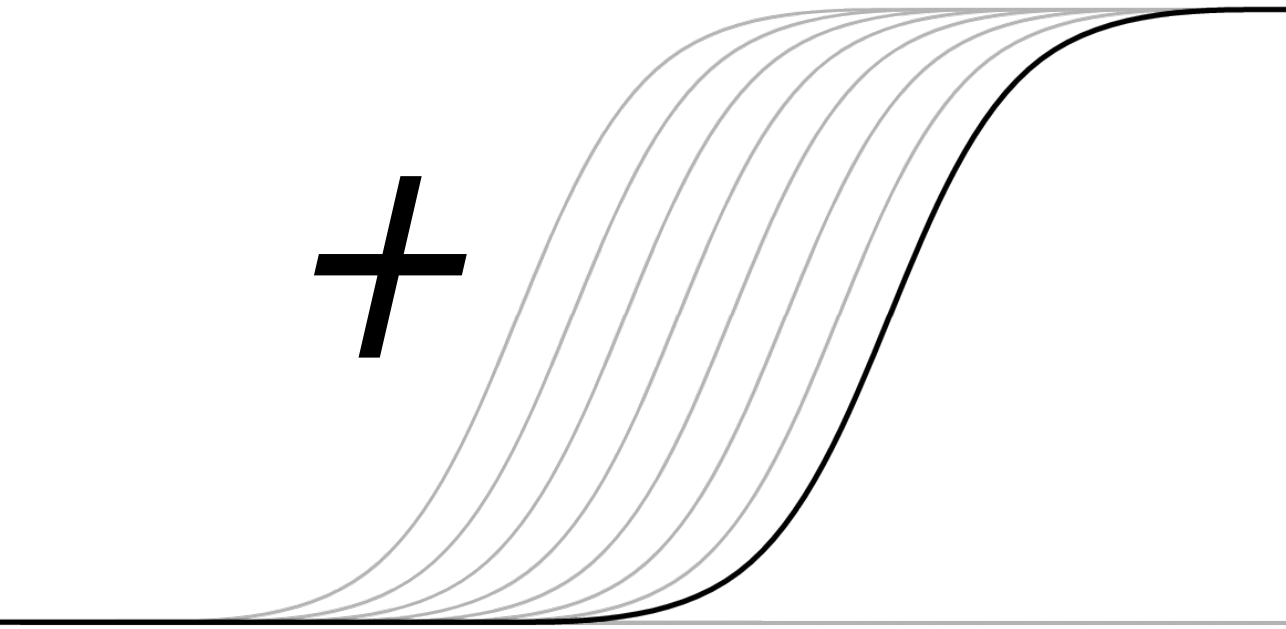
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Addenda



Nederlandse samenvatting

Doel van het proefschrift

Het doel van het in dit proefschrift beschreven onderzoek was om tijdens acute donornier afstoting biomarkers te identificeren die de respons op steroïden behandeling en niertransplantaatoverleving kunnen voorspellen.

Patiënten met chronische nierinsufficiëntie ervaren een progressief verlies van de nierfunctie over een periode van maanden of jaren. Deze chronische achteruitgang van de nierfunctie kan leiden tot eindstadium nierfalen, een aandoening waarbij de nieren niet meer in staat zijn om voldoende bloed te filteren en het lichaam vloeistoffen en schadelijke afvalstoffen behoudt. Deze volledige of bijna volledige uitval van nierfunctie is permanent en vereist gewoonlijk nierfunctievervangende therapie. De geprefereerde therapie voor patiënten die lijden aan eindstadium nierfalen is niertransplantatie. In tegenstelling tot andere nierfunctievervangende therapieën, zoals hemodialyse en peritoneaaldialyse, resulteert niertransplantatie in volledige vervanging van de autochtone nierfuncties. Daarnaast levert niertransplantatie superieure patiëntoverleving en een toename in levenskwaliteit. Ondanks het succes van niertransplantatie belemmert een tekort aan donoren het aantal transplantaties dat jaarlijks kan worden uitgevoerd. Deze beperking benadrukt de noodzaak om de lange termijn overlevingskans van het transplantaat te verbeteren en nadelige transplantatie uitkomsten te voorkomen.

Het optreden van acute afstotingsreacties gericht tegen de donornier is een van de belangrijkste risicofactoren voor adversieve transplantatie uitkomsten. Acute rejectie episodes zijn geassocieerd met een verminderde kans op transplantaatoverleving en de ontwikkeling van chronische transplantaat dysfunctie. Na de diagnose van een acute rejectie episode blijft het echter lastig om aan de hand van klinische parameters en histopathologische bepalingen het risico op nadelige transplantatie uitkomsten te voorspellen. De beschikbaarheid van biomarkers kan aanvullende parameters bieden voor de beoordeling van het risico op transplantaatverlies en de respons op anti-rejectie behandeling. Hoewel er verscheidene markers voor transplantatie uitkomst zijn voorgesteld, maakt de heterogeniteit in transcriptionele regulatie die in nierbiopten is waargenomen de interpretatie van deze bevindingen lastig.

Optimalisatie van moleculaire technieken gebruikt voor het meten van biomarkers

Moleculaire technieken zijn vitaal voor het identificeren van biomarkers voor transplantatie uitkomst. Moleculaire onderzoeksmethoden, zoals kwantitatieve polymerase-kettingreactie (qPCR) en microarray analyse, bieden de mogelijkheid om snelle, sensitieve, en specifieke bepalingen te doen voor het detecteren van verschillen in genexpressie tussen patiëntengroepen. De betrouwbaarheid van moleculaire technieken kan beïnvloed worden door de kwaliteit en kwantiteit van ribonucleïnezuur (RNA), dat is geïsoleerd van patiëntmonsters. Biopten van de donornier zijn de meest waardevolle patiëntmonsters na niertransplantatie. Histopathologische evaluatie van biopten kan informatie verschaffen over de locatie, ernst en karakteristieken van ontstekingsinfiltraten in de donornier. Deze informatie is belangrijk voor diagnose van de oorzaak van transplantaat dysfunctie. Daarnaast vormen nierbiopten een belangrijke bron van patiëntmateriaal voor RNA-extractie en daaropvolgende genexpressie analyses. De tijdens nierbiopsie verkregen weefselkernen zijn echter relatief klein; gemiddeld 1 millimeter in diameter en maximaal 1 centimeter lang. De gelimiteerde beschikbaarheid van biopsiemateriaal en de hoge sensitiviteit van moleculaire technieken benadrukken het belang om de opbrengst en kwaliteit van RNA te optimaliseren en om degradatie van RNA te minimaliseren.

Hoofdstuk 2 behandelt allereerst het onderzoek naar het effect van de opslag van patiëntmateriaal op RNA degradatie, vervolgens de vergelijking van de efficiëntie van verschillende procedures voor het verkrijgen van RNA en cDNA en daarna de impact van RNA degradatie op genexpressie analyse. Om de nierbiopten te behouden voor de uiteindelijke biomarkerontwikkeling, hebben we perifere bloed mononucleaire cellen (PBMC) gebruikt als modelsysteem voor het optimaliseren van de moleculaire technieken.

Impact van materiaalopslag op RNA integriteit

Ondanks dat opslag van patiëntmateriaal vaak noodzakelijk is voor het verkrijgen van een betekenisvol patiëntencohort, kan de opslag mogelijk ook nadelige effecten hebben op de integriteit van het RNA in het materiaal. Onze bevindingen tonen aan dat de RNA integriteit gewaarborgd blijft tijdens conventionele procedures voor het opslaan van perifere bloedcellen, waarbij de cellen worden bevroren tot -180ºC en op een later tijdstip weer worden ontdooid. Daarnaast kunnen ontdooide cellen

opnieuw worden opgeslagen in RNA-preserverende reagentia voor RNA-extractie op een later tijdstip. Onze bevindingen bevestigen dat het invriezen van patiëntmateriaal een goede methode is voor het conserveren van RNA integriteit.

RNA-extractie en cDNA-synthese

Naast de opslag van patiëntmateriaal kunnen ook de moleculaire procedures, gebruikt voor RNA-extractie en de hierop volgende cDNA-synthese, de betrouwbaarheid van genexpressie analyses beïnvloeden. Vergelijking van vijf RNA-extractie protocollen toont aan dat alle procedures een vergelijkbare opbrengst en RNA kwaliteit opleveren, waarbij de extractie protocollen zich alleen onderscheiden via lichte verschillen in qPCR sensitiviteit en gebruiksgemak. Vergelijk van cDNA-synthese protocollen onthult dat het SuperScript III protocol de hoogste cDNA opbrengst biedt. Robuuste cDNA-synthese verhoogt de sensitiviteit van genexpressie metingen.

Impact van RNA degradatie op genexpressie analyse

Evaluatie van RNA kwaliteit is een cruciale stap voor betrouwbare kwantificatie van genexpressieniveaus. De integriteit van RNA-monsters is een belangrijk element voor het algehele succes van op RNA gebaseerde analyses. Ons onderzoek naar de impact van RNA integriteit op genexpressie analyse toont aan dat RNA degradatie een negatief effect heeft op mRNA expressieniveaus. Gebruik van referentiegenen voor de normalisatie van genexpressie data maakt het mogelijk om betrouwbare mRNA niveaus te meten in RNA-monsters met matige degradatie. Echter, het gebruik van deels afgebroken RNA kan resulteren in suboptimale qPCR conditie en leiden tot verlaagde sensitiviteit.

Naast de impact van RNA degradatie op de stabiliteit van mRNA expressie metingen hebben we de stabiliteit van microRNA transcripten onderzocht. Uit onze studies blijkt dat microRNA expressieniveaus relatief stabiel blijven in gedegradeerd RNA. Kwantificatie van microRNA transcripten kan betrouwbaar worden uitgevoerd in ernstig aangetaste RNA-monsters. Deze observaties tonen de potentie voor het meten van microRNA expressieniveaus in patiëntmonsters met een verhoogde kans op RNA degradatie, zoals urinemonsters.

Response op hoge dosis corticosteroïden therapie voor de behandeling van acute rejectie

Een van de belangrijkste parameters die transplantaatoverleving na niertransplantatie bepaalt, is de gevoeligheid van de patiënt voor steroïden therapie tijdens acute donornier afstoting. Ongeveer 25 tot 30% van de eerste afstoting episodes kan niet worden onderdrukt met enkel corticosteroïden behandeling. In deze gevallen van steroïden resistentie heeft de patiënt een sterkere behandeling met antithymocytenglobuline (ATG) nodig voor het onderdrukken van de acute rejectie episode. Het onvolledig herstel van de donornierfunctie in steroïden resistente rejectie kan leiden tot progressie van chronische transplantaatschade en heeft een nadelig effect op transplantatie uitkomst.

Het therapeutische effect van synthetische glucocorticoiden (GC) voor de behandeling van acute donornier afstoting, zoals prednison en methylprednisolon, wordt vooral toegeschreven aan hun anti-inflammatoire en immunosuppressieve effecten. Het brede spectrum aan immuun modulerende effecten van GC komt voort uit een complex systeem van moleculaire mechanismen.

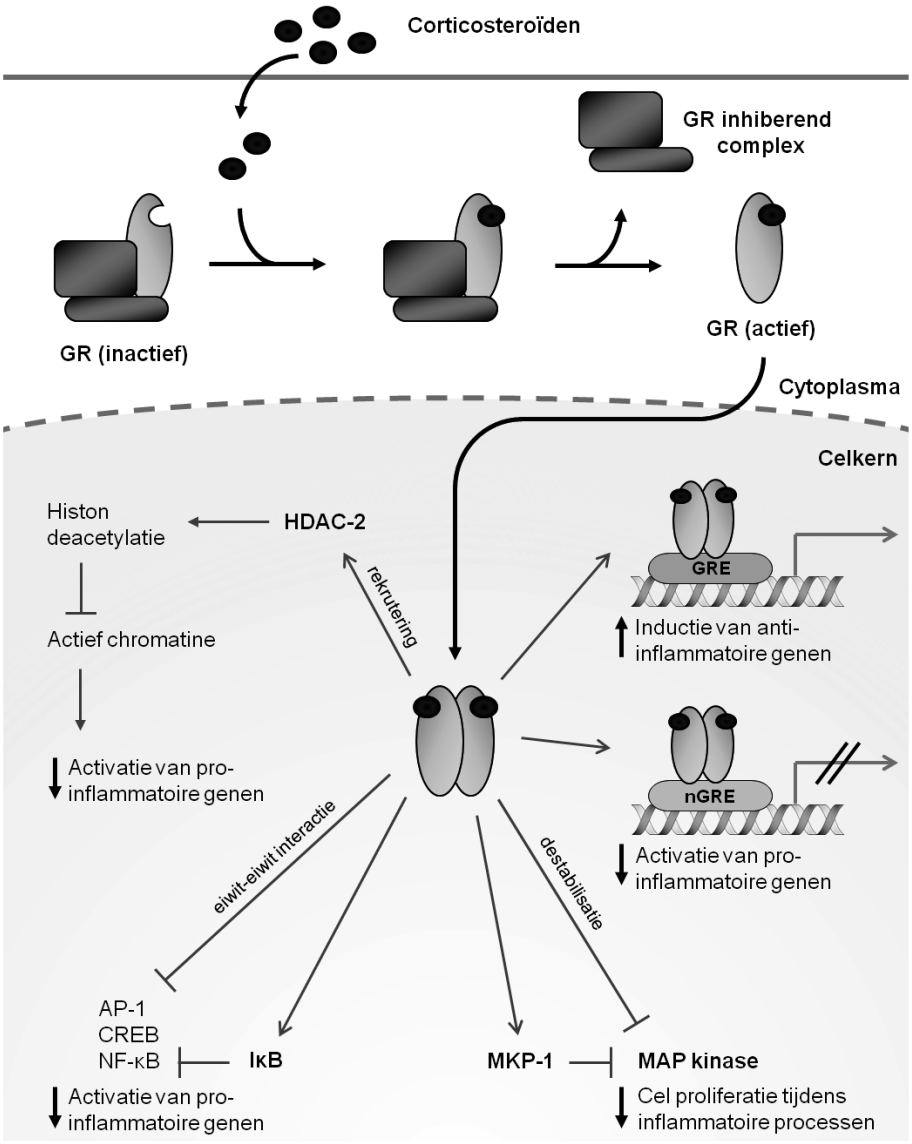
Complexiteit van corticosteroïden signalering

De effecten van GC worden aangestuurd door de intracellulaire glucocorticoid receptor (GR), welke door bijna alle menselijke cellen tot expressie wordt gebracht. Door alternatieve gensplitsing kunnen twee isovormen van de GR ontstaan, genaamd GRα and GRβ. De overwegend geëxpresseerde GRα wordt geactiveerd door GC binding en zorgt voor de meeste immunomodulatoire effecten, terwijl de GRβ isovorm mogelijk leidt tot inhibitie van GC binding. Het functionele belang van de GRβ isovorm is echter nog niet vastgesteld.

In ligand-vrije vorm is de cytoplasmatische GRα gebonden aan een inhiberend eiwitcomplex (zie figuur 1). Deze associatie stabiliseert de hormoonresponsieve vorm van de receptor en inhibeert lokalisatie naar de celkern. GC diffuseren door het celmembraan en binden aan de GR. Na deze ligand-geïnduceerde activatie dissocieert de GR van het inhiberende eiwitcomplex en ondergaat conformationele veranderingen. Dit resulteert in een snelle translocatie van het GC-GR complex naar de celkern, waar het gentranscriptie reguleert via zowel directe als indirecte signaleringsroutes (zie figuur 1). GR-dimeren binden via zogeheten “zinc-finger” motieven, specifieke DNA-bindende motieven met stabiliserende zinkionen, aan

glucocorticoid response elementen (GRE) in de promotorregio's van specifieke immuungenen. Deze interactie rekruteert transcriptionele co-activatoren en transcriptie-eiwitten naar de startlocatie voor gentranscriptie. De co-activatoren induceren histone acetylatie in het DNA en daarop volgend de transcriptie van anti-inflammatoire genen. Minder vaak gaat het GC-GR complex interacties aan met negatieve GRE (nGRE), welke resulteren in de onderdrukking van pro-inflammatoire genen.

Het belangrijkste effect van corticosteroïden is de indirecte suppressie van pro-inflammatoire genen, die geactiveerd worden tijdens acute transplantaat afstoting (zie figuur 1). De GC-GR complexen interfereren met activerende transcriptiefactoren, zoals nuclear factor-κB (NF-κB), activator protein-1 (AP-1), en cyclic AMP-responsive element-binding (CREB), en hun transcriptionele co-activatie moleculen. Daarnaast zorgt GR voor verhoogde transcriptie van IκB en MAP kinase phosphatase (MKP)-1, welke respectievelijk leiden tot inhibitie van NF-κB en mitogen-activated protein (MAP) kinase. Verder leidt activatie van de GR tot rekrutering van histone deacetylase (HDAC)-2 naar geactiveerde inflammatoire genen, wat resulteert in deacetylatie van nucleaire histonen en de inhibitie van gentranscriptie. Deze indirecte signaleringsroutes van het GC-GR complex onderdrukken de transcriptie van pro-inflammatoire moleculen, zoals cytokines, chemokines, adhesie moleculen, inflammatoire enzymen en receptoren. Veranderingen in de moleculaire mechanismen van GC-signalering kunnen leiden tot steroïden resistentie.



Figuur 1. Complexiteit van corticosteroïden signaleringsroutes.

Moleculaire biomarkers voor steroïden resistentie

Voorspelling van steroïden resistentie kan onnodige blootstelling aan hoge dosissen corticosteroïden verminderen en onomkeerbare schade aan nefronen in de donornier, die optreedt tijdens de periode waarin de acute afstoting onderbehandeld wordt met enkel steroïden, voorkomen. Beide effecten kunnen van invloed zijn op de lange-termijn transplantatie uitkomst.

Voor het identificeren van cellulaire en moleculaire markers, die geassocieerd zijn met steroïden resistentie tijdens acute donornier afstoting, hebben we retrospectieve studies uitgevoerd in een groot cohort van niertransplantatie patiënten met een eerste acute rejectie episode. In hoofdstuk 3 evalueren we de genexpressie van een breed panel van immunologische markers in niertransplantaten. Het geselecteerde panel van immuun-gerelateerde genen, welke markers bevat die in eerdere studies zijn geassocieerd met gevoeligheid voor steroïden, reflecteert het volledige immuunrepertoire dat in het transplantaat aanwezig kan zijn. Het panel bevat cytokines, chemokines, en oppervlakte- en activatie-markers van verscheidene celtypen, zoals T-cellen, macrofagen en B-cellen. Onze studie toont aan dat verschillen in expressie profielen gemeten in niertransplantaten een reflectie geeft van de variabiliteit in de response op anti-rejectie behandeling met steroïden. We hebben gevonden dat de combinatie van T-cel activatie markers CD25:CD3e ratio en lymfociet activatie gen-3 (LAG-3) een verbeterde prognostische waarde biedt voor het bepalen van respons op steroïden in vergelijking met conventionele klinische parameters en histopathologische bepalingen. Deze twee signaaltransductie moleculen zijn betrokken bij de regulatie van T-cellen: CD25, de α-subunit van de IL-2 receptor (IL-2R), is een belangrijke regulator van T-cel overleving en proliferatie, terwijl het activatie-geïnduceerde LAG-3 betrokken is bij de negatieve regulatie van homeostase en T-cel functie. Hoge expressie van cytotoxische T-cellen is in eerdere studies gekoppeld aan resistentie tegen steroïden behandeling van acute donornier afstoting. Daarnaast zijn de karakteristieken van de T-cel, door verschillen in reacties op IL-2, gecorreleerd met steroïden resistentie.

Deze bevindingen wijzen erop dat de oorzaak van steroïden resistentie zich mogelijk in specifieke geactiveerde T-celpopulaties kan bevinden. De prognostische waarde van de T-cel activatie markers word echter verstoord door heterogeniteit in transcriptionele regulatie in de biopten met acute donornier afstoting. Deze observatie is mogelijk een reflectie van de complexiteit in de mechanismen betrokken bij de response op steroïden therapie. Daarentegen kan het ook zijn dat de verschillen in T-cel eigenschappen, die zijn waargenomen tussen patiënten met steroïden resistente en steroïden responsieve acute afstoting, het resultaat zijn van andere veranderingen in steroïden signalering.

Om verder inzicht te krijgen in de onderliggende mechanismen van steroïden resistentie, en om nieuwe moleculaire markers geassocieerd met steroïden resistente acute afstoting te identificeren, hebben we genoomwijde expressie profilering uitgevoerd. In hoofdstuk 4 laten we zien dat relatief hoge expressie van metallothioneins (MT) in de donornier tijdens acute afstoting geassocieerd is met steroïden resistentie. Zeven leden van de MT-1 gen familie worden significant hoger tot expressie gebracht tijdens steroïden resistente donornier afstoting. MT expressie word voornamelijk gedetecteerd in geactiveerde macrofagen en tubulaire epitheelcellen in de nier. Deze bevindingen zijn in lijn met eerdere bevindingen in longtransplantatie patiënten, waar verhoogde percentages van MT-positieve macrofagen werden gevonden in transbronchiale biopsie weefselmonsters van patiënten met steroïden resistente acute donorlong afstoting. MT zijn cysteïne-rijke eiwitten die betrokken zijn bij de homeostase van biologisch essentiële metalen, waarvan de regulatie van zinkionen de belangrijkste is. Door te functioneren als een zinkdonor of zinkontvanger kunnen MT de cellulaire zinkdistributie reguleren. Verhoogde MT expressie in de donornier kan resulteren in het verwijderen van zinkionen, die normaal worden gebruikt in GC-signalering. Het kan leiden tot verwijdering van zinkionen uit de zinc-finger motieven, waardoor de binding van GR aan GREs voorkomen wordt en de immunomodulerende effecten geïnhibeerd worden. Een andere GC-signaleringsroute die beïnvloed kan worden door MT is de zink-afhankelijke recrutering van HDAC-2 door het GC-GR complex. Verhoogde MT expressie kan mogelijk leiden tot inhibitie van de anti-inflammatoire effecten van dit proces.

Multivariate analyse van de expressie profielen heeft onthuld dat de combinatie van MT-1 met CYP4A11, TIMP1 en F2R het best voorspellende model vormt voor steroïden resistente acute donornier afstoting. Dit multivariate MT model biedt een aanzienlijk verbeterde prognostische waarde voor de beoordeling van responsiviteit op steroïden therapie in vergelijking met conventionele parameters en biedt een iets hogere voorspellende waarde dan het multivariate T-cel activatie model dat in hoofdstuk 3 is gevonden. Combinatie van de twee modellen resulteert in een superieur voorspellend model met MT-1, TIMP1, F2R, CD25:CD3e ratio en LAG-3 als onafhankelijke covariaten. Hieruit blijkt dat de modellen elkaars prognostische waarde versterken en er geen verband is tussen de waargenomen verschillen in T-cel kenmerken en de regulering van zinkionen in de donornier.

Naast MT-1 en de T-cel activatie markers bevat het gecombineerde multivariate model twee andere nieuwe markers voor steroïden resistentie: tissue inhibitor of metalloproteinase-1 (TIMP1) en coagulatie factor II receptor (F2R). TIMP1 is een cysteïne-rijk eiwit, dat matrix metalloproteinases remt via de coördinatie van zinkionen. Vergelijkbaar met MT kan TIMP1 mogelijk de zink-afhankelijke anti-inflammatoire effecten van het GC-GR complex verminderen door de intracellulaire zinkconcentraties te reguleren. Daarnaast hebben recente studies TIMP1 geïmpliceerd in de regulatie van celgroei en apoptose, wat de effecten van GC-signalering kan beïnvloeden. F2R is een regulator van talrijke intracellulaire signaleringsroutes, waaronder de NF-κB en MAP kinase routes. Verschillen in F2R expressie kunnen mogelijk de pro- en anti-inflammatoire effecten van GC beïnvloeden. Verder onderzoek is nodig om de specifieke mechanismen te ontrafelen waarmee MT-1, TIMP1 en F2R invloed hebben op de respons op corticosteroïden therapie.

Verder is ons onderzoek gericht op de rol van DNA variaties in genen betrokken bij glucocorticoïden signalering en medicijn metabolisme als predisponerende factoren voor steroïden responsiviteit. Enkel-nucleotide polymorfie in deze genen kan leiden tot verschillen in respons op steroïden behandeling. In hoofdstuk 5 laten we zien dat steroïden responsiviteit tijdens acute donornier afstoting alleen afhankelijk is van genetische variatie in CYP3A5, een enzym dat betrokken is bij het metabolisme van ongeveer 50% van alle medicijnen. Een adequate reactie op steroïden behandeling is geassocieerd met expressie van de CYP3A5*1 genvariant in het donorweefsel. Dragers van de CYP3A5*1 variant hebben hogere expressie van het CYP3A5 enzym in vergelijking met individuen die homozygoot zijn voor de veel voorkomende CYP3A5*3 genvariant. Deze expressie wordt voornamelijk gedetecteerd in ontstekingscellen en tubulaire epitheelcellen in de donornier. Een mogelijke verklaring voor deze waargenomen correlatie kan zijn dat CYP3A5*1 expressie in het transplantaat leidt tot verbeterde omzetting van methylprednisolon in een effectievere metaboliet, wat het immunosuppressieve effect van de steroïden behandeling kan verhogen. Een andere mogelijkheid is dat de hogere metabolische CYP3A5*1 variant toxische bijwerkingen van hoge steroïden concentraties in het transplantaat voorkomt, terwijl de anti-inflammatoire effecten van de therapie bewaard blijven. Verder onderzoek is nodig om inzicht te krijgen in het mechanisme voor de correlatie tussen CYP3A5 en de respons op steroïden behandeling.

Klinische implicaties van biomarkers voor response op steroiden therapie

Onze studies tonen aan dat steroïden resistentie een complexe en multifactoriële aandoening is, waarbij zowel immunologische en niet-immunologische factoren betrokken kunnen zijn. Onderzoek naar immuun-gerelateerde biomarkers demonstreert dat zowel T-cellen en macrofagen een belangrijke rol spelen bij de respons op steroïden therapie. Niertransplantatie patiënten met een steroïden resistente acute rejectie episode hebben hogere expressieniveaus van T-cellen met karakteristieke activatie markers vergeleken met patiënten die goed reageren op steroïden behandeling. Daarnaast blijken macrofagen, afkomstig van de transplantaat ontvanger, een belangrijk component te zijn van het immuun-infiltraat tijdens acute afstoting. Hierbij correleert het niveau van MT-expresserende macrofagen in het infiltraat met de respons op steroïden behandeling. Verder is er, ondanks aanwijzingen in eerdere studies, geen correlatie gevonden tussen B-cel infiltratie en de respons op steroïden. Gecombineerd toont deze data aan dat steroïden resistentie gevestigd is in specifieke celpopulaties en geen kenmerk is van alle lymfocyten. Deze bevindingen kunnen sturing geven aan de toekomstige therapeutische benaderingen voor de behandeling van steroïden resistente acute donornier afstoting.

Een nieuwe bevinding van ons onderzoek is dat regulatie van zinkionen een belangrijke rol speelt in de reactie op steroïden behandeling. De response op steroïden therapie tijdens acute donornier afstoting correleert met de expressie van MT-1 familieleden en TIMP1, die via hun cysteïne rijke molecuulstructuur betrokken zijn bij de regulatie van intracellulaire zinkconcentraties. Verhoogde expressie van deze moleculen kan de beschikbaarheid van zinkionen, vereist tijdens steroïden signalering, verlagen. Dit kan leiden tot inactivatie van de DNA-bindingscapaciteit van het GC-GR complex en remming van HDAC-2 werving, wat vervolgens de immunomodulerende effecten van de steroïden therapie vermindert. Niertransplantaat ontvangers, die tijdens acute afstoting hoge niveaus van MT en TIMP1 tot expressie brengen in de donornier, kunnen mogelijk profiteren van extra zink inname voor een optimale GC-signalering. Een tweede niet-immunologische factor, die GC-signalering kan beïnvloeden, is de inductie van metabole veranderingen in de toegediende methylprednisolon. Expressie van genetische variaties in het metabolisme gen CYP3A5 in de donornier kan de metabole capaciteit van CYP3A5 wijzigen en mogelijk de respons op steroïden therapie tijdens acute donornier rejectie verbeteren.

Samengevat, onze studies hebben meerdere markers onthuld, die geassocieerd zijn met steroïden resistente acute donornier afstoting. De aanwezigheid van meerdere mechanismen die ten grondslag liggen aan steroïden resistentie is waarschijnlijk bepalend voor de beperkte voorspellende kracht van op zichzelf staande markers. Daarnaast kan de geobserveerde moleculaire heterogeniteit onder biopsie weefselmonsters ook verklaren waarom het moeilijk is gebleken om de prognostische waarde van eerder voorgestelde biomarkers te bevestigen. Wij demonstreren dat een multivariaat voorspellingsmodel, waarin biomarkers zijn opgenomen die betrekking hebben tot verschillende aspecten van GC-signalering, een superieure prognostische waarde biedt voor de beoordeling van steroïden respons ten opzichte van zowel conventionele parameters als losstaande biomarkers. Deze multivariate benadering kan worden gebruikt om patiënten te identificeren, die niet reageren op behandeling met steroïden en die kunnen profiteren van directe behandeling met ATG. Echter, de specificiteit en sensitiviteit van ons multivariate model liggen onder de honderd procent voor het voorspellen van steroïden resistente acute afstoting. Dit suggereert dat aanvullende, nog te identificeren factoren invloed hebben op de respons op hoge dosis steroïden behandeling van acute donornier afstoting.

Voorspelling van niertransplantaatoverleving: expressie van S100 moleculen

Gebruikmakend van de huidige diagnostische procedures is het lastig om onderscheid te maken tussen patiënten met een hoog of laag risico op nadelige transplantatie uitkomsten. Incorporatie van biomarkers kan bijdragen tot de voorspelling van 1) het risico op ontwikkeling van acute afstoting en 2) of een dergelijke acute rejectie episode na verloop van tijd leidt tot transplantaatverlies. Twee potentiële biomarkers voor transplantatie uitkomst zijn S100A8 en S100A9. Deze leden van de S100 familie van calciumbindende eiwitten worden voornamelijk tot expressie gebracht in myeloïde cellen en zijn in eerdere studies geïmpliceerd als mogelijke biomarkers voor een verscheidenheid aan acute inflammatoire aandoeningen, waaronder complicaties bij orgaantransplantatie.

Hoofdstuk 6 behandelt ons onderzoek naar de potentie van in de donornier gemeten expressieniveaus van S100A8 en S100A9 als prognostische markers voor ongewenste uitkomsten na niertransplantatie. Eerdere studies hebben laten zien dat verhoogde serumspiegels van S100A8 en S100A9 mogelijk het optreden van acute donornier afstoting kunnen voorspellen. In lijn met deze observaties toont

onze studie aan dat donornierbiopten met histologisch bewezen acute afstoting verhoogde expressie van S100A8 en S100A9 hebben ten opzichte van biopten zonder tekenen van acute afstoting. Onze bevindingen tonen dat de expressieniveaus van S100A8 en S100A9 in de donornier biomarkers zijn voor de ontwikkeling van acute niertransplantaat afstoting.

Verdere analyse van S100 expressie binnen de patiëntenpopulatie met histologisch bewezen acute afstoting demonstreert dat relatief hoge S100A8 en S100A9 expressie tijdens acute afstoting correleert met betere transplantaatoverleving over de tijd. Deze waarnemingen bevestigen data afkomstig van een eerder patiëntencohort. De transplantaatoverleving van patiënten met hoge S100 expressie is vergelijkbaar met de transplantaatoverleving van ontvangers die geen acute rejectie episode hebben ervaren. Diverse studies hebben aangetoond dat het optreden van acute afstoting tijdens de eerste paar maanden na transplantatie is geassocieerd met een verhoogd risico op transplantaatverlies. Onze bevindingen suggereren dat de expressieniveaus van S100A8 en S100A9 gemeten in de donornier tijdens acute afstoting prognostische informatie geeft over lange termijn transplantaatoverleving.

Om inzicht te krijgen in het mechanisme, waarmee deze moleculen hun biologische effecten uitoefenen tijdens acute donornier afstoting, hebben we de associatie van S100 expressie met de expressie van pro- en anti-inflammatoire factoren onderzocht. Patiënten met relatief hoge S100 expressie en gunstige transplantatie uitkomst tonen verhoogde expressie van immuunregulerende markers en tegelijkertijd lage expressie van markers die nierschade en inflitratie van T-cellen in de donornier reflecteren. Deze bevindingen zijn in lijn met observaties in eerdere studies. Karakterisering van de S100 positieve celpopulatie onthult dat S100A8 en S100A9 tot expressie worden gebracht door een subset van CD68 positieve myeloïde cellen in de nier. Hoewel CD68 algemeen wordt beschouwd als een selectieve monocyten/macrofagen marker, kan het ook worden geëxpresseerd door andere myeloïde celtypes, zoals granulocyten en dendritische cellen. De S100 positieve cellen lijken een subgroep van myeloïde cellen te vertegenwoordigen, die andere immuunregulerende effecten heeft op de immuunrespons dan eerder beschreven CD163 positieve anti-inflammatoire macrofagen. Op basis van deze bevindingen stellen wij dat de overlevingskans van de donornier afhangt van de aanwezige myeloïde cel subgroepen in het transplantaat tijdens acute afstoting en dat hoge expressie van S100 positieve cellen leidt tot lokale regulatie van de immuunrespons en een vermindering in donornierschade.

Klinische implicaties van S100 moleculen als biomarkers voor transplantaat-overleving

Onze studie toont aan dat het bepalen van S100A8 en S100A9 expressie in donornierweefsel klinisch relevant is en gebruikt kan worden voor het inschatten van het risico op een nadelige transplantatie uitkomst. Screening van de S100 expressieniveaus in de donornier kan een complementaire aanpak bieden voor het diagnosticeren van acute donornier afstoting en om te bepalen of een dergelijke acute rejectie episode na verloop van tijd tot transplantaatverlies leidt. De omvang van S100 expressie tijdens acute afstoting biedt daarnaast inzage in de immunologische mechanismen, die betrokken zijn bij de acute rejectie episode. We tonen aan dat hoge expressie van S100 positieve myeloïde cellen in de donornier leidt tot een verhoging in lokale immuunregulatie en een daling van het totale T-cel infiltraat in het transplantaat. Deze verschuiving naar regulering vermindert de intensiteit van de acute ontstekingsreactie gericht tegen de donornier, die op zijn beurt gereflecteerd wordt door een vermindering van donornierschade en gunstige langdurige transplantatie uitkomst. Onze hypothese is dat ontstekingsinfiltraten tijdens acute afstoting, die relatief lage aantallen S100 positieve myeloïde cellen bevatten, minder adequaat opgelost worden en/of voor langere tijd in het transplantaat stand houden.

Dus, samengevat, de identificatie van S100A8 en S100A9 als voorspellende markers voor ongunstige transplantatie uitkomst kan leiden tot betere controle- en interventiestrategieën na niertransplantatie en bijdragen tot een betere lange termijn donornier uitkomst.

Toekomstperspectieven: mogelijke immuunmechanismen van S100A8 en S100A9

De immuunmechanismen achter de effecten van S100 moleculen op transplantatie uitkomst zijn nog onbekend. S100A8 en S100A9 versterken mogelijk de activering van de nicotinamide adenine dinucleotide fosfaat (NADPH) oxidase in fagocyterende cellen zoals monocyten en macrofagen. Activatie van NADPH oxidase leidt tot vorming van reactieve zuurstofradicalen, signaalmoleculen die immuunsuppressie kunnen induceren via de inductie van regulatoire T-cellen. Essentieel bij de vorming en activering van NADPH oxidase is arachidonzuur. S100A8 en S100A9 binden arachidonzuur en brengen het over naar het membraangebonden NADPH oxidase complex. Verhoogde expressie van S100A8 en S100A9 tijdens acute donornier afstoting zou daarom kunnen leiden tot verhoogde productie van reactieve zuurstofradicalen

door myeloïde cellen en de inductie van regulatoire immuunmechanismen. Deze lokale immuunregulerende effecten van S100 moleculen kunnen donornierschade beperken en verlies van het transplantaat voorkomen. Verdere studies zijn nodig om op te helderen via welke mechanismen S100A8 en S100A9 de immuunrespons moduleren tijdens acute donornier afstoting.

Conclusie

De in dit proefschrift gepresenteerde resultaten demonstreren het potentieel van moleculaire en cellulaire biomarkers voor de diagnose en voorspelling van de uitkomst van acute donornier afstoting. We demonstreren dat resistentie tegen steroïden therapie een complexe en multifactoriële aandoening is, waarbij zowel immunologische en niet-immunologische factoren betrokken kunnen zijn. De respons op steroïden therapie voor de behandeling van acute niertransplantaat afstoting correleert met het expressieniveau en de karakteristieken van T-cellen en macrofagen infiltraten in de donornier. Deze bevindingen geven aan dat steroïden resistentie zich in specifieke celpopulaties bevindt en geen kenmerk is van alle lymfocyten. Daarnaast kunnen zinkregulatie en het metabolisme van geneesmiddelen een rol spelen in de respons op steroïden therapie tijdens acute donornier afstoting. Verhoogde expressie van zinkregulerende moleculen kunnen de zink-afhankelijke anti-inflammatoire effecten van corticosteroïden verminderen, terwijl genetische variaties in metabolisme genen predisponeren voor responsiviteit voor steroïden therapie tijdens acute afstoting. Verder demonstreren onze bevindingen dat een multivariaat voorspellingsmodel, met biomarkers die gerelateerd zijn aan verschillende aspecten van GC-signalering, de beste prognostische waarde biedt voor de beoordeling van response op steroïden therapie. Deze prognostische waarde was beter dan die van conventionele klinische parameters en histopathologische beoordeling. Ten slotte demonstreren we dat beoordeling van S100A8 en S100A9 expressie in donornierweefsel gebruikt kan worden als een indicatie voor het optreden van acute afstoting en voor het beoordelen of een acute afstotingsepisode na verloop van tijd kan leiden tot niertransplantaatverlies.

Curriculum vitae

Niels Vincent Rekers werd geboren op 20 juli 1983 in Haarlem. Na het behalen van zijn HAVO diploma in 2000 aan het St. Antonius College te Gouda, begon hij aan de opleiding biologie en medisch laboratoriumonderzoek aan de Hogeschool Leiden. Daar rondde hij in 2004 zijn bachelor-opleiding af in de afstudeerrichting proefdierkundig onderzoek, met een afstudeeronderzoek en scriptie aan de Endocrinologie afdeling van het Leids Universitair Medisch Centrum (LUMC) te Leiden. Hierop volgend is hij begonnen aan de opleiding biomedische wetenschappen aan de Universiteit van Leiden, waar hij in 2008 cum laude zijn master-opleiding voltooide met onderzoeken en scripties aan de afdeling Medische Farmacologie van het Leiden / Amsterdam Center for Drugs Research (LACDR) te Leiden en de afdeling Immunohematologie en Bloed Transfusie van het LUMC. Voor zijn ontdekking dat de vermindering in cognitief vermogen van muizen met type 1 diabetes veroorzaakt wordt door excessieve activatie van glucocorticoid receptoren, gedaan tijdens zijn onderzoek aan het LACDR, werd hem de S.E. de Jongh Medal 2007 toegekend. Gedurende de laatste drie jaar van zijn studie was Niels werkzaam als student assistent op de afdeling Neurofysiology van de Universiteit van Leiden. In 2008 begon hij aan zijn promotie-onderzoek op de afdeling Immunohematologie en Bloed Transfusie van het LUMC, onder begeleiding van prof.dr. F.H.J. Claas, prof.dr. J.W. de Fijter, dr. M. Eikmans en dr. I.M. Bajema. Tijdens zijn promotie-onderzoek maakte hij van 2008 tot 2011 deel uit van het bestuur van korfbal vereniging CKV Reeuwijk, waarbij hij van 2009 tot 2011 als secretaris lid was van het dagelijks bestuur. In 2009 ontving Niels voor zijn onderzoek een Young Investigator Award tijdens het American Transplant Congress 2009 in Boston. De resultaten van zijn promotieonderzoek staan beschreven in dit proefschrift. Sinds 26 augustus 2013 heeft Niels Rekers zijn loopbaan voort gezet met een post-doctoraal fellowship bij de Type 1 Diabetes groep van Matthias von Herrath in het Novo Nordisk Research Center in Seattle.

Niels Vincent Rekers was born on 20 Juli 1983 in Haarlem, the Netherlands on Juli 20, 1983. After graduating secondary school in 2001 at St. Antonius College in Gouda, he went on to study biology and medical laboratory research at Leiden College. He completed his bachelor studies in the field of laboratory animal research in 2004, with a graduation research project and thesis at the department of Endocrinology of the Leiden University Medical Center (LUMC), Leiden, the Netherlands. Subsequently, he went on to study biomedical sciences at the Leiden University. In 2008 he obtained his master degree with honours, with research projects and theses at the department of Medical Pharmacology of the Leiden / Amsterdam Center for Drugs Research (LACDR), Leiden, and the department of Immunohematology and Blood Transfusion of the LUMC, respectively. He was awarded the S.E. de Jongh Medal 2007 for his discovery that cognitive deficits in type 1 diabetes mice are caused by the excessive activation of glucocorticoid receptors. During the last three years of his studies, Niels worked as a student assistant at the department of Neurophysiology of the Leiden University. At the end of 2008 he started his PhD research under supervision of prof. F.H.J. Claas, prof. J.W. de Fijter, dr. M. Eikmans en dr. I.M. Bajema at the department of Immunohematology and Blood Transfusion of the LUMC. From 2008 to 2011 he was a board member of korfball club CKV Reeuwijk. In 2009 he was awarded a Young Investigator Award at the American Transplant Congress 2009 in Boston. The results of his PhD research are described in this thesis. Since August 2013 Niels has joined the Type 1 Diabetes research group of Matthias von Herrath of the Novo Nordisk Research Center, Seattle, with a two-year post-doctoral fellowship.

Abbreviations

ABCB1	ATP-binding cassette B1; P-glycoprotein
AEC	3-amino-9-ethylcarbazole
AP-1	activator protein-1
AR	acute rejection
ATG	anti-thymocyte globulin
ATP	adenosine triphosphate
AUC	area under the curve
BP	biological process
Bx	biopsy
CAN	chronic allograft nephropathy
CBP	CREB-binding protein
CC	cellular component
CI	confidence interval
CREB	cyclic AMP-responsive element-binding
CTD	chronic transplant dysfunction
CYP3A5	cytochrome P450 3A5
DAB	3,3'-diaminobenzidine
DBD	DNA-binding domain
DCs	dendritic cells
DGF	delayed graft function
DIG	digoxigenin
DMSO	dimethylsulfoxide
EGF	epidermal growth factor
F2R	factor II receptor
FBS	fetal bovine serum
FCS	fetal calf serum
FKBP	FK506-binding protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATM	glycine amidinotransferase
GC	glucocorticoids
GLCCI1	glucocorticoid-induced transcript 1 gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	gene ontology

GR	glucocorticoid receptor
GRα	glucocorticoid receptor α
GRβ	glucocorticoid receptor β
GREs	glucocorticoid response elements
GRP-1	GR-interacting protein 1
HAECs	human aorta endothelial cells
HDAC	histone deacetylase
HLA	human leukocyte antigen
Hsp	heat shock protein
HUVECs	human umbilical vascular endothelial cells
IFTA	interstitial fibrosis and tubular atrophy
IκB	inhibitor of κB
IL	interleukin
IL-2R	IL-2 receptor
IMDM	Iscove's modified Dulbecco's medium
KIM-1	kidney injury molecule 1
LAG-3	lymphocyte activation gene-3
LBD	ligand-binding domain
LNA	locked nucleic acids
LPS	Lipopolysaccharides
MΦ	macrophages
MΦ (stim)	stimulated macrophages
MAP	mitogen-activated protein
M-CSF	Macrophage colony-stimulating factor
MF	molecular function
MKP	MAP kinase phosphatase
MMP	matrix metalloproteinases
MP	methylprednisolone
MPK-1	mitogen-activated protein kinase phosphatase-1
MT	metallothioneins
NADPH	nicotinamide adenine dinucleotide phosphate
nGRE	negative GRE
NF-κB	nuclear factor-κB
NGS	normal goat serum

NK cells	natural killer cells
NP	Non-progression
NR1I2	nuclear receptor 1I2; pregnane X receptor
NR3C1	nuclear receptor 3C1; glucocorticoid receptor
NS	Not significant
NTD	N-terminal domain
OR	odds ratio
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PHYH	phytanoyl-CoA hydroxylase
PR	Progression
PRA	panel reactive antibodies
Pre-Tx	pretransplant
prot. Bx	protocol biopsy
PTC	peritubular capillaries
PTECs	proximal tubular epithelial cells
PV	predictive value
PXR	pregnane X receptor
qPCR	quantitative PCR
RIN	RNA integrity number
ROC	receiver operating characteristics
ROS	reactive oxygen species
RQI	RNA quality index
rRNA	ribosomal RNA
RT	Room temperature
SAR	subclinical acute rejection
SEM	standard error of the mean
SNP	Single nucleotide polymorphism
SRC-1	steroid receptor co-activator-1
TCMR	T-cell mediated rejection
TIMP1	tissue inhibitor of metalloproteinase-1
Tx	transplantation

List of publications

1. **Rekers NV**, Bajema IM, Mallat MJ, Anholts JD, de Vaal YJ, Zandbergen M, Haasnoot GW, van Zwet EW, de Fijter JW, Claas FH, Eikmans M. Increased metallothionein expression reflects steroid resistance in renal allograft recipients. *Am J Transplant*; 2013;13(8):2106-18.

2. Kuijk LM, Verstege MI, **Rekers NV**, Bruijns SC, Hooijberg E, Roep BO, de Gruijl TD, van Kooyk Y, Unger WW. Notch controls generation and function of human effector CD8+ T cells. *Blood*; 2013;121(14):2638-46.

3. Eikmans M, **Rekers NV**, Anholts JD, Heidt S, Claas FH. Blood cell mRNAs and microRNAs: optimized protocols for extraction and preservation. *Blood*; 2013;121(11):e81-9.

4. **Rekers NV**, Bajema IM, Mallat MJ, Zuidwijk K, Anholts JD, Goemaere N, Haasnoot GW, van Groningen MC, van Kooten C, de Fijter JW, Claas FH, Eikmans M. Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment. *Transplantation*; 2012;94(6):596-602.

5. Revsin Y, **Rekers NV**, Louwe MC, Saravia FE, De Nicola AF, de Kloet ER, Oitzl MS. Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice. *Neuropsychopharmacology*; 2009;34(3):747-58.

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