Molecular and genetic markers for the prediction of kidney transplant outcome

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General introduction

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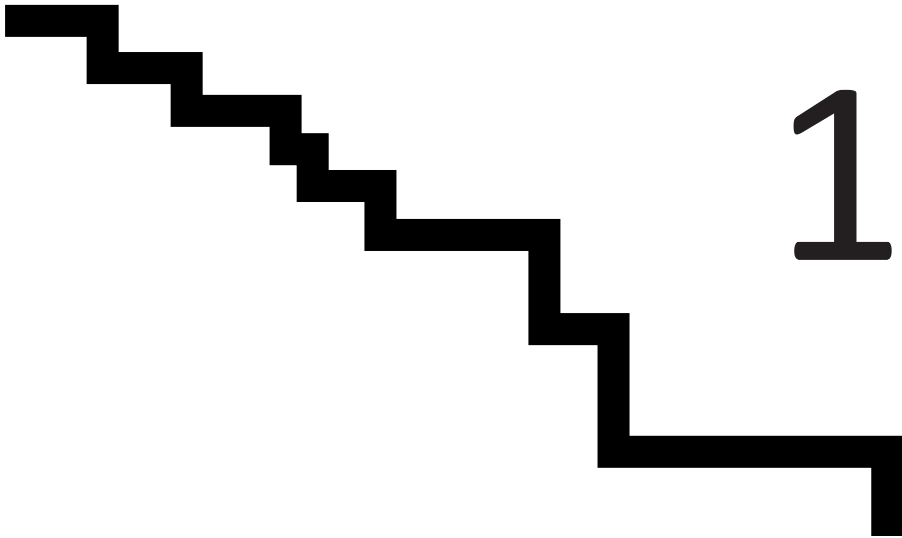

Content

Kidney transplantation
Complications of kidney transplantation
Rejection of the renal allograft
Hyperacute rejection
Acute rejection
Chronic allograft dysfunction
Diagnosis of allograft rejection
Serum creatinine
Histologic classification
Treatment of allograft rejection
Pulse corticosteroid therapy
Lymphocyte depleting antibodies
Treatment of antibody mediated rejection
Molecules involved in allograft rejection
Innate immunity: Toll-like receptors
The complement system
Apoptosis in the kidney
Endothelium-epithelium related genes
Risk assessment of transplant outcome by molecular tools
TCMR related genes
Myeloid related S100 proteins
Risk assessment of steroid resistance
Genetic risk factors for acute rejection
Genome-wide association study (GWAS)
Aim of this thesis

Kidney transplantation

Patients with diabetes mellitus, glomerulonephritis, or polycystic kidney disease may develop chronic kidney disease. If the decline of renal function continues, the patient can progress to end-stage renal disease (ESRD), associated with failure to remove excess fluid and waste products from the blood (1). Patients with permanent kidney failure require dialysis or kidney transplantation. Kidney transplantation is considered the favored treatment for patients suffering from ESRD, since successful transplantation is associated with longer survival and improved quality of life (2).

In 1954, Joseph Murray and his colleagues in Boston performed the first successful kidney transplant between identical twins (3). However, immunological rejection was observed when kidneys were transplanted from genetically non-identical donors. The introduction of the immunosuppressive drugs azathioprine and corticosteroids enabled kidney transplantation from unrelated donors with, originally, a success rate of 50% at 1 year (4). In the 1980s, graft survival at 1 year was higher than 80% due to the introduction of cyclosporine (4). Even with the current immunosuppressive medication, rejection remains a risk factor for adverse graft outcome (5).

The role of human leukocyte antigens (HLA) as strong histocompatibility antigens became apparent from skin grafting experiments in the early 1960s, which showed that grafts from HLA identical siblings had a longer survival than grafts from HLA mismatched siblings (6). Similarly, HLA matching had a beneficial effect on kidney graft survival. In order to enable HLA matching in unrelated transplants, Jon van Rood in Leiden founded in 1967 the international organ exchange organization Eurotransplant (7). The clear relationship between well matched HLA and lower incidence of acute rejection and longer graft survival was established. Following studies showed that not only HLA-A and -B matching but also HLA-DR matching had a strong beneficial impact on the outcome of cadaveric renal transplantations, and this led to strategies to allocate deceased donor kidneys to fully HLA-DR compatible patients (8-10). Although the introduction of more potent immunosuppression has diminished the beneficial effect of HLA matching on graft survival, fully HLA matched grafts still have a superior survival. This might be a reason to decrease the immunosuppression in fully HLA matched transplants as current immunosuppressive drugs are not specific and are often associated with side-effects (11).

Complications of kidney transplantation

Surgical complications may occur after kidney transplantation such as bleeding, thrombosis, urine leak or risk of infection. Due to the improvement of surgical techniques, the incidence of these complications is currently low (12). However, delayed graft function (DGF) and allograft rejection are regularly seen after transplantation.

Delayed graft function, defined as the need for dialysis within the first week after transplant, is an early complication after deceased donor transplantation (13). DGF is strongly associated with the incidence of acute rejection and chronic allograft nephropathy (14, 15). DGF is generally a consequence of acute kidney injury because of ischemia and reperfusion injury (IRI). Ischemia, e.g. deprivation of oxygen, results in exhaustion of adenosine triphosphate (ATP). Depletion of ATP destroys the homeostasis by accumulation of metabolic intermediates of glycolysis and increasing the osmolar load of cells, and it results in ischemic edema and subsequent necrosis and apoptosis (16). Numerous studies showed that brain death causes increased expression of proinflammatory cytokines, complement components, cell adherence molecules, and inflammatory cells in tissues (17-20). Reperfusion injury develops after a period of ischemia when blood supply to the tissue is affected. The rapid burst of reactive oxygen species and release of proteolytic enzymes following reperfusion lead to apoptotic and necrotic cell death (16). Damage associated molecules, such as high-mobility group box-1 (HMGB1), heat shock proteins (HSP), genomic double-stranded DNA, are released and bind to innate immune receptors leading to downstream signaling through NF-kB and subsequently initiation of inflammation (21-23). The damage of endothelial and epithelial cells may result in DGF affecting early and late graft function.

Rejection of the renal allograft

Despite the improvement of HLA typing techniques, facilitating better matching and the introduction of more potent immunosuppressive drugs, allograft rejection still occurs; although the incidence has diminished. Rejection can be categorized according to the time of occurrence: hyperacute rejection, acute rejection, and chronic allograft nephropathy (24). This thesis mainly focuses on factors related to the occurrence of acute renal allograft rejection.

Hyperacute rejection

Hyperacute rejection of renal allograft occurs within minutes to hours after reperfusion. This type of rejection results from preformed antibodies, induced by pregnancies, previous blood transfusion and previous transplants, which are directed against either the donor HLA or blood group antigens (25, 26). Antibody deposit on the microvasculature endothelium leads to activation of the classic complement cascade, which result in endothelial cell necrosis, thrombotic occlusion, and graft failure (27). Since the introduction of serological crossmatch testing before transplantation, hyperacute rejection has been largely eliminated (28, 29).

Acute rejection

Acute cellular rejection is the most common form of acute rejection and usually occurs within 6 months of post transplantation. It is primarily a T cell mediated immune response

against donor HLA expressed on the transplanted organ. The frequency of allo-reactive T cells is 1-10%, involving both naïve and memory T cell populations (30). The host's T cell can recognize alloantigen by three distinct mechanisms. Direct allorecognition is the direct interaction between the TCR on recipient T cells and mismatched HLA antigens on donor derived APCs. Indirect allorecognition plays a dominant role in initiating allograft rejection. In indirect recognition, donor derived antigens are processed and presented by recipient APCs to the recipient CD4 T cells (31). Recipient DC can also bind intact donor HLA, often in the form of exosomes, which leads to priming of recipient T cells via the semi-direct pathway (32). The B7 molecules (CD80 and CD86) present on APCs provide the costimulatory signal for T cell activation. The HLA peptide-TCR interaction and costimulation signal result in upregulation of IL-2 and CD25, which promote cell progression and differentiation. The activated T cells can migrate across peritubular capillaries and penetrate the tubules, leading to destruction of the tubular epithelial cells (cytotoxicity) and production of inflammatory cytokines and chemokines (24). CD8+ T cells release perforin and granzyme B that induce target cell apoptosis, and CD4+ T cells secrete TNFα that triggers apoptosis of endothelial and tubular cells (24). Other cells bearing chemokine receptors such as monocytes and myeloid DCs, which infiltrate the graft, also contribute to acute rejection (33, 34). Approximately 75% of patients with preexisting DSA are diagnosed with ABMR within 1 year (35).

Antibody mediated rejection (ABMR) can be already seen within the first year after transplantation (36, 37). ABMR is mediated by preexisting or *de novo* donor specific antibodies (DSA) that normally target the HLA displayed on donor endothelium or non-HLA antigens such as MICA (MHC class I polypeptide-related sequence A) and endothelial cell specific antigen (38, 39). Antigen and antibody interaction results in antibody-dependent cellular cytotoxicity and complement activation, both leading to lysis of the target cells. Endothelial cell injury result in platelet aggregation and recruitment of leukocytes via cytokines and chemokines (IL-1a, IL-8, and CCL2), and chemoattractants such as C3a and C5a. These phenomenons may eventually lead to graft failure (27).

Signs of acute TCMR and ABMR may be seen at the same time (mixed rejection) (40). Rejections can also occur beyond 6 months post transplantation, and is then often termed as chronic allograft rejection.

Chronic allograft dysfunction

Kidney transplants with progressive decline of renal function were formally characterized as chronic allograft nephropathy (CAN), a term which was later replaced by interstitial fibrosis and tubular atrophy (IF/TA) (41). Both alloimmune injury and non-immune injury such as calcineurin inhibitor toxicity, polyomavirus infection, and glomerular / vascular diseases can lead to IF/TA (41). Chronic ABMR, characterized by circulating DSA, microcirculatory lesions, and C4d deposition in the peritubular capillaries, is the major cause of late allograft failure. This late rejection process may also involve cell-mediated graft injury. Recipients with a

negative crossmatch test but with donor specific HLA antibodies (DSA) have an increased risk for graft failure (42). Compared to patients with preexisting DSA ABMR, patients with *de novo* DSA ABMR had an inferior graft survival (35). The production of *de novo* DSA was 51% in recipients with graft failure compared to 2% in patients with a stable graft function(43).

Diagnosis of allograft rejection

The current diagnosis of renal allograft rejection mainly relies on clinical monitoring, such as serum creatinine, proteinuria, and confirmation by histopathologic lesions in the kidney transplant biopsy. Molecular assessment of biopsy samples provides added value to facilitate histologic interpretation.

Serum creatinine

Creatinine is a waste molecule generated via catabolism of phosphocreatine, which is formed in the muscles. The production of creatinine is proportional to the muscle mass and varies with dietary intake of creatine. Circulating creatinine is freely filtered by glomeruli at a constant rate and excreted in the urine. An increased level of serum creatinine reflects a decreased glomerular filtration rate caused by a variety processes including allograft rejection, acute tubular injury, medication toxicity, and nephropathy from virus infection (44). Therefore, serum creatinine is the main marker used to monitor kidney function.

Histologic classification

Histological assessment of a core biopsy from the renal allograft is performed to distinguish acute rejection from other causes of decreased graft function. In 1991, international consensus criteria for histology diagnosis were proposed for the first time in Banff, Canada. These are updated and refined every two years (45). The Banff classification system was established to standardize the renal biopsy interpretation, which is applied as current golden standard (the Banff 97 criteria) for diagnosis of renal allograft rejection**.**

Treatment of allograft rejection

In spite of using potent induction and maintenance immunosuppressive therapy, rejection may still occur in renal transplantation recipients. Treatment of acute cellular rejection consists of pulse corticosteroid for the first rejection episode and lymphocyte-depleting antibodies (OKT3 or ATG) for severe rejections or steroid resistant rejections.

Pulse corticosteroid therapy

The treatment of acute renal rejection using prednisone was firstly reported in 1960 (46). The temporarily improved renal function raised the interest in pulse corticosteroid therapy as acute rejection treatment. Subsequently, Starzl showed that the acute rejection can regularly be reversed by addition of high doses of prednisone and actinomycin C (47). Since then, the

pulse prednisone approach turned into the principal treatment of acute rejection. As a high dose of oral prednisone was associated with a high risk of gastrointestinal complications and infection, a high dose of intravenous methylprednisolone was implemented as a successful therapy to reverse acute rejection, as this was associated with fewer side effects (48-50).

Lymphocyte depleting antibodies

Steroid resistant rejection is considered when the patient does not respond to steroid pulse therapy (serum creatinine does not return to below 1.2 fold of baseline level) within two weeks after the start of the treatment, leading to requirement of ATG treatment (51, 52). Approximately 30% of transplant recipients with acute rejection show no response to steroid treatment and require a more rigorous therapy. This type of rejection is termed as steroid resistant rejection. By the early 1960s, administration of the anti-lymphocytes serum was shown to prolong renal allograft survival in dogs (53). Subsequently, this kind of immunosuppressive therapy was applied in man to prevent organ rejection (54). Antithymocyte globulin (ATG) for clinical use is a polyclonal antibody directed against human T cells. The immunosuppressive effect of ATG is mainly due to the depletion of T cells via complement dependent lysis and T cell apoptosis. In addition, ATG may interfere with DC functions and it induces apoptosis in B cells (55). This antibody therapy is an effective treatment of acute rejection but it is associated with severe complications, such as fever, chill, leukopenia, and infection (56). At the moment, ATG is used for severe acute rejection and steroid resistant rejection.

The use of monoclonal antibodies targeting the cell surface markers of lymphocytes represents an alternative approach to deplete lymphocytes. Mouse-derived antibody against the CD3 molecules (OKT3) is used for blocking T cell function, and was found to be effective for induction of immunosuppression and treatment of steroid resistant rejection (57-59). However, treatment with OKT3 often leads to severe side effects, such as cytokine release syndrome, nephropathy, and cancer induction (60, 61). Basiliximab is a chimeric (mouse/human) antibody against human IL-2 receptor on the surface of T cells and it completely inhibits lymphocyte activation. Basiliximab combined with standard immunosuppressive therapy significantly reduces the incidence of acute rejection without increasing adverse effects after kidney transplantation (62). A humanized monoclonal antibody called Alemtuzumab is directed against CD52 on mature lymphocytes, and is an effective therapy for treating steroid resistant rejection, having greater beneficial effects than ATG treatment (63-65).

Treatment of antibody mediated rejection

Treatment of ABMR is aimed at the removal of preformed antibodies and elimination of B cells. Plasmapheresis rapidly removes existing antibodies and is considered a standard therapeutic strategy for ABMR, even though conflicting effects are reported (66). Whereas,

the DSA rebound after plasmapheresis therapy was well described and required additional strategy to decreases DSA production (67). It was reported that the combination of plasmapheresis and intravenous immunoglobulin (IVIG) leads to a better one year graft and patient survival than plasmapheresis alone (68). Numerous studies reported that the treatment of combination of plasmapheresis, IVIG, and rituximab (antibody against CD20) lead to superior graft survival rates compared to single treatment (69-71). Eculizumab (anti-C5 monoclonal antibody) for inhibition of terminal complement activation was used for treatment of ABMR (72, 73), but a large study is essential to confirm its beneficial effect. Currently, the treatment of choice for ABMR is the combination of plasmapheresis, IVIG, corticosteroids, and rituximab (74).

Molecules involved in allograft rejection

Several factors have shown to influence the occurrence of acute rejection, including DSA, HLA compatibility, DGF, the type of donor, recipient and donor age, and immunosuppressive therapy. Alterations in gene expression levels are often associated with the occurrence of acute rejection. This thesis mainly focuses on innate immune related genes, apoptosis related genes, endothelium-epithelium related genes.

Innate immunity: Toll-like receptors

The innate immune system, an evolutionarily conserved system, is an important component of the nonspecific defense against invading pathogens. It provide immediate defense against infectious pathogen but not a long lasting immunity. Besides the anatomical barriers, the innate immune system prevents infection via the complement system and cellular responses by macrophages, dendritic cells (DCs), and natural killer (NK) cells. Macrophages and DCs carry pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), which are molecular motifs conserved within many classes of pathogens but absent in vertebrates; and damage-associated molecular patterns (DAMPs), which are molecules released from damaged tissue and cells (75, 76). The PRRs include Toll-like receptors (TLRs), intracellular nucleotide-binding oligomerization domain-like receptors (NLRs) and RIG-I-like receptors (RLRs). This thesis focuses on the role of TLRs in kidney transplantation.

TLRs play a key role in innate immunity. TLRs are a family of transmembrane proteins recognizing PAMPs and mediating signal transduction via the transcriptions factors that induce expression of proinflammatory cytokines and chemokines (77, 78). So far, ten distinct TLRs have been identified in man, which can be largely divided into two subgroups depending on their cellular location. TLR1, TLR2, TLR4, TLR5, and TLR6 are located on the cell surface and recognize microbial membrane component such as lipopolysaccharide (LPS), lipoproteins, and flagellin. TLR3, TRL7, TLR8, and TLR9 are exclusively located in intracellular vesicles such as endosomes, lysosomes, and they recognize microbial nucleic acids such as CpG DNA, dsRNA, and ssRNA.

TLRs have extracellular recognition domains containing leucine rich repeats and an intracellular Toll/IL-1 receptor (TIR) signaling domain (79). When exposed to PAMPs, the TLRs form heterodimers and homodimers. Binding of triacylated lipopeptide induces the formation of heterodimers of TLR2 and TLR1, whereas binding of the diacylated lipopeptide leads to formation of TLR2 and TLR6 heterodimers (80, 81). Flagellin binding leads to formation of TLR5 homodimers (82). For TLR4 to recognize LPS it requires its co-receptor, myeloid differentiation factor 2 (MD2). TLR4-MD2 interactions lead to the formation of the hetero-tetrameric complex, (83). TLR3 directly recognizes dsDNA and forms a homodimer. The endosomal TLR7-TLR9 exist as stable preformed dimers (84). Binding of CpG DNA or RNAs results in a conformational change in the TLR dimer interface, which is essential for downstream signaling (85).

Dimerization of TLRs brings the cytosolic TIR domains into close proximity, which serves as scaffold protein for downstream signal molecules. All TLRs except for TLR3 require the adaptor protein: myeloid differentiation primary response protein 88 (MYD88) contains a death domain and TIR domain-containing adaptor protein (TIRAP) act as a bridging adaptor protein (86). During signal transduction, MYD88 recruits the IL-1R-associated kinase (IRAK) family and assembles the hetero-complex, which is required for activation of MAPK and NF-kB. The MAPK pathway can activate various transcription factors, including activator protein 1 (AP-1). Activation of the MYD88 dependent pathway, results in translocation of NF-kB and subsequently upregulates proinflammatory cytokines and chemokines (87). TLR3 signals through adaptor protein TIR domain-containing adaptor protein inducing IFNβ (TRIF). TLR4 also can signal via the TRIF related pathways, which requires the bridging adaptor protein TRIF-related adaptor molecules (TRAM) (88). The TRIF-dependent pathway mediated by TLR3 and TLR4 activates IRF3 and NF-kB (89). Activation of IRF3 results in the production of interferon-β that has a crucial role in antiviral immune responses. Thus, the signal transduction by TLRs is important for the proinflammatory pathway, and it bridges innate and adaptive immunity.

TLRs (2 and 9) are also considered important sensors of many extracellular and intracellular DAMPs, including HMGB1, uric acid (MSU), heat shock proteins (HSPs), S100 proteins, defensins, hyaluronic acid, fibrinogen, and chromatin (90). Self dsRNA or ssRNA released from damaged cells can be recognized by TLR3 or TLR7/TLR8. Additional DAMP receptors are the receptor for advanced glycation end-products (RAGE) that binds to HMGB1 and S100 protein, CD2 that recognizes galectins, and integrin that binds to extracellular components (90). TLR2 or TLR4 deficient mice are protected against kidney dysfunction and neutrophil accumulation after IRI (91, 92). In human studies, the expression of HMGB1 and TLR4 was significantly increased in deceased donor grafts. Recipients with a donor graft containing loss of function variants in TLR4 had a lower expression of proinflammatory genes MCP-1 and TNFα and higher expression of anti-inflammatory heme oxygenase 1, associated with an increased rate of immediate graft function (93). TLR2 and TLR4 expression was

found to be affected by renal ischemia-reperfusion and associated with poor early kidney allograft outcome (94-96).

The complement system

The complement system is part of the innate immune system and includes more than 30 plasma and membrane proteins. These attracts mononuclear phagocytes and enhance phagocytosis to remove microbes and dead cells. There are three distinct pathways of complement activation: the classical, alternative, and lectin pathway. The classical pathway is initiated by formation of the C1 complex ($C1q:C1r_2:C1s_2$) which directly binds to the antigen-antibody complex. Activated C1s enzyme cleaves C4 and then C2 to form the C3 convertase (C4b2a). The mannose-binding lectin (MBL) pathway is very similar to the classical pathway. MBL specifically binds to mannose residues present on many pathogens and forms the MBL complex with MASP-1 and MASP-2, homologues to C1r and C1s, leading to cleavage of C4 and C2. The alternative pathway is activated by spontaneous hydrolysis of C3. Once C3b covalently binds to a pathogen or cell surface, factor B binds to C3b and cleaved by factor D, which results in formation of C3 convertase (C3bBb). C3 convertase cleaves C3 to generate the C5 convertase (C4b2a3b or C3bBbC3b), which cleaves C5 and releases the C5b. C5b subsequently bind to C6, C7, and C8 to form the complex that inserts into the cell membrane and induces C9 polymerization (10-16 molecules) and pore formation known as the membrane-attack complex (MAC). The disruption of the cell membrane by MAC results in the loss of cell homeostasis and the eventual destruction of the cell or pathogen (97).

The role of complement activation is to promote phagocytosis and removal of pathogens and cell debris. Opsonization is mainly mediated by C3b, which binds to the surface of pathogens and is recognized by complement receptors on the phagocytes. C4b has a minor effect on opsonization (97). In addition, the small fragments generated during the complement activation cascade, such as C3a, C4a and C5a, have an important function to induce local inflammation. These molecules induce smooth muscle contraction, vasodilation, and enhance the vascular permeability similar to an anaphylactic shock, and are therefore referred to as anaphylotoxins. C3a and C5a can also induce the expression of adhesion molecules on endothelial cells and the release of histamine from activated mast cells. C5a could bind to G proteins and function analogous to chemokines to promote neutrophils and monocytes to adherence to endothelial cells and migrate toward the inflammation site and increase their phagocytosis ability (97).

Complement regulators are a family of proteins (including CR1, CD46, CD55, CD59, factor H) which negatively regulate complement activation. CD55 only has the decay accelerating activity that accelerates the C3 and C5 convertases. CD46 only has the cofactor activity for inactivation of C3b and C4b via cleavage by serum factor I. CR1, also known as CD35, has both decay accelerating activity and cofactor activity, but it has a limited tissue distribution. CD59 prevents C9 polymerization and formation of MAC. Factor H is a soluble

glycoprotein with both properties, to ensure that the complement system does not damage host tissue. This thesis only focuses on the membrane complement regulators.

The role of the complement system in the pathogenesis of IRI and allograft rejection has extensively been studied. The expression of complement components is significantly increased already before implantation in deceased donor kidneys compared to living donors (98, 99). Zhou showed that C3-, C5-, C6-deficient mice are protected from IRI. Reconstitution of C6 in C6-deficient mice restores the IRI, suggesting that formation of MAC may account for the renal injury (100). Steven Sack's group transplanted C3-deficient or wild type kidneys into MHC mismatched mouse recipients. Recipients of C3-deficient kidneys had long term graft function, suggesting that expression of C3 is crucial for IRI and acute rejection (101). Knocking out or inhibiting MBL in mice protects against renal IRI (102, 103). Factor B deficient mice IRI are also protected from renal IRI, suggesting the involvement of the alterative pathway in complement activation (104). Transplantation of a donor heart that is deficient of CD55 results in a much stronger complement activation in the transplant (105). Blocking of C5a receptor improves graft function after IRI, indicating that anaphylotoxins (C5a) are involved in renal IRI (106). All these studies indicate that the complement activation plays an important role in IRI.

Numerous studies in mice have investigated the effect of complement components on the activation of the adaptive immune system. Absence of C3a signaling in DCs, either by C3a receptor-deficiency or C3aR antagonist treatment, will decrease the expression of MHC II and costimulatory molecules, and consequently leads to reduced allo-specific T cell responses (107). Also, deficiency of the C5a receptor in both recipients and donors is associated with a reduction of the allo-specific T cell immune response and prolonged graft survival (108, 109). Absence of C5a signaling leads to increased expression of TGF-beta, which triggers the $CD4^+$ T cells to differentiate into $Foxp3^+$ Treg cells and Th17 cells (110). Monocyte-derived DC stimulated with C3a and C5a show an increased ability for allo-stimulation through NF-kB signaling (111). C5a binds to its receptor on T cells, which will lead to increased T cell expansion through diminished T cell apoptosis (112). These studies show that complement fragments play a vital role in adaptive immune response.

Complement also has an effect on antibody production, since depletion of C3 suppresses thymus-dependent antibody generation (113). C3 deficient mice transplanted with wild type bone marrow are able to produce antibodies upon a viral infection (114). In MHC mismatched skin transplantation, C3 deficient mice demonstrated an impaired IgG response and a decreased range of IgG isotypes (115). Thus, B cell maturation and antibody secretion require complement activation.

Apoptosis in the kidney

Renal IRI causes cell death by necrosis and apoptosis (116, 117). Kerr et al. reported apoptosis as a mechanism of cell death, which is different from necrosis with respect to acute tissue injury (118).

The major B-cell lymphoma 2 (BCL2) family members, pro-apoptotic protein BCL2-associated X protein (BAX) and anti-apoptotic protein BCL2, play an important role in apoptosis activation. Cell death signals activate Bcl-2 homology domain 3 (BH3), which result in conformational changes of BAX and BCL2. The membrane-integrated BAX assembles into a homo-oligomeric pore that permeabilizes the mitochondrial membrane to release pro-apoptotic factors such as cytochrome c (119). The changed BCL2 can bind to BAX and inhibit the oligomerization process (119). The cytosol cytochrome c binds to apoptotic protease-activating factor-1 (APAF1) in order to form a heptameric protein ring termed apoptosome, which activates initiator caspase-9 and then activates executioner caspse-3 (120). Caspases in turn degrade a series of cellular components and orchestrate cell demolition (120). Thus the balance between death or survival upon stimulation seems to be determined by the BAX:BCL2 ratio (121, 122). In addition, renal tubular cell apoptosis also can be activated by extrinsic pathways. The extracellular ligands such as TNF or Fas ligand interact with the cell surface death receptors, and they lead to formation of death inducing signaling complex (disc) that recruit and activate caspase-8. The activated caspase-8 could either cleave BCL2 family members and induce mitochondrial stress, or directly activate caspase-3 to promote apoptosis (120, 123). In apoptosis, the chromatin forms dense crescent-shaped aggregates lining the nuclear membrane. Nuclear membrane invagination results in segmented nucleus. Subsequently convolution of the plasma membranes leads to a cluster of membrane bound segments and apoptotic bodies, which contain cellular organelles (118). The apoptotic cells are rapidly phagocytized by macrophages without generating inflammation (118, 124).

Apoptosis and DNA fragmentation are often observed after reperfusion (125, 126), but the apoptosis of tubular cells is initiated during the normothermic ischemia, and is characterized by activated BAX and decreased anti-apoptotic proteins BCL2 and cFLIP (121). Apoptosis of tubular cells contributes at least part to acute and chronic renal allograft rejection (127, 128). Phagocytosis of apoptotic cells by macrophages does not stimulate inflammation. However, if the apoptotic body membranes becomes permeabilized, this can switch the macrophage response to proinflammatory. The responsiveness of macrophages to apoptotic cells mainly depends on the balance of anti- and pro-inflammatory signals (129).

Endothelium-epithelium related genes

Vascular endothelial cells serve as the interface between the blood and tissue and mediate coagulation and inflammation. Glomerular endothelial cells are remarkably flattened and highly fenestrated, which allows exchange of large molecules (130). Peritubular capillaries surround the vasa recta, allowing reabsorption of oxygen and nutrients, and they secrete certain mineral ions. Therefore, maintenance of endothelial integrity is critical for renal function and graft survival. Ozdemir et al reported that patient with mild microvascular destruction respond more frequently to steroid treatment than patient with severe

microvascular endothelium destruction (131). Various studies showed that vascular rejection is associated with inferior kidney transplant outcome (131-133). Endothelial cell specific transcripts can be identified using microarray techniques, and include amongst others CD31, cadherin 5, and von Willebrand factor (134). These transcripts are involved in blood vessel formation and cellular adhesion and may reflect the quality of endothelium and kidney function.

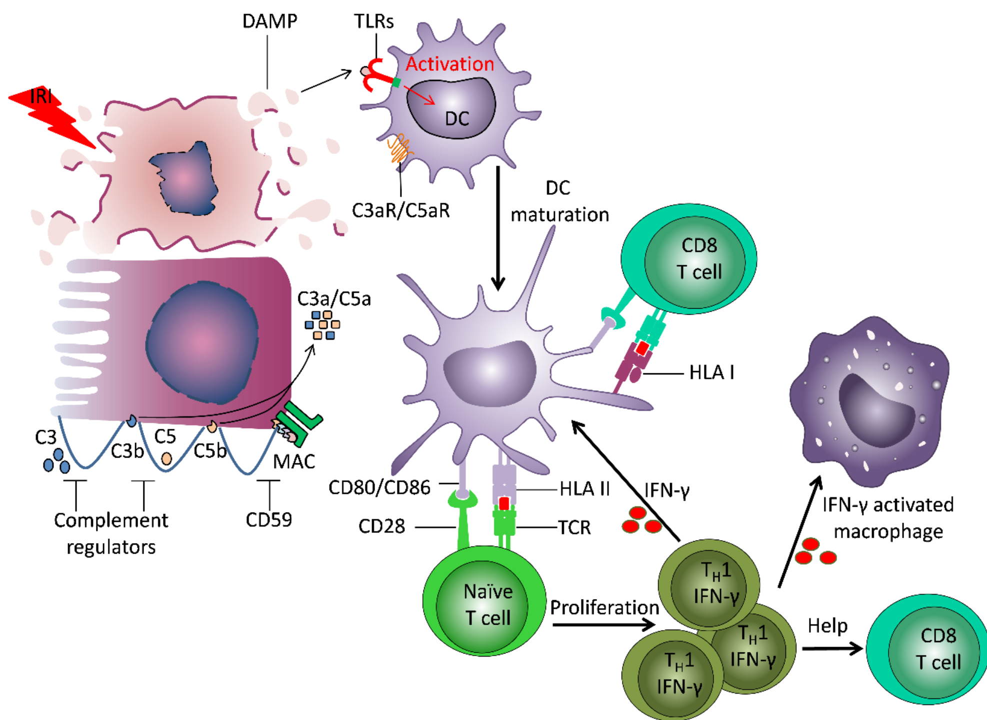

Figure 1. Danger signals in transplant immune activation. Ischemia reperfusion injury (IRI) leads to induction of necrosis of tubular cells and release of damage associated molecular patterns (DAMPs), which are normally hidden within intact cells. DAMPs binds to Toll-like receptors (TLRs) on dendritic cells (DC) and induce DC activation and maturation. The matured DC present donor derived antigen and co-stimulatory molecules to naïve T cells, which drive T cell differentiation into IFN-γ producing T_H1 cells. IFNγ can stimulate maturation of other DCs, induce macrophage activation and recruitment, and direct differentiation of CD8+ T cells. The recipient DCs are also able to capture donor HLA class I and stimulate recipient CD8+ T cells. IRI can lead to induction of a local increase of complement component 3. Cleavage of C3 by the alterative pathway results in C3b deposition on the cell membrane and complement cascade activation. The small fragments C3a and C5a, released during complement activation, have pro-inflammatory effects. The formation of membrane attack complex (MAC) leads to target cell lysis and release of DAMPs.

Risk assessment of transplant outcome by molecular tools

To overcome the limitations of histologic assessment, molecular diagnostic tools were developed and improved in quest of better precision. Philip Halloran's group has developed machine learning classifier algorithms to assess the probability of TCMR and ABMR using microarray (135, 136). TCMR expression profiles reflect APC activation and costimulation, T cell signaling, and IFN-γ related effect (137). ABMR specific transcripts are mainly expressed in endothelium, epithelium, and NK cells, and many of these are induced by IFN-γ (135). A molecular score, reflecting the probability of TCMR or ABMR, was assigned by the classifier algorithms based on these rejection specific transcripts. The same research group has developed a molecular microscope diagnostic system (MMDx) for real time assessment of kidney transplant rejections, whereby the biopsy samples are processed in a 29 hours procedure (138). MMDx diagnosis for the ABMR and TCMR showed 77% of balanced accuracy versus histology diagnosis, but clinicians agreed with molecular assessment (87%) more than with histology diagnosis (81%). All the reports signed out by trained observers based on the molecular score had more than 90% agreement. Therefore, the molecular diagnosis certainly can contribute to clinical management (138).

TCMR related genes

Identification of TCMR specific molecular markers using microarray assays is of importance to add extra values for histologic assessment and predict graft outcome. Sarwal et al firstly revealed the heterogeneity in acute rejection based on differences in immune activation and inflammatory cell composition (139). Reeve et al identified a series of transcripts that are differently expressed in TCMR versus other indication biopsy samples, including CD8a, CD96, CD28, BTLA, IFN-γ (136). Another prospective study confirmed that the TCMR score generated using the specific transcripts is associated with TCMR lesions (140).
These transcripts mainly reflect T cell immunity, APC activation, IFN-γ effects, and parenchymal injury. Assessing the molecular profile of rejection may contribute to a more reliable diagnosis, especially in ambiguous biopsies.

Myeloid related S100 proteins

Patients with a high expression of myeloid related S100A9 in acute rejection biopsy samples show a better long term graft survival than patients with low expression (141, 142). Calprotectin, the heterodimer complex formed by S100A8 and S100A9, is abundantly expressed in myeloid cells. Calprotectin acts as a biomarker of inflammatory bowel disease, as increased number of neutrophils infiltrate in the bowel (143). In addition, the expression levels of calprotectin also correlate with autoimmune diseases (144-147). Ryckman et al. showed that S100A8/S100A9 are involved in neutrophil activation and migration to inflammatory site (148). Extracellular S100A8 and S100A9 proteins can bind to TLR4 or the receptor for advanced glycation end products (RAGE), expressed on macrophages or

endothelial cells, leading to the production of proinflammatory cytokines and chemokines via the NF-kB signaling pathway (149, 150). Furthermore, S100 proteins exhibit calcium and zinc dependent antimicrobial activity effects (151-153).

On the contrary, other studies reported that S100A8 and S100A9 are involved in accumulation of myeloid-derived suppressor cells (MDSC) that play an anti-inflammatory role in the adaptive immune response. Chen et al. demonstrated that S100A9 can inhibit the dendritic cells differentiation and accumulate MDSC in tumor-bearing mice (154). Sinha et al. reported that S100A8/A9 could bind to cell surface glycoprotein receptors on MDSC and enhance MDSC migration through NF-kB pathway (155). MDSC can also secrete S100A8/A9 proteins that form an autocrine feedback loop for their accumulation (155). Zhao et al. proposed S100A9 as a novel maker of human monocytic MDSCs (156). In human kidney transplantation, MDSC present in the recipients can expand regulatory T cells *in vitro* (157, 158). In addition, recipients with a higher frequency of MDSC at time of rejection experienced better long term graft outcome compared to recipients with lower numbers of MDSC (158).

S100A8/A9 deficient mice show enhanced renal dysfunction, sustained inflammation, and increased fibrosis during tissue repair process after IRI, suggesting that S100A8/A9 play an important role in macrophage mediated renal repair (159). Immunofluorescence staining of the tissue biopsy showed that of the S100A9+ cell population 97.2% was positive for pan-macrophage marker CD68 and 77.8% positive for HLA-DR, but that only 35.6% and 25.9% was positive for macrophage type 2 marker CD163 and granulocyte marker CD66b, respectively. These results indicate that the infiltrated S100A9+ myeloid cells represent a distinct macrophage subset with immune regulatory capacity (141). In addition, extracellular S100A8 and S100A9 inhibit the maturation of monocyte derived dendritic cell *in vitro*, which subsequently leads to a reduced T cell proliferation and IFN-γ production in mixed lymphocyte reactions.

Risk assessment of steroid resistance

Accurate prediction of steroid resistance using indication biopsies enables application of the appropriate immunosuppressive therapy, which prevents irreversible nephron damage that otherwise would develop during the period of suboptimal steroid treatment. Histological evaluation of kidney biopsies is used to assess steroid resistant rejection. Acute rejection with endarteritis and sticking of mononuclear cell to endothelial cells predict steroid resistance (133). Haas et al. reported that severe acute vascular rejection (type 2B) is associated with steroid resistant rejection and long term clinical outcome (132). C4d deposition in peritubular capillaries (PTC), a maker of DSA formation and complement activation, is associated with steroid resistant rejection (160-162). However, Botermans et al. could not confirm the correlation between C4d staining and steroid resistance in early acute rejection episodes (163).

Presence of inflammatory cells, such as B cell, macrophages, NK cells, and cytotoxic T cells is correlated with worse response to steroid therapy. Several studies showed that the dense CD20+ B cells infiltrates in biopsy samples predicts steroid resistance (139, 164, 165), whereas recent studies showed inconsistent results (166-168). The presence of CD68 positive macrophages in glomeruli and interstitium correlates with steroid resistant rejection (169, 170) and is associated with intimal arteritis and C4d deposition (171-173). Rejection with a predominance of cytotoxic T cells in the glomeruli and extensive staining of mononuclear granulysin is indicative for steroid resistance (174, 175). A subsequent study confirmed that patients with steroid resistant rejection display increased mRNA levels of cytotoxic T cell- and NK cell markers (139). Increased expression of the T cell activation markers CD25:CD3 ratio and LAG-3 (51) and of Fas ligand (176) in kidney biopsies are predictive of steroid resistant rejection. In contrast, a relatively high mRNA level of FOXP3 in urinary cells is predictive of the reversal of acute rejection by steroids (177). Rekers et al. showed that high tissue expression of metallothioneins, which are zinc-binding proteins, predict steroid resistance (178). These findings indicate that several factors play a role in steroid resistant rejection of kidney transplants.

Genetic risk factors for acute rejection

The role of HLA molecules in the field of transplantation has been widely appreciated: better matching leads to better graft function. Possible associations between clinical outcome and non-HLA polymorphisms in genes encoding cytokines, chemokines, toll-like receptors, ficolins, and complement components, have been investigated in many studies (93, 179-184). Some studies show a significant association between candidate SNPs and transplant outcome, but validation of the same SNPs in follow-up studies often led to inconsistent results. The inconsistent results may be due to differences in population composition and characteristics, inadequate sample size, lack of statistical correction for multiple testing, and lack of validation in an independent cohort. Thus large international collaboration study is required to establish the role of non-HLA polymorphisms in the field of kidney transplantation.

Genome-wide association study (GWAS)

GWAS represent an unbiased approach to analyze millions of SNPs scattered across the genome, GWAS may also provide a robust genomic platform to characterize genetic risk factors of adverse transplant outcome. The advantages of GWAS in kidney transplantation will be described in chapter 6.

Genomic research in transplantation is more complicated than genomic research of common diseases, because it involves the interaction between the recipient and the donor graft. The human H-Y antigen, a male donor allograft to a female recipient, is associated with elevated risk of graft loss after kidney transplantation (185, 186). The effect of non-HLA

antigens such as MICA, G protein-coupled receptors (GPCRs), vimentin, angiotensin II type 1 receptor (AT1R), and perlecan in kidney transplant has been summarized in a previous review (187). A small pilot study showed that the number of amino acid mismatches in trans-membrane proteins is negatively correlated with long term allograft function, independent of HLA matching and donor age (188). The combined analysis of recipient and donor genomes, such as homozygous loss–of-function variants and nonsynonymous SNP mismatching, may provide new insight into the mechanism of rejection.

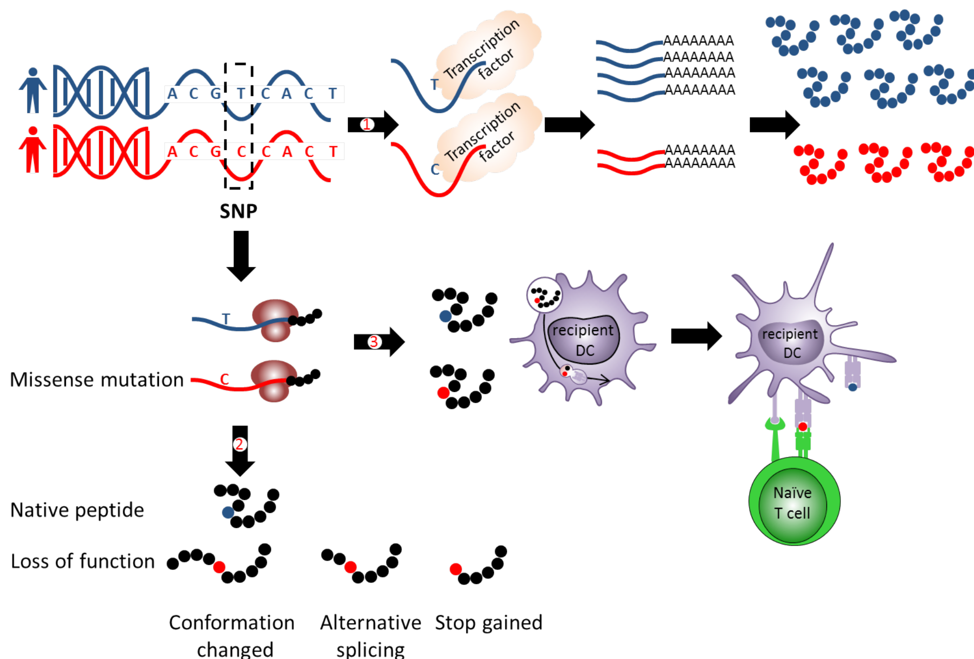

Figure 2. The role of single nucleotide polymorphism in the alloimmune response. SNPs may affect alloimmune responses through multiple mechanisms. Firstly, SNPs located in the promoter of genes may affect the binding of transcription factors, which subsequently alter gene expression of immune-related molecules. Secondly, missense SNPs may lead to a protein confirmation change, alternative splicing, or gain of stop codon, leading to a loss of function. Thirdly, missense SNPs may result into amino acid substitution of encoded proteins that generate polymorphic epitopes. Recipient DCs process and present such mutated proteins in the form of peptides to CD4+ T cells and initiate an alloimmune response.

Aim of this thesis

The aim of this thesis was to identify molecular markers and genetic variants associated with adverse transplant outcome and investigated the immune regulatory effect of S100 calcium binding proteins.

In this thesis, quantitative real time PCR was used to identify the molecular markers associated with kidney transplant outcome. **First**, we compared commercial SYBR green master mixes and optimized qPCR protocols in order to obtaining a high sensitivity and specificity of the assays to be used in the other chapters (**chapter 2)**.

To investigate molecular markers associated with IRI, steroid resistance and adverse graft outcome, a large cohort study of kidney transplant patients with acute rejection was studied. In **chapter 3** we tried to answer the question whether innate immunity, complement and apoptosis related makers, associated with IRI, can predict long term graft survival when measured at the time of acute rejection. In **chapter 4** we tried to assess the risk of patients with acute rejection and predict the steroid resistance by analyzing a number of endothelial-epithelial cell and TCMR related makers in biopsy samples. In **chapter 5** we tried to identify the genetic risk factors associated with biopsy proven acute rejection, based on a genome wide association study of more than 300 donors and recipients. In **chapter 6** we tried to answer the question whether the genomic missense SNP mismatching between donor and recipient has any effect on kidney transplant outcome. The advantages and constraints of GWAS in kidney transplantation are discussed in **chapter 7**.

In **chapter 8** we provided evidence that calcium binding proteins S100A8 and S100A9, which have been show to predict a favorable graft outcome after acute rejection, have an immune regulatory effect in myeloid cells.

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